ISTITUTO SUPERIORE DI SANITÀ

Third National Research Program on AIDS
Progress report

Istituto Superiore di Sanità
Rome, February 26 - March 2, 2001

Edited by
Centro di coordinamento, organizzazione e verifica
dei progetti per la lotta all'AIDS

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Parole chiave: AIDS: Programma nazionale di ricerca, Ricerca sanitaria, Sanità pubblica

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INDEX

Extramural Research Projects ***pg.***

Epidemiology and health care **1**
(Scientific Coordinator: *Giovanni REZZA*)

Pathology, clinic and therapy of AIDS **33**
(Scientific Coordinator: *Stefano VELLA*)

Pathogenesis and immunity for individuation of new chemotherapy, immunotherapy and vaccine prevention targets **147**
(Scientific Coordinator: *Paola VERANI*)

Opportunistic infections and tuberculosis **265**
(Scientific Coordinator: *Antonio CASSONE*)

ISS Research Projects

Epidemiology and health care **313**
(Scientific Coordinator: *Giovanni REZZA*)

Pathology, clinic and therapy of AIDS **319**
(Scientific Coordinator: *Stefano VELLA*)

Phatogenesis and immunity **353**
(Scientific Coordinator: *Paola VERANI*)

Development of a vaccine against AIDS **375**
(Scientific Coordinator: *Barbara ENSOLI*)

Opportunistic infections and tuberculosis **383**
(Scientific Coordinator: *Antonio CASSONE*)

Author Index **395**

The National research program on AIDS

(Extramural research projects)

Project

EPIDEMIOLOGY AND HEALTH CARE

Scientific Coordinator: **Giovanni REZZA**

Projects financed N° 18

ROLE OF CCR5-Δ32, CCR2-64I AND SDF1-3'A ALLELES IN A POPULATION OF HIV-1 PATIENTS WITH KNOWN SEROCONVERSION DATE

Stefano Corvasce, Renata Mazzucchelli, Lorenzo Dehò, Javier Cibella, Maurizio Bada, Laura Papagno, Daniela Senese, Rossella Velleca, Giuseppe Ippolito, Mauro Moroni, Antonella d'Arminio-Monforte, Patrizio Pezzotti, Maria Dorrucci, Giovanni Rezza and Claudia Balotta for the I.Co.N.A. Study Group.

RATIONALE: Insights in the field of HIV-1 pathogenesis came from the discovery that viral entry requires the use of coreceptors, identified as α– and β–chemokine receptors. The epidemiological studies on common polymorphisms of these coreceptors or their ligands did not lead to unequivocal results regarding the role of CCR5-Δ32, CCR2-64I and SDF1-3'A mutant alleles.
We aimed at addressing the role in the natural history of HIV-1 disease of CCR5-Δ32, CCR2-64I and SDF1-3'A alleles, both singularly or in combination, by studying their prevalence and the correlation between different allelic conditions, CD4 cell counts and HIV-1 RNA levels in plasma, in a population of HIV-1 patients with known date of seroconversion.
METHODS: We analysed 665 HIV-1 infected subjects, of whom 393 belonging to I.Co.N.A and 248 to I.S.S.C. cohort. Twenty-four subjects belonged to both the cohorts. As controls, 117 healthy blood donors (HCs) were studied. CCR5, CCR2 and SDF-1 genotyping was performed by specific real-time PCR-based TaqMan® allelic discrimination assays using ABIPrism™ 7700 Sequence Detection System. TaqMan assay uses the 5'→3' nuclease activity of Taq DNA polymerase to cleave a nonextendible hybridization probe during the extension phase of PCR. The TaqMan probe is added directly to the PCR mix, and conditions are virtually identical to those that are established for a standard PCR. As Taq DNA polymerase extends from the PCR primers, it cleaves the TaqMan probe only when it is hybridized to the target, separating the reporter dye from the quencer dye.
Date of seroconversion, values of CD4+ cell counts and HIV-1 RNA level for each patient were obtained from I.Co.N.A. and I.S.S.C. databases.
RESULTS: The table shows the prevalence of each genotype and the frequency of CCR5-Δ32, CCR2-64I and SDF1-3'A alleles in HCs and HIV-1 positive population.

	CCR5			CCR2			SDF-1		
	WT	HE	HO	WT	HE	HO	WT	HE	HO
HCs (n=117)	104 88.9%	12 10.3%	1 0.8%	93 79.5%	24 20.5%	-	69 59.0%	44 37.6%	4 3.4%
Allelic frequency		0.060			0.102			0.222	
HIV-1 patients (n=665)	567 85.3%	98 14.7%	-	499 75.0%	156 23.5%	10 1.5%	385 57.9%	230 34.6%	50 7.5%
Allelic frequency		0.074			0.132			0.248	

DISCUSSION: Our data indicate that the prevalence of CCR5-Δ32 allele is lower in Italy compared to Northern European countries. This is in agreement with a recent large genetic study that demonstrated the absence of Δ32 allele in native Africans, American Indians and East

Asians. Of note, the Δ32 allele is distributed following a North to South gradient across Eurasia. The allelic frequency found in our population (.060) is similar to those detected in the Mediterranean area (.077, .063, .045, .044 in Slovenia, Turkey, Bulgaria and Greece, respectively), probably reflecting a strong selective pressure caused by a past pandemic event due to major pathogen(s). Both the prevalences and the allelic frequencies of CCR2 and SDF-1 mutant alleles are similar to those detected by previous surveys worldwide. The specific role of each protective allele in determining delay of HIV-1 disease progression will be analysed by considering the degree of immune depletion, HIV-1 replication and AIDS as end-points in a time-depending survival analysis. The multivariate analysis will indicate the relative risk of progression in HIV-1 patients stratified according to both single and combined CCR5, CCR2 and SDF-1 genotypes.

N° dell'Accordo di Collaborazione: 20C.1

ANTIBODIES AGAINST KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) NUCLEAR ANTIGENS AND DEVELOPMENT OF KAPOSI'S SARCOMA (KS) IN ITALIAN PATIENTS RECEIVING HEART ALLOGRAFT TRANSPLANTATION.

Carlo Parravicini, MD[1], Giovanna Bestetti MD[2], Chiara Scalamogna, MD[2], Sara Calattini, BSc[2], Morena Galazzi[2], Francesca Poli, MD[3], Girolamo Sirchia, MD[3], Massimo Galli, MD[2], Mauro Moroni, MD[2], and Mario Corbellino, MD[2]

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Background.. In previous studies we have shown that in subjects receiving kidney transplantation, the incidence of KS (I-KS) is strictly related to the prevalence of KSHV infection in organ donors (D) and recipients (R) and that virus reactivation is the most frequent pathogenetic event in the development of I-KS. However, there is no present consensus as to the most prevalent pathogenetic event underlying KS development in solid-organ transplant patients.
Objective and Methods. We investigated the role of KSHV in the pathogenesis of I-KS among subjects undergoing heart transplantation. Antibodies against KSHV LNA and Orf65 were sought for in D and/or R sera obtained at the time of transplantation from 9 subjects with I-KS. In addition, serological tests were performed in 100 patients in the waiting list for heart transplantation and in 358 organ donors referring to the Nord Italian Transplant Program during the year 1996.
Results. In all 9 I-KS cases examined, the recipient was found to be seropositive for KSHV, while the tests were negative in the 7 donors for whom serum samples were available. Among patients in the waiting list, 5/100 had detectable antibodies against KSHV. Finally, 14/358 (3,9%) organ donors were found to be seropositive.
Conclusions. In immunosuppressed heart transplant recipients of Northern Italy, development of KS is associated to serologic evidence of KSHV infection which is in most cases detectable in the recipient at the time of organ transplantation, prior to initiation of immunosuppressive treatment. These findings are similar to those encountered in renal transplant patients. However, different rates of I-KS in solid-organ transplant patients receiving different organs might be ascribed to distinct immunosuppressive and prophylactic strategies which deserve additional studies.

N°. dell'Accordo di Collaborazione. N 20C.2

DETERMINANTS OF ACCESS TO TREATMENT FOR HIV INFECTION AMONG DRUG USERS

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Introduction

An increased survival of persons with AIDS, a decreased incidence of AIDS and longer incubation period have been observed, since 1996. These epidemiological changes are at least partly attributed to the availability of new powerful treatments for HIV infection and opportunistic infections. The availability of these treatments has determined a socioeconomic discrimination in survival of persons with AIDS which was not present before the introduction of such treatments. Drug users seem to be particularly vulnerable to this discrimination. Several studies carried out in the States show that drug users had less benefit in terms of reduced morbidity and mortality as compared to non drug users. Obstacles to access to treatment for HIV infection may be attributed to drug users themselves, to care providers and to the organisational characteristics of the health services.

Objectives

The general aim of the study is to analyse frequency of access to treatment for HIV infection among HIV positive drug users.
Specific objectives of the project are:

- To measure occurrence of access to health services for HIV infection among HIV positive drug users
- To analyse knowledge of HIV positive drug users on availability and accessibility of antiretroviral therapy
- To analyse possible determinants of access to treatment for HIV infection among HIV positive drug users
- To analyse attitudes and knowledge of health personnel dealing with drug users towards strategies for HIV testing, for offering other diagnostic procedures and antiretroviral therapies themselves.

Methods

Cross-sectional survey among drug users reporting HIV positive status recruited in Rome in three treatment services for drug addiction (ASLRME), one street unit (Villa Maraini) and at entry in one prison (Rebibbia). The estimated size of the study population is 600.
An anonymous questionnaire to collect essential information, among drug users, on socio-demographic characteristics, health status, knowledge of and access to treatments for HIV infection, has been drafted.
A total of 10 interviewers have been selected and trained to administer the questionnaires to the subjects enrolled in the study.
Moreover all health personnel of participating services are included together with all the Directors of public treatment services for drug use in Rome.
An anonymous questionnaire is also used for interviewing health personnel to retrieve information on: characteristics of protocols for HIV testing, protocols for referral to further investigations and inclusion criteria for antiretroviral treatment.

Preliminary results

The final study protocol has been developed.
In order to validate the draft questionnaire, a pilot study has been carried out. Twenty eight drug users have been enrolled at the above mentioned centres. The pilot study showed that the interview, lasting about 15 minutes was well accepted; some small changes in the questionnaires have been done.

Accordo di Collaborazione N. 20C.3

PHYSICAL HEALTH STATUS IS PREDICTIVE OF SURVIVAL IN HIV+ PATIENTS

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Objective: To evaluate the predictive value of physical and mental health on clinical progression and death of HIV-infected patients in the era of new antiretroviral therapy (ART).
Methods: Prospective, multicenter, cohort study on Italian HIV+ patients at any stage of disease. From October 1997 to May 1998 patients were consecutively enrolled in 7 Italian Clinical centers. The validated Italian version of the MOS-HIV Health Survey was used as tool for the study. It evaluates 11 health-related quality of life (HRQoL) domains: pain, physical functioning, role functioning, social functioning, mental health, vitality, health distress, cognitive functioning, general health perception, health transition, overall QoL. Two summary scores for physical health (PHS) and mental health (MHS) were obtained. A symptom list was added to the original version. Questionnaire administration was planned every 6 months.
Results: 809 patients were enrolled into the study. Mean age: 35.6 years, females: 32%, intravenous drug users: 48%, 33% CDC group C. Median CD4+ cells count: 259/μl; people with undetectable HIV RNA: 32%. 90% of patients were taking ART, of whom 56% a protease inhibitors (PIs)-containing regimen. After a median of 697 days (25^{th}-75^{th} percentiles: 637-762), 95 AIDS-related events or death were observed. At Cox model, after adjusting for age, gender, HIV transmission modality, CD4+, HIV RNA, time on ART, interactions among the most important variables, PHS (OR 0.8 for any 5-points increment; 95% CI 0.8-2.9; p=0.001), CDC group C (OR 3.6; 95% CI 2.0-6.4; p<0.001), and CD4<200/μl (OR 2.1; 95% CI 1.2-3.7; p=0.0l) were related to clinical progression or death. Conclusions: Physical health status is one of the strongest predictors of clinical progression in HIV+ patients at any stage of HIV disease. Implementation of health status assessment in clinical practice is strongly warranted both in diagnostic and therapeutical algorithms. Each survival analysis in HIV+ patients should be adjusted for health status variables. Health status may become a useful surrogate markers of clinical progression in poor resources settings such as those of developing countries.

Accordo di Collaborazione N. 20C.4

DEATH RATES FOR AIDS AND OTHER DISEASES IN A COHORT OF 3925 INTRAVENOUS DRUG USERS RECRUITED IN MILAN SINCE 1980. TEMPORAL TRENDS FROM 1980 TO 2000.

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Objective of this investigation was to study the trend in mortality in a cohort of 3925 intravenous drug users (IVDU) enrolled in Milan between November 1980 and December 1989. The cohort was composed of 3053 men and 872 women with a mean age at enrollment of 26 years. The enrolled subjects voluntarily attended four public services for treatment of drug-dependence in Milan. The vital status of enrolled subjects was ascertained every year at registry offices of municipalities of residence. Causes of death were assessed by reviewing death certificates, clinical records and autopsy reports. Mortality was analyzed in terms of death rates per 1000 person/years. Expected deaths were calculated from mortality of the general population of the same sex and age living in Milan in 1991.
From 1980 to June 2000 a total of 1471 deaths were registered in the cohort, representing about the 40% of the enrolled cohort. The average overall mortality during the entire period of observation was equal to 34.1 per 1000 person years. AIDS was the principal cause of death and accounted for most of the excess of mortality of this population.The table presents the cause specific mortalities per 1000 person-years and the ratio of observed to expected deaths.

Year	AIDS	Overd.	Infect.Dis.	Cancer	Liver Cirrhosis	Violence accidents	Other	Unkn.	p-y	exp/obs	mortality rates
80	- (0,00)	3 (20,07)	- (0,00)	- (0,00)	- (0,00)	- (0,00)	- (0,00)	- (0,00)	149,44	17,6	20,07
81	(0,00)	5 (5,89)	3 (3,54)	- (0,00)	2 (2,36)	6 (7,07)	1 (1,18)	2 (2,36)	848,53	19,4	22,39
82	(0,00)	12 (8,86)	- (0,00)	- (0,00)	- (0,00)	4 (2,95)	- (0,00)	3 (2,22)	1354,11	11,8	14,03
83	1 (0,57)	9 (5,15)	3 (1,72)	- (0,00)	- (0,00)	5 (2,86)	- (0,00)	3 (1,72)	1747,41	9,9	12,02
84	2 (0,95)	14 (6,62)	4 (1,89)	- (0,00)	- (0,00)	5 (2,36)	1 (0,47)	1 (0,47)	2114,95	10,2	12,77
85	3 (1,24)	13 (5,38)	2 (0,83)	1 (0,41	2 (0,83)	2 (0,83)	1 (0,41)	4 (1,66)	2416,55	9,1	11,59
86	14 (5,17)	16 (5,90)	- (0,00)	1 (0,37)	4 (1,48)	5 (1,84)	2 (0,74)	1 (0,37)	2710,42	12,0	15,86
87	23 (7,94)	26 (8,97)	2 (0,69)	1 (0,35)	3 (1,04)	- (0,00)	2 (0,69)	3 (1,04)	2898,49	15,5	20,70
88	39 (13,11)	38 (12,77)	1 (0,34)	- (0,00)	6 (2,02)	9 (3,02)	- (0,00)	2 (0,67)	2975,95	23,8	31,92
89	41 (13,93)	37 (12,57)	2 (0,68)	1 (0,34)	5 (1,70)	4 (1,36)	1 (0,34)	3 (1,02)	2943,94	23,6	31,93
90	65 (23,17)	41 (14,61)	4 (1,43)	1 (0,36)	4 (1,43)	3 (1,07)	- (0,00)	3 (1,07)	2805,84	31,9	43,12
91	74 (27,92)	35 (13,21)	3 (1,13)	3 (1,13)	2 (0,75)	10 (3,77)	2 (0,75)	4 (1,51)	2650,41	36,7	50,18
92	79 (31,28)	26 (10,30)	3 (1,19)	- (0,00)	7 (2,77)	6 (2,38)	2 (0,79)	1 (0,40)	2525,49	35,8	49,10
93	96 (40,50)	7 (2,95)	8 (3,38)	1 (0,42)	5 (2,11)	4 (1,69)	2 (0,84)	4 (1,69)	2370,27	39,0	53,58
94	107 (47,92)	6 (2,69)	2 (0,90)	2 (0,90)	3 (1,34)	4 (1,79)	2 (0,90)	3 (1,34)	2232,87	41,7	57,77
95	117 (56,16)	6 (2,88)	1 (0,48)	3 (1,44)	5 (2,40)	5 (2,40	2 (0,96)	4 (1,92)	2083,43	48,5	68,64
96	88 (45,03)	8 (4,09)	4 (2,05)	3 (1,54)	6 (3,07)	6 (3,07)	1 (0,51)	1 (0,51)	1954,27	40,9	59,87
97	37 (20,22)	8 (4,37)	5 (2,73)	2 (1,09)	7 (3,83)	1 (0,55)	5 (2,73)	1 (0,55)	1829,76	23,6	36,07
98	15 (8,48)	8 (4,52)	4 (2,26)	2 (1,13)	4 (2,26)	1 (0,57)	1 (0,57)	- (0,00)	1769,81	12,0	19,78
99	13 (7,55)	7 (4,07)	8 (4,65)	3 (1,74)	11 (6,39)	2 (1,16)	1 (0,58)	1 (0,58)	1721,21	15,4	26,73
00*	4 (3,77)	2 (1,89)	7 (6,60	2 (1,89)	3 (2,83)	1 (0,94)	1 (0,94)	1 (0,94)	1060,04	10,5	19,81

°° first 6 months

Starting from 1996 we observed a dramatic decrease in mortality for AIDS which was the leading cause of death since 1989. The observed to expected ratio for overall deaths was always greater than one for the whole period of observation. However the increased risk of death in last years of observation tended to level off to values similar to those of pre AIDS era. Deaths for causes different from AIDS (in particular liver cirrhosis, infectious diseases other than AIDS and AIDS-related infections and cancer) showed a trend of increase in the last calendar years. From ’96 to ’99 the mortality rate for overdose was stably > 4 per 1000 py, suggesting the persistance of iv-drug abuse in a valuable number of subject included in the cohort even 10 or more years after the first conctact with the public services.
In conclusion, HIV related deaths are dramatically and constantly declining in this cohort. However, our results show a relevant reduction of life expectancy of people who are or were former drug users due to causes different from AIDS.

N°. dell'Accordo di Collaborazione: 20C.5

THE ITALIAN REGISTER OF LONG TERM NON PROGRESSORS. RATES AND DETERMINANTS OF PROGRESSION IN PATIENTS RECRUITED AS OF DECEMBER 2000

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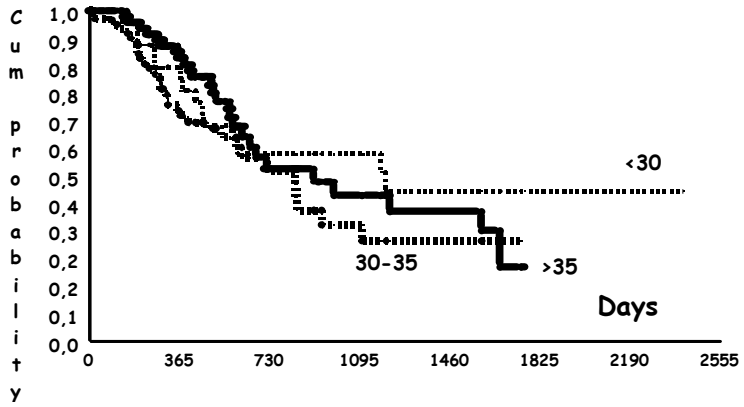
*ITBA-CNR, Milan; [§]Institute of Infectious Diseases, University of Milan

The Italian Register of Long Term Non Progressors includes 230 consecutive patients seen in 16 Italian centers from January 1993 to December 2000. Inclusion criteria were CD4+ cell count greater than or equal to 500 per µl, absence of present or past AIDS defining events, no antiretroviral therapy and a documented duration of the infection of seven or more years. Data of follow-up visits were available for 174 patients who are included in the present analysis. The large majority (81%) of these subjects (127 men and 47 women with a median age of 33 years) acquired the infection via intravenous drug use. They had a median CD4+ cell count at enrollment was 745 per µl and in 56 subjects HIV-RNA was not detectable at inclusion visit. During a median follow-up period of 23 months (range 1-80), 3 patients died (not for AIDS related reasons), 5 showed a clinical progression, 67 showed a decrease of CD4+ under 500 /µl (followed by a clinical progression in 4/67). 52 patients started an antiretroviral therapy (ART), 22 of them after an increase of viremia values and the remaining 30 after a decease of CD4+ under 500 per µl or after the occurrence of AIDS defining events. In a survival analysis, we considered as end points either the decrease of CD4+ cells count under 500 /µl or the occurrence of an AIDS defining events. Patients who did not show any end point were censored at their last follow-up visit or at the time of ART initiation. The analysis showed that the cumulative probability of reaching the end point in 7 years of follow-up was 70%. CD4+ at enrollment was a predictor of short and long term progressions, viral load predicted only progressions occurring within one year from inclusion whereas age at enrollment did not influence the risk of progression (figure).
Our results suggest that a relevant proportion of these patients did not show signs of progression after seven years of follow-up and that CD4+ are the most important prognostic predictors, even in patients who are resistant to the progression of the disease.

Survival with more than 500 CD4 by age at enrollment



Register partecipants are: Cossarizza, V. Borghi (Modena);G. Carosi, S. Casari, M. Cristini (Brescia); F. Chiodo, M. Borderi (Bologna); F. Gritti F. (Bologna); Montella, F. Lauria (Roma); G. Poli, F. Moretti (Milano); F Milazzo (Milano); F Suter (Bergamo); G. Monolo (Magenta); G. Di Perri, A. Sciandra (Torino); E. Concia (Verona); S. Pasquinucci, E. Raise (Venezia)

N°. dell'Accordo di Collaborazione: 20C.7

THE SURVEILLANCE OF THE INCIDENCE OF HIV INFECTION AMONG INJECTING DRUG USERS IN NORTHERN ITALY (1993-1999).

Nicolosi A, Agostara F, Aliprandi C, Albini-Riccioli, Arrigoni E, Brianza E, Brignolo, Brivio B, Bruni C, Buratti A, Camisani A, Campana M, Campione G, Cardia A, Carulli B, Cefis M, Chiesa A, Cinquegrana A, Colaianni A, Colleoni P, Corréa Leite ML, Cozzolino E, Cuorti M, De Camillis, De Micco G, Dettori, Di Fazio C, Donadeo, Elba E, Erpoli P, Fasoli MG, Fea M, Fonzi S, Gaggini, Heydari A, Iacopino B, Lucchini A, Maffezzoni N, Magnone A, Mammoliti G, Mangili R, Marino V, Merlo M, Mezzanotte C, Moioli R, Mollica R, Pedrazzani G, Pennisi, Perotti P, Prete A, Priora C, Quaresima S, Reina F, Resentini M, Riboldi F, Roberto G, Sabbatini A, Sbarbati, Secchi E, Stefano V, Tinghino B, Tonini G, Valsecchi D, Velati C, Villa M, Vincenzo S,Vitali R, Zanini MT.

Objective: To monitor and describe the time trends of the HIV epidemic among intravenous drug users (IDUs) attending drug dependence treatment centers (DDTCs) in Lombardy and Northern Italy.
Methods: Incidence rates were estimated by using data of the cohort of seronegative IDUs attending DDTCs from Lombardy and limiting areas enrolled in the NISDA study. The base population was composed of all IDUs attending the DDTCs of the cities of Abbiategrasso, Angera, Arcisate, Bergamo, Bollate, Brescia, Busto Arsizio, Cittiglio, Corsico, Crema, Cremona, Darfo, Desenzano, Domodossola, Gallarate, Gardone Val Trompia, Gorgonzola, Limbiate, Lodi, Melegnano, Merate, Milano, Montichiari, Monza, Pavia, Ponte San Pietro, Rho, Rozzano, Sant'Angelo Lodigiano, Saronno, Sesto Calende, Somma Lombardo, Sondrio, Tradate, Treviglio, Trezzo d'Adda, Varese, Verbania, Vizzolo Predabissi who were seronegative at their first conctact with the DDTC between 1993 and 2000. All the followed subjects have repeatedly been tested for HIV. Incidence rates (IR) of HIV infection and their confidence intervals (CI) were calculated by the person-years (PY) method and expressed as number of cases per 1000 person-years at risk. Annual incidence rates are represented as moving averages .
Results: Between 1993 and 1999, 135 seroconversions were observed in 7,945 subjects followed for 19,671 PY, for an incidence rate of 6.9 per 1000 PY (95% CI 5.8-8.2). Ninety seroconversions were observed among 6,563 males (PY=16,447) for an incidence of 5.5 (95% CI 4.4-6.8) and 45 seroconversions among 1,382 females (PY=3,224) for an incidence of 14.0 (95% CI 10.3-18.7). Among males, HIV incidence was 4.5 (95% CI 2.7-7.1) under the age of 25 years and 5.8 (95% CI 4.5-7.3) over the age of 25 years. Among females, HIV incidence was 21.1 (95% CI 13.4-31.7) under the age of 25 years and 10.3 (95% CI 6.5-15.7) over the age of 25 years.
Conclusions: The speed of the epidemic of HIV infection among intravenous drug users in Northern Italy has been stable between 1993 and 1999. The incidence rates among females are about 2 times higher than rates among males among subjects 25 years old and older, and 4 times higher among subjects younger than 25. This pattern is likely to be due to the reduction in the parenteral in favor of the heterosexual mode of HIV transmission.

Accordo di collaborazione scientifica n. 20C.8.

STUDIES ON HIV-1 CERVICOVAGINAL SHEDDING

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Introduction

The study on the presence of HIV-1 in cervicovaginal secretions has important implications inherent both sexual and mother-to-child transmission and may contribute to the assessment of preventive (pharmacological and/or vaccine-based) strategies.

Here we report the definitive data of a study aimed to evaluate qualitative and quantitative aspects of HIV-1 shedding in cervicovaginal secretions and its clinical and immunological correlates.

Patients, material and methods

We enrolled in the study 105 HIV-1 seropositive women, referring to the Clinic of Infectious Diseases, University of Bari. During gynaecological examinations, from each patient blood samples and cervicovaginal lavages (CVL) were obtained. CVL were performed by irrigating the fornix and the vaginal walls with 3 ml of sterile PBS. Blood and CVL samples were processed within three hours from collection and aliquots of peripheral blood mononuclear cells, plasma and CVL cells and fluid were stored frozen at –70 °C until use. Such a material was used in the various phases of the study for different purposes: HIV-1 RNA detection and quantification (by commercially available RT-PCR, Amplicor HIV-1 Monitor test, Roche), RNA and DNA sequencing (by direct sequencing of the entire protease gene and the first 220 codons of the RT gene), detection of HIV-1 specific IgG and secretory IgA (by a commercially available Immunoblot assay, Chiron RIBA HIV-1/HIV-2), detection of proinflammatory cytokines and beta-chemokines (TNF-a, IL-1b, MIP-1a, MIP-1b, RANTES).

Results and Discussion

We demonstrated that HIV-1 cervicovaginal shedding is a common event during HIV-1 infection: it occurs at all stages of the disease in roughly 85% of women, the amount of virus detectable is not related to the degree of immunedeficiency and is not influenced by plasma viral loads. Although CVL viral loads are generally lower than in the corresponding plasma, in 20% of cases 10-100 folds higher viral loads are detectable in CVL, suggesting a local, sustained, replication of the virus. Symptomatic genital infections are associated with an increase in viral replication, but a rapid reduction in CVL HIV loads is observed when infection is cured; the mere presence of potentially pathogenic agents, in the absence of inflammatory signs and symptoms, instead, is not related to an increase in HIV-1 shedding.

HIV-1 specific antibodies (both IgG and secretory IgA) are detectable in CVL, but at low concentration and with narrow specificity; their role in influencing HIV shedding and consequently the infectivity of cervicovaginal secretions is thus probably limited. Beta-chemokines, as well, potent inhibitors of HIV-1, are produced by cervicovaginal mucosa, but the levels of these substances detectable in CVL are too low to suggest an “in vivo” inhibitory effect on viral replication at the genital site.

Antiretrovirals are generally effective in reducing viral load in CV secretions, with a CV response to the treatment that reflect plasma response: however a few cases were observed, where HIV replication and shedding persisted at the genital site, in spite of the absence of detectable virus in plasma: this phenomenon, even if of limited size, may be very relevant in sexual and mother-to-child transmission events.

HIV-1 variants carrying mutations correlated with resistance to antiretroviral drugs can be frequently detected in CV secretions of women under treatment, and cases of viral variants with mutations conferring simoultaneous resistance to multiple drugs (NRTI, NNRTI and PI) were documented in extensively treated women: the possibility of female-to-male and mother-to-child transmission of such drug-resistant viruses and the potential clinical consequences must be therefore carefully taken in account and deserve further investigations.

Accordo di Collaborazione n. 20C.9.

HOSPITAL ACQUIRED BLOODSTREAM INFECTIONS IN HIV + PATIENTS

Petrosillo N, Viale P, Arici C, Bombana E, Bonaventura ME, Casella A, Cesari L, Colombini P, Coronado O, Cristini F, De Gennaro M, Dodi F, Ferri F, Gabbuti A, Gattuso G, Irato L, Lazzari D, Maggi P, Martini L, Nicastri E, Pallavicini F, Pan A, Pantaleoni M, Porcasi R, Preziosi G, Scarabaggio T, and Ippolito G.

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Background: The most distinctive feature of nosocomial infectious risk in HIV-infected patients is the high incidence of nosocomial bloodstream infections (N-BSI) that range from 23.9% to 36% of all nosocomial infections. This finding led us to asses the frequency of N-BSI, to identify prevalent etiologic agents and to define associated risk factors. Methods: A prospective, multicentre study involving 17 centres was conducted in HIV-infected patients with advanced stage disease (B3, C1-3 by CDC classification). Demographic data, neutrophil and CD4+ counts, Viral load value, Severity Index for Adults with AIDS (SIAA). Karnofski score, information on invasive procedures, chemoprophylaxis, occurrence of BSI and all stages of sepsis and outcome, were recorded. Sepsis was identified according to ACCP/SCCM Consensus Conference. To examine possible risk factors for BSI and to obtain adjusted estimates of Odds Ratios while accounting for all possible risk factors, univariate analysis and multiple logistic regression model were used.
Results: As a June 2000 a total of 1379 hospital admissions of 1036 patients and 26461 hospital days were reported. Median length of stay was 14 days (mean 20 $\pm$ 21). The risk factors for HIV infection regarded injecting drug use for 881 admissions (63,9%), 398 of these (45,1%) with an history of active drug abuse within the last 30 days. During the 1379 admissions, a total of 65 N-BSI among 61 patients occurred. The incidence rates were 2.45 per 1000 patient days and 4.7 per 100 admissions. Of the 65 BSI, 38 were sepsis, 5 severe sepsis and 8 septic shock. In 14 cases there were no signs of SIRS. Twenty-four BSI were secondary and 41 were primary. The remote foci of infection in secondary BSI were mainly represented by skin and soft tissue infections (6 cases), pneumonia (5), and urinary tract infections in catheterized patients (5). Twenty-nine out of the 41 primary BSI were classified as CVC related (CR-BSI), with an overall device-associated infection rate of 9.6 x 1000 device days. Out of the 29 CR-BSI, 15 were related to short-term catheters (15/88; 17.04%) and 14 to long-term catheters (14/88; 15.9%). The device-associated infection rate was 10.7 and 8.8 per 1000 CVC device days among short term catheters and long term catheters, respectively (p= 0.59). Gram positive organisms accounted for 67.1% of microrganisms isolated in all BSI and 72.4% of CR-BSI. The most common organisms isolated in BSI were S. aureus (29,8%), Coagulase Negative Staphylococci (29,3%) and Candida spp (14,9%). The following variables were associated with the occurrence of N-BSI in univariate analysis: active injecting drug use, Karnofski Performance Score < 40, CVC inserted and length of hospital stay, WBC < 2000 $x10^6$/l, SIAA Score class 3, Total Parenteral Nutrition. Co-trimoxazole prophylaxis also resulted inversely significantly associated to the occurrence of N-BSI. When inserted in a multivariate logistic regression model, active injecting drug use, Karnofski Performance Score < 40, CVC inserted and length of hospital stay remained significantly associated with NBSI. The median lengths of stay were 29 days (mean 38 $\pm$ 37) and 13 days (mean 19 $\pm$ 19) for patients with and without N-BSI respectively (p <.00001). Mortality rate was 24.6% among patients with N-BSI and 7.2% among those without NBSI (p <.00001). Mortality rates were 29.3% in case of sepsis , 40% in severe sepsis and 75% in septic shock. Conclusions. The incidence of BSI among HIV-infected patients in advanced stage disease is noteworthy and closely related to CVC use, representing an important issue in the overall management of HIV patients. Of note is the high incidence of S. aureus infections supporting prior observations about its particular relevance in HIV infected patients.

Accordo di Collaborazione N. 20C.10

NATURAL HISTORY OF HIV INFECTION IN THE ERA OF COMBINATION ANTIRETROVIRAL THERAPY.

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Summary

Background: The introduction of the highly active antiretroviral therapies (HAART) has changed the natural history and the epidemiology of HIV infection. Starting from 1996, in fact, a dramatic reduction of the incidence of AIDS and a strong improvement of survival of people with HIV infection and AIDS were observed. A decline of the number of accesses to hospitalisation and of the length of stay was also a consequence of the improvement of quality of life of HIV-infected people due to the use of HAART.
Objective: We want to evaluate temporal changes in the natural history of HIV infection using a seroprevalent cohort of HIV infected people of Lazio Region, with an estimated date of seroconversion. In particular our aim is to estimate the temporal trend of progression to AIDS and survival, to evaluate differences in the incidence of specific AIDS-defining illnesses, to study differential access to care with respect to different variables, in particular social class.
Methods: A data collection form was prepared to collect the following information about the members of the cohort: information about hepatitis infections, haematologic variables, modality of transmission. Furhermore, for each six-month period from 1995 to 2000 we want to collect information on: eventual AIDS-defining illnesses (primary or secondary), use and kind of antiretroviral therapy, use and kind of prophylaxis to opportunistic infections, date and number of CD4 cell count, date and count of viral load. We want to trace the whole retrospective history of each members of the cohort, looking for them in the archives of all the eight clinical centers collaborating at the study, which are the main centers of the Lazio region and are attended by more than 90% of the HIV infected people of the region.
Results: We tested the data collection form at two of the eight clinical centers participating in the study.

Accordo di Collaborazione N. 20C.11

LIVER FIBROSIS PROGRESSION IS RELATED TO CD4+ CELLS DEPLETION IN PATIENTS WITH HEPATITIS C AND HUMAN IMMUNODEFICIENCY VIRUS COINFECTION

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Background: End stage liver disease is becoming one of the most frequent cause of morbility and mortality in Human Immunodeficiency Virus (HIV) infected patients. Hepatitis C Virus (HCV) and HIV share common transmission pathway and HCV is the most common cause of liver damage in HIV seropositives. HCV infection show an accelarated progression towards cirrhosis in HIV-HCV coinfected subjects. Natural history of hepatitis C is characterised by a silent progression of liver fibrosis that brings towards cirrhosis, liver failure and death no more than 20-30% of patients after 10-30 years. Liver fibrosis progression is accelerated by many factors such as older age at infection, male sex and alcohol use. Hiv infection is associated with several causes of liver damage (drugs toxicity, liver involvement in opportunistic infections) and associated with Hepatitis B and Delta co-infection. It is still not cleasr if HIV per se, and not these causes of liver damage associated with HIV infection, is responsible for the accelerated course of HCV infection. The knowledge of the relationship between HIV infection, immune depletion and fibrosis progresion may open the way to startegies aimed to slow liver fibrosis progression in HIV-HCV coinfected patients. Objective: To evaluate the relationship between liver fibrosis and CD4 depletion in Human Immunodeficiency Virus (HIV) seropositives with hepatitis C virus (HCV) coinfection. Design:Evaluation of liver biopsies obtained from intravenous drugs users with or without HIV coinfection Patients:Two hundred and four HCV infected patients, 84 with and 120 without HIV coinfection without any other cause of liver damage including past or present opportunistic infections, drugs used for their treatment and prophylaxis, past or present treatment with antiretrovirals, HBV and/or HDV coinfection.. Intervention. Blind evaluation of the stage of fibrosis according to a reproducible and validated histological 4 stage classification system. Main outcome measures: The relationship between the satge of liver fibrosis and CD4 levels was analysed taking into account the variables known or suspected to influence liver fibrosis progression by using polytomous logistic regression. Results: Twenty-four patients (11%) showed many fibroous septa with (5%)or without (6%) cirrhosis (METAVIR stage F3-F4), 56 (27%) had few fibrous septa and 124 (60%) had no fibrous septa. An asssociation was found (OR 3.2 p=0.037) between CD4 lymphocyte counts lower than 500/mmc and the presence of many fibrous septa (F3-F4 stages), independently from other factors, including age, HIV infection, estimated duration of HCV infection, alcohol use, sex and age at estimated first exposure. Conclusions: CD4 cells depletion seems to be independently related to the severity of liver fibrosis in chronic hepatitis C. This results suggest the hypothesis that earlier start of antiretroviral combination therapy, could slow liver fibrosis progression by mantaining CD4 counts higher than 500.

N°. dell'Accordo di Collaborazione.20C12.

LIFE THREATENING HEPATOTOXICITY DURING COMBINATION ANTI RETROVIRAL TREATMENT: INCIDENCE, RISK FACTORS, HISTOLOGY AND LONG TERM OUTCOME

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Objective : To assess the incidence, etiology histology and long term outcome of life threatening hepatotoxicity (LTH) during combination antiretroviral treatment (ART).Design: Prospective cohort study. Patients: Seven hundred fifty five HIV naives seropositives consecutively undergoing combination ART from January 1997 to June 1998.
Interventions: Liver enzymes and liver function tests were tested at the 1st and then at least every 4 months. Liver biopsy and 2 years follow up was proposed to all patients with LTH. A second liver biopsy was performed in consenting patients after 2 years Liver biopsies were blindly evaluated by a single pathologist. Main outcome measures: LTH was defined as «de novo» occurrence of liver failure or increase of liver enzymes of at least 10 times over baseline or 5 times if markedly abnormal. Results. Twenty six cases of LTH were observed with an incidence of 4.2 per 100 person/years. Twenty five out of twenty six were infected by HCV. Risk factors for LTH were: history of injection drug use (Odds Ratio, OR 7.6), age younger than 35 (OR 4.7), anti hepatitis C and Delta viruses reactivities (ORs 6 and 4.6), baseline abnormalities of liver function tests (ORs 5.1 for hypertransaminasemia, 3.7 for hyperbilirubinemia, and 5.8 for prolonged prothrombin time). Liver histology showed exacerbation of viral hepatitis in all of 16 biopsied patients. Variations of aminotrasferases and CD4 cells were significantly correlated in patients with LTH (correlation coefficient: $p<0.001$). Death occurred during follow up in 7 out of 26 patients all showing liver failure at occurrence of LTH and baseline CD4 lower than 200/mm3 (vs 42% of pts. alive at 18 months $p=0.01$). ART was re-started in the remaining 19 after 3 months wash out; 7 out of 19 showed LTH relapse. In 5 out of 7 ART was started a third time after alpha interferon pre-treatment and LTH relapse was not observed. Liver biopsies obtained in 15 patients after two years of follow up did not show evolution of liver fibrosis or worsening of hepatic inflammatory activity. Conclusions LTH seems to be mainly related to exacerbation of viral hepatitis due to immune restoration that can cause irreversible liver disease in patients with severe immune depletion. Careful monitoring and earlier starting of antiretroviral treatment are priorities in the management of patients with viral hepatitis. Wash out from ART may decrease the rate of LTH relapse and interferon pre-treatment, in patients with HCV coinfection, may prevent LTH relapse and is not associated with evolution of liver disease at least in the short term.

N°. dell'Accordo di Collaborazione.20C12

THE IMPACT OF DIFFERENT CARE PROFILES ON SURVIVAL AND COST OF PEOPLE WITH AIDS AND ANALYSIS OF ASSOCIATION BETWEEN SOCIO ECONOMIC STATUS AND CARE PATTERNS

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The introduction of new highly active antiretroviral therapies (HAARTs) has deeply modified clinical pattern and care burden of AIDS disease, decreasing use of hospitalisation and increasing demand of other approaches, more based on ambulatory and home care. Description and analysis of these new care patterns and study of their spread, effects and costs have therefore become a priority for planning of health services in order to respond to needs of the always increasing prevalent people with this disease.
Moreover, in spite of presence of numerous scientific efforts towards rationalization and homogeneity of therapeutical and care approaches for people with AIDS (pwA), persisting heterogeneity of clinical management or individual access to care have been hypothesized as accounting for the differential subgroup survival, as frequently found according to socioeconomic status (1,2,3).
Our study is aimed at evaluating the association between social position of pwA and profiles of received care and also to study the effect of these patterns on survival and costs of pwA.
The study population is a retrospective cohort of 338 AIDS adult cases, residing in the municipality of Rome, diagnosed from 1st January to 31st December 1997 and notified at the AIDS Surveillance System of the Agency of Public Health of Lazio region. For each case, information on sex, age at diagnosis, place of living, fiscal code, marriage status, probable risk factor of HIV, hospital of diagnosis, date of diagnosis, AIDS defining illness and CD4 cell count at the time of diagnosis were extracted from notification records of the Surveillance System. For each individual, neighbourhood socioeconomic status was defined on the basis of a SES index developed for Rome census blocks and categorized into four levels (level I = highest socioeconomic status) (4). Vital status of each case included in the cohort was verified on 30th June 1999, through search of individuals within archives of Registry Office of the Municipality of Rome. For the period starting from date of diagnosis to death or end of follow up (30th June 1999), every hospital admission of each case was searched through record linkage with Hospital Information System of Agency of Public Health of Lazio region, with fiscal code as a linkage key and information on date of admission and discharge, type of hospitalisation (ordinary or day hospital), main and secundary diagnosis, interventions and procedures, DRG and related tariffs were extracted. Then we selected for the analysis only hospital admissions with date of discharge not before date of diagnosis; hospitalisations with AIDS unrelated diagnosis were excluded. We performed a preliminary descriptive analysis of the database and analysed distributions of hospital admission percentages and proportions of follow up spent in hospital, defined as individual ratio between hospital length of stay and total survival from diagnosis to death or the end of follow up, by socioeconomic status, stratifying according to CD4 counts at diagnosis.
Study cohort is characterized by 73.1% males, 17.7% persons aged under 30 and 29.6% more than 40 years old; 48.5 % of the cohort includes individuals infected because of being intravenous drug users; heterosexual and homosexual transmission was the cause of infection for respectively 29.3% and 14.5% of the study population. The three main infectious disease

hospitals accounted for 88.5% diagnosed cases. Distribution of study individuals by CD4 cell count at diagnosis shows 30.5% with less than 25 CD4, but also 35.2 % with more than 100 CD4 cells. Ten percent of study cohort is characterized by the highest social category, while 31.7 % is in the lowest socioeconomic class.
After two years from diagnosis, proportion of pwA still alive is 61%.
From diagnosis to death or end of follow up we found 1,565 hospital admissions, ordinary and day hospital, for the study population. Within the most and less severe groups at diagnosis (respectively 0-25, >200 CD4 categories), the highest hospital rates were found within the highest social class; medians of proportions of follow up times spent in hospital show an increasing trend when social position decreases. Higher hospital rates within lowest social class were observed for individuals with 25 to 200 CD4 at diagnosis . Medians of proportions of hospitalised follow up seemed to be always higher within individual with lowest social position, for all severity categories at diagnosis with the exception of people with more than 25 and less than 100 CD4.

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N°. dell'Accordo di Collaborazione: 20C.13

PRIMARY HIV INFECTION REGISTER

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RATIONALE

Surveillance of the HIV epidemic is based on the AIDS cases reported to the Italian National Institute of Health. Given the long incubation period of AIDS, this method is able to describe an epidemiological reality ten years old. From studies on sentinel population it is only possible to estimate differences in prevalence, but no information can be obtained on the number of new infections and on the route of transmission. The identification of Primary HIV Infection (PHI) cases is therefore crucial to obtain up-to-date information on HIV infection dynamics, as the distribution of the risk factors reported by the enrolled PHI cases will describe the route of HIV infection at the present time . Primary prevention campaigns and mathematical and statistic models to predict the future of the epidemic can be designed on the present epidemiology. Being characterised by mild symptoms, PHI is difficult to diagnose screening tests alone are not useful so only "active" case finding may provide an accurate method to identify PHI.
Moreover each new case can be informed about primary prevention methods in order to limit the diffusion of the virus and last but not least the person can be treated immediately according to the international guidelines recommending a quick and strong intervention.

METHODS

a) Case finding

In order to identify PHI cases all participating Centres ha**ve** set up a referral system for everyone whose HIV test scores: borderline positive,WB indeterminate profile (2 positive bands, one env gp160,gp120 and/or p.24), HIV p24 Ag positive (Screening negative) or has detectable viral RNA. Those patients will be referred from the laboratory to the infectious disease specialist who will investigate if the person fulfils the criteria for PHI. For the purposes of the study a PHI case is a person showing 1 laboratory + 1 clinical of the following conditions
Lab: borderline positive
WB indeterminate profile (2 positive bands, one env gp160,gp120 and/or p.24) HIV p24 Ag positive (Screening negative) *Clinical*: negative HIV test during the preceding 6 months probable exposure to HIV in the previous 70 days acute retroviral syndrome

b) Register of PHI

Subjects fulfilling criteria for PHI will be asked to participate in the study and those willing **are** interviewed immediately and reported as probable cases to the Co-ordinating Centre. For those refusing to participate, only sex, age and reported risk factor , if any, will be recorded . The Co-ordinating Centre will review each case and, if all infection parameters are confirmed, **it will** include it in the Register.

RESULTS

PHI Cases

Years	1992-95	1996-98	1999-2000
N°	**18**	**75**	**25**
% Male	**89**	**77**	**57**
%Female	**11**	**23**	**43**
Age avr	**32**	**37**	**26**
Risk Factors			
Heterosexual	**33**	**55**	**63**
Homosexual	**56**	**22**	**23**
IVDU	**11**	**8**	**4**

As to December 2000, 118 patients fulfilling PHI case definition have been enrolled
Gender and risk factor for HIV infection observed in PHI cases reflect the dynamics of national AIDS showing a high but constantly decreasing percent of cases among IVDU(1992-95 11%, 1996-98 4% 1999-2000 6%) and homosexual (1992-95 56%, 1996-98 22% 1999-2000 23%) and a increase of cases due to heterosexual contact. As a result in our population has been observed a progressive increase of the percent of women infected.
The decrease in the average age of people affected by PHI shows that information campaigns on HIV infection prevention are still needed.
When sought, HIV-RNA has been constantly found in CLF of patient enrolled in the registry, suggesting a local HIV production. This supports the opinion that an early treatment with drugs able to cross the ematoncefalic barrier should be taken into account.

Accordo di collaborazione scientifica: 20C.14

SHEDDING OF HHV-8 IN WOMEN WITH HIV INFECTION

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Some modalities of HHV-8 transmission are still to be clarified, particularly as regards the role of heterosexual contacts and the female-to-male route. To evaluate to what extent HHV-8 may be sexually transmitted through heterosexual intercourse, we studied cervical smears of women with HIV infection attending the gynaecological outpatient clinic of the National Institute for Infectious Diseases Lazzaro Spallanzani, Rome. Serum samples and cervical smears were taken from 107 women consecutively enrolled between October 1999 and June 2000 and who signed the informed consent. Demographic, clinical and laboratory data were collected through a standardised questionnaire. Antibodies to lytic antigens of HHV-8 were detected using an immunofluorescence assays (IFA) based on BCBL-1 cell line: samples reactive at >1:20 dilution were considered positive. The 107 HIV-infected women had a median age of 35.8 years (range 24-49), and they acquired HIV infection through injection of intravenous drugs or through heterosexual intercourse. Overall, 17 (15.9%) of the 107 women participating at the study were seropositive for anti-HHV8 antibodies; HHV-8 seroprevalence was not associated with age, number of CD4+ cells or with viral load. Two out of the 17 women seropositive for HHV8 antibodies (11.8%) had HHV8-DNA sequences detectable (by means of the PCR) in the cervical smear. Our data are consistent with those of a previous study in indicating that the detection of HHV8 sequences in cervicovaginal secretions of HHV8-positive women is rare. This finding suggests a low efficiency for the female-to-male sexual transmission of HHV8.

Accordo di collaborazione: 20C.15

HIV/HCV COINFECTION AND CANCER RISK AMONG FORMER INTAVENOUS DRUG USERS

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Infection with hepatitis C virus (HCV) is commonly associated with HIV infection, particularly among intravenous drug users (IDU). Now that the life expectancy of HIV-positive patients is increasing, such patients are more likely to face the complications of HCV infection. A lack of association between HCV infection and the risk of non-Hodgkin lymphomas (NHL) has been already reported among HIV-positive individuals, but the role of HCV coinfection on the overall cancer risk of HIV-infected individuals is still to be defined. Thus, in this study we longitudinally assessed the cancer risk of IDU according to HIV and/or HCV infection. The study comprised 5,220 former IDU (4,100 men and 1,120 women), with a median age of 26,6 years (range 15.0-49.2) who resided, between 1982 and 1999, in the San Patrignano Community (a residential community for drug cessation located in Rimini, North Italy) for a median period of 2.4 years. The person-years (py) of observation were computed from the date of admission in the community to the date of cancer diagnosis, or of death, or of living the community, or to 30/09/1999. Cancer incidence rates (IR) per 1,000py were separately computed according to HIV and/or HCV infection: rate ratios (RR) for these IR and their 95% confidence intervals (CI) were used to estimate the cancer risk associated with HCV infection. At enrolment, 21.7% of former IDU were HIV-positive and 77.4% were HCV-positive: 86.2% of the HIV-positive IDU were also HCV-positive (i.e., 977 individuals were positive for both HIV and HCV antibodies). Overall, the 5,220 former IDU were followed up for 15,715 py (11,994 py in men and 3,721 py in women): during such period, 32 cancers were diagnosed (IR=2.0/1,000py). Among HIV-negative IDU, the presence of HCV infection did not modify the cancer risk (IR=0.7/1,000 py in both HCV-negative and HCV-positive IDU), whereas some differences seemed to emerge among HIV-positive IDU (IR=9.5/1,000py in HCV-negative and 5.2/1,000 py in HCV-positive). Such discrepancy was largely attributable to an excess of diagnoses of NHL among HIV-positive/HCV-negative IDU: when cancers other than NHL were considered, the IR turned out to be very similar in the two groups (RR=1.2, 95% CI:0.6-2.1). Since the study is based on a short period of observation, these results should be interpreted with caution. However, they seem to confirm that HCV infection does not increase the risk of NHL in HIV-positive individuals, and suggest a lack of association also with the risk for other cancers.

Accordo di collaborazione: 20C.15

ITALIAN REGISTER FOR HIV INFECTION IN CHILDREN

Tovo PA [(a)], de Martino M, Gabiano C [(a)], Galli L, for the Italian Register for HIV infection in children.

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At the latest analysis of "Italian Register for HIV infection in children" 5,515 subjects were enrolled. Of the 1397 HIV-1-infected children, 150 received blood transfusions, 1218 were born to seropositive mothers, 10 in an endemic country, 16 had an indeterminate risk factor, and 2 children had a HIV-1-negative mother, but a seropositive father. One HIV-2-infected child acquired the infection from the mother (other 2 HIV-2-exposed infants seroreverted). Of the remaining 4116 children, 3610 seroreverted and were taken to be uninfected, and 506 were still in an indeterminate infectious status.

A few targeted analyses were completed in the last year. As regards the relation between HIV and cancers, 35 infected children developed 36 malignancies at a median age of 5.6 years (range 0.3 to 13.1 years). The cumulative incidence of HIV-related tumors was 4.18 per 1000 children/year among vertically infected children. The death rate was 54.4 per 1000 children/year. Asymptomatic or paucisymptomatic and immunocompetent children also developed cancers. The most common tumor was non-Hodgking's lymphoma (NHL), with primary involvement of the CNS in 25% of cases. Many children with NHL responded satisfactorily to chemotherapy. Nine of thirteen treated with a standard protocol remained in complete remission a median 48 months after treatment.

To assess the effect of antiretroviral therapy on decreasing mortality, a total of 1142 perinatally infected children, born between November 1980 and December 1997, were evaluated. Children were stratified in three categories, according year of birth and calendar year: before 1990, between 1990 and 1995, and after 1995. The cumulative survival probability by birth-cohort and calendar period was estimated by Kaplan-Meier curves. Survival was longer in children born after 1995 (RH of death = 0.39, 95% Cl 0.15-0.96) as well as in those in the calendar period 1996-1998 (RH of death = 0.65, 95% Cl 0.45-0.95) than in the birth-cohort and calendar period 1981-1995 ($p<0.01$). The RH of death in children receiving double or triple combination therapy was 0.62 (95% Cl 0.43-0.90) and 0.33 (95% Cl 0.15-0.73), respectively, compared to no therapy. We can conclude that survival of HIV perinatally infected children significantly improved in 1996-1998, due to the introduction of combined antiretroviral therapy.

Our data were also included in an individual patient data meta-analysis co-ordinated by NIH (USA) to test whether the duration of ruptured membranes is associated with an increased risk of transmission; 4721 deliveries were assessed. The risk of vertical HIV transmission increased approximately 2% with an increase of one hour in the duration of ruptured membranes [adjusted odds ratio = 1.02 for each 1 hour increment]. There were no significant interactions of duration of ruptured membranes with study cohort or with any other covariates, except maternal AIDS. These results support the importance of duration of ruptured membranes as a risk factor for vertical transmission of HIV and suggest a diagnosis of AIDS in the mother at the time of delivery may potentiate the effect of duration of ruptured membranes.

N°. dell'Accordo di Collaborazione 20C.16

VERTICAL TRANSMISSION OF HIV INFECTION: NATURAL HISTORY AND RISK OF TRANSMISSION

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In 1984 we started in Padova a prospective study on children born to HIV positive mothers, whose infection status was known during pregnancy. The objective of this study was to evaluate the natural history of HIV infection in children with particular attention to the risk-factors for mother-to-child transmission. Because the transmission rate was lower than expected, in 1985 a multicentre European study was set up (the European Collaborative Study - ECS), a EEC concerted action co-ordinated in London by Prof.C.Peckham. Several paediatric and obstetric centres are involved in this network (7-12-14-23), however the "nest" of the ECS is formed by the Paediatric Department of Padova, Edinburgh, Berlin, Madrid, Valencia, Barcelona, Geneva, Amsterdam and Stockholm.
In Padova by December 2000 about 410 children born to HIV positive mothers have been followed up. Among them, 262 were studied prospectively and enrolled in the ECS.
In the previous studies we have already delineated the mother-to child transmission rate (1-3-5-6-8-10-12-14-22-25-31), the related risk factors (2-7-14-23), the characteristics of certain clinical conditions (26-28-29) the natural history of HIV infection in the child, including the effect on antiretrovirals, (4-6-27) and the prognostic role of CD4 + lymphocytes (8-11).
A joint analyses with the French Collaborative Study was undertaken in order to, ultimately, define the natural history of vertically transmitted HIV infection. More than 4000 mother-infants pairs have been evaluated (31).
In collaboration with the Institute of Oncology of the University of Padova we studied the correlation between the biological characteristic of the virus and the clinical progression (13-18-19-20-21-24). At present we are focusing on the pathogenetic aspects of HIV transmission and disease in children (20-24-30).
Recently we described the first case of vulvar carcinoma in a girl with HIV infection, showing a long term survival as compared with what previously observed in adults (42)
The longitudinal design of the ECS provides the opportunity to describe family circumstances and social care of children born to HIV positive mothers (33). Moreover we were able to compare hospitalisation rates of infected and uninfected children (32).
Information relating to disclosure of infection status in families affected by HIV was collected as part of the ECS. Disclosure of both the child's and parent's infection status was rare and found to be associated with child's age in both cases (44)
In order to evaluate the relationship between viral load and MTCT we studied 282 mother-infant pair enrolled in the ECS. Results showed that high viral load is associated with increased transmission, however the association is stronger in children born < 37gestational weeks, in those whose mother did not receive antepartum ZDV and in those with a lower CD4 cell count.(38). To assess the association between type and timing of initiation of antiretroviral therapy in pregnancy and duration of pregnancy data from 3920 mother-child pair from the ECS and the Swiss cohort have been studied. The prematurity rate was 17%. 23% of women received ART during pregnancy. Exposure to monotherapy was not associated with prematurity. Women on combination therapy were twice as likely to deliver prematurely as those starting therapy in the third trimester (OR 2.17; 95%CI 1.03-4.58) (41).
In order to have an overview of the treatment of children with HIV infection in Europe, in collaboration with the PENTA network and the Italian Multicentre Study, a questionnaire was submitted to 70 paediatric centres from 13 European countries. Data on a total of 1772 children were reported. Double
therapy is the intial therapy fro about 50% of children. In Europe the use of NNRTI is still limited (30%). (43)

After the results of ACTG 076 and other trial with antiretrovirals, showing a marked decrease of vertical transmission rate by zidovudine, several studies have been planned in order to evaluate the combination of several interventions (mode of delivery, antiviral combination therapy, Nevirapine study etc.) to decrease HIV transmission rate. We are involved in the studies co-ordinated by the Institute Mario Negri in Milan, and MRC HIV clinical trials centre in London.
Following reports from the French Collaborative Study on some cases of Mitochondrial Myopathy in uninfetced children exposed to ARV in pregnancy, We collected information on 1113 uninfected children (278 exposed to ARVs). Information included reports of any clinical symptoms or sign that occurred in the first months of life. Only one child had a CNS syndrome unlikely to be related to mitochondrial dysfunction.
In order to evaluate the significance of a rise of lactic acid in children exposed we performed this assay in 11 children unifected children born in Padova in the last trimester. Six out of eleven children have level of AL above 2.5 mmol/l. Further studies are ongoing to elucidate this abnormalities.

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HTLV-1 PREVALENCE IN HIGH RISK IMMIGRANTS COMING FROM ENDEMIC REGIONS

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Aim: immigrants represent about 2% of the present Italian population. Many of them (more than 50%) came from areas in which HTLV-1 and/or HTLV-2 infections are endemic (Africa, Asia and Central/South America).
We performed a study for establishing the prevalence of HTLV-1 infection among HIV-1 positive and/or at high behavioural risk immigrants coming from HTLV-1 endemic areas, in order to prevent possible HTLV-1 associated pathologies (Adult T-cell leukemia, ATL or Tropical spastic paraparesis/HTLV-associated myelopathy, TSP/HAM) and the spread of the infection through sexual, parenteral or perinatal routes.
Patients and methods: a consecutive series of 82 HIV-1 infected or at risk individuals was screened by ELISA for the detection of anti-HTLV-1/2 antibodies. Confirmation and HTLV-1/HTLV-2 discrimination were obtained by means of Western blot and PCR. Phylogenetic analysis was performed on a sequence of 658 bp in length of the provirus LTR.
Results: among 82 subjects, 50% were born in Central/South America, 30% in Africa and about 3% in Asia. The remaining subjects came from European countries.
Six (7.3%) individuals showed serum HTLV-1/2 antibodies. WB and PCR showed HTLV-1 infection in 5 (6.1%) and HTLV-2 infection in 1 (1.2%) individual. Subjects born in Central/South America showed the highest prevalence of HTLV-1 (4/38, 10.5%) and HTLV-2 (1/38, 2.6 %), and all of them were transsexual sex workers. One HTLV-1 patient had TSP/HAM, while the HTLV-1 infected patient born in Africa was affected by ATL.
Phylogenetic analysis of the LTR sequences showed that all the isolates were of HTLV-1a (Cosmopolitan) genotype, but American individuals carried the subtype A (Transcontinental), while African patient had the subtype C.
Conclusions: our data showed a wide diffusion of HTLV-1 infection among individuals at high risk coming from endemic regions, in particular from Central/South America. In all cases the genotype and subtype of the isolates corresponded to that endemic in the origin area. Further studies occur to clarify whether the high prevalence observed in South-American subjects is rather due to a high prevalence of the infection than to the high sexual exposition of the transsexual sex workers.

ACCORDO DI COLLABORAZIONE N° 20 C.18

INCIDENCE OF PERIPHERAL NEUROPATHY (PN) AND HTLV-ASSOCIATED MYELOPATHY (HAM) IN HIV-1/HTLV-2 COINFECTED PATIENTS.

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Aim: to elucidate whether HTLV-2 may affect progression to AIDS or development of specific neurological diseases in HIV-1/HTLV-2 coinfected patients we performed a longitudinal study on a cohort of dually infected patients.
Patients and Methods: 1152 HIV-1 positive patients were screened for HTLV-1/2 antibodies by ELISA test from January '93 to May '97. Reactive samples were confirmed by Western blot. A DNA sequence included in the tax region of HTLV-2 was detected in patients PBMCs by PCR. HIV-1/HTLV-2 coinfected individuals without signs of AIDS were included in a cohort study. Clinical and laboratory controls were performed every three months. For each patient two HIV-1 positive but HTLV-2 negative subjects matched for age, HIV-1 clinical stage, sex gender, CD4 cells count and year of first HIV-1 positive test, were enrolled as controls. Survival analysis was performed by Kaplan-Meier; relative hazard (RH) to develop PN was calculated by Cox regression.
Results: 95 out of 1152 (8.2%) HIV-1 positive patients showed HTLV-2 infection by serological and molecular methods. 33 of them were free of AIDS at the moment of the cohort enrolment and 30 were followed up for a mean period of 41.7 months (range: 9-78 months). 60 matched controls were also followed up for the same period of time. No significant differences were observed between patients and controls as regards CD4 cell decline, progression to AIDS or AIDS mortality. On the contrary, HTLV-2 coinfected individuals had an increased risk to develop peripheral neuropathy (RH 3.3, 95% CI 1.3-8.0 - p=0.009) than controls. Subjects with HIV-1 infection alone that developed PN were treated with antiretroviral drugs when showed neurological symptoms; on the contrary, 4 of 11 HTLV-2 coinfected individuals were naive for antiretroviral therapy when developed PN.
One HIV-1/HTLV-2 dually infected individual showed signs of myelopathy after three years of observation. HTLV-2 antibodies were detected in the patient CSF by Western blot. The patient died for neurological complications about 6 months after the diagnosis of HAM.
Conclusions: our data suggest that HTLV-2 does not influence AIDS progression, and support the hypothesis of a etiologic role of HTLV-2 in the PN affecting HIV-1 positive individuals. The development of HAM in a coinfected patient raises questions about the frequency and the possible therapies of such a condition.

ACCORDO DI COLLABORAZIONE N° 20 C.18
Titolo del progetto: "Sorveglianza della coinfezione e delle patologie indotte da HTLV-2 e HTLV-1 in pazienti con infezione da HIV-1"
Responsabile: dr. Gianguglielmo Zehender

THE NATIONAL ANONYMOUS UNLINKED HIV SEROSURVEY AMONG ITALIAN NEWBORNS

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Objectives: To analyze temporal and geographical trends of HIV infection among parturients in Italy. To monitor trends of HIV infection among newborns. To provide data to monitor HIV vertical transmission rates at population level.
Methods Anonymous unlinked testing for the presence of anti-HIV antibodies of neonatal blood collected for routine screening of metabolic diseases. Samples are collected in each of Italy's 20 Regions during the last three months of the year.
Results: : Analysis of 1998 data show an interruption of the decreasing trend in HIV prevalence recorded between 1994 and 1997 (table). Moreover 1998 data confirm, significant differences in prevalence among different Regions and, in each Region, between metropolitan and non-metropolitan areas.

Year	Samples tested	HIV+ samples	HIV prevalence ‰	Estimated n. of HIV+ births
1990	97.658	121	1,18	670
1991	143.280	143	1,00	563
1992	152.972	130	0,84	474
1993	161.812	156	0,97	532
1994	149.600	153	1,03	548
1995	150.855	131	0,87	458
1996	100.426	74	0,74	391
1997	97.080	57	0,58	311
1998	**95550**	**81**	**0,85**	**409**

A method for estimating vertical transmission rates was validated using data for those born in 1990-94, i.e. before antiretroviral prophylaxis of vertical transmission came into use.

Conclusion: : There is an apparent reversal of the decreasing time trend in HIV prevalence among women giving birth in Italy that needs to be confirmed. These data can be useful to monitor time trends in vertical transmission rate and, indirectly, the impact of interventions aimed at reducing vertical transmission rates from 1995 onward.

Grant n. 20C/1.3

RATE OF EXPOSURE AND RISK OF INFECTION FROM BLOODBORNE PATHOGENS IN HOSPITAL WORKERS

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To analyze the rate of exposure and the risk of infection with bloodborne pathogens from all sources and, specifically, from HIV-infected sources among hospital workers, we analyzed occupational exposure data collected over a 5-year period (1994-1998) in 18 Italian acute-care, urban hospitals including infectious disease units participating in the multicenter prospective study on occupational risk of HIV infection (SIROH). Rates of exposure by job category and work area were calculated using occupational exposures as numerator, and full-time equivalents (FTE) as denominator. The relative frequencies, trends, and characteristics of percutaneous exposures were assessed according to the serostatus of the source patient for HIV and, as a control, HCV. We used the number of prevalent AIDS cases in each hospital's region as a denominator to calculate rates of HIV exposure per 100 prevalent AIDS cases. During the study period, a total of 10,988 percutaneous and 3,361 mucocutaneous exposures were reported. The highest rate of percutaneous exposure/100 FTE was observed among general surgery (11%) and general medicine (10.6%) nurses, the lowest among infectious diseases (1.1%) and laboratory (1%) physicians. The highest rate of mucocutaneous exposure was observed among midwives (5.3%) and dialysis nurses (4.7%), the lowest among pathologists (0%). Inadequate sharps disposal and the prevalence of sharps in the working unit influence the risk to housekeepers. The highest combined HIV exposure rates were observed among nurses (7.8%) and physicians (1.9%) working in infectious disease units. The highest rates of high-risk percutaneous exposures per 100 FTE were again observed in nurses regardless of work area, but this risk was higher in medical areas than in surgery (OR 2.14, 95% C.I. 1.87-2.45; $p< 0.0001$). Frequency and rate per 100 prevalent AIDS cases of HIV exposures decreased by 40% (from 4.3% to 2.6%, and from 1.0% to 0.6%, respectively; $p<0.001$), mainly those related to the insertion or manipulation of peripheral vascular access devices (from 7.2% to 4.8%; $p=0.05$). Exposure risk is related to job tasks as well as to the type and complexity of care provided in different areas, while HIV exposure risk mainly relates to the prevalence of HIV-infected patients in a specific area. The number of accident-prone procedures, especially those involving the use of hollow-bore needles, performed by job category influence the rate of exposure at high risk of infection. HAART's benefits have changed the complexity of care required and, therefore, the number and type of procedures performed on HIV patients that place the HCW at risk of injury. The observed reduction in HIV exposures, particularly those at high risk, combined with standard precautions, new safety devices, the low infectivity of patients receiving HAART, and post exposure prophylaxis should drastically reduce the risk of acquiring HIV in the health care setting.

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PREVALENCE TREND AND CORRELATES OF HHV-8 INFECTION AMONG HIV INFECTED INDIVIDUALS.

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To assess the circulation of HHV-8 infection over the years, two seroprevalence surveys were conducted, testing sera from HIV infected persons recruited 10 years apart (206 individuals in 1986-88 period and 177 individuals in 1998 year). For all patients, antibodies to HCV, HBV and HHV-8 lytic antigens were evaluated. HHV-8 seroprevalence was higher among individuals recruited in 1998 (31.6%) compared to those visited in the late 80s (14.6%), with a 3-fold increase of risk of HHV-8 infection.

HBV infection was significantly associated with HHV-8 infection (OR=2.2, 95%CI: 1.3-3.6), whereas HCV infected individuals had a lower probability of having HHV-8 antibodies (OR=0.3, 95%CI: 0.2-0.6). After controlling for gender, HCV seropositivity and age, men-to-men sex, time of recruitment, and exposure to HBV, were independently associated with HHV-8 infection.

HHV-8 seroprevalence appears to be increased in recent years among this population group suggesting an increased circulation of the virus among HIV infected individuals.

Progetto 1; COLLABORAZIONE N°: 20C/1.6.

INCROCIO SISTEMATICO DEI DATI DEL SISTEMA DI SORVEGLIANZA NAZIONALE PER L'AIDS E DEI REGISTRI TUMORI ITALIANI.

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L'incrocio dei dati dei Registri Tumori (RT) con quelli del Registro nazionale AIDS (RAIDS) è stato, e rimane, di grande importanza per studiare, a livello di popolazione, le caratteristiche dei tumori nei soggetti con infezione da HIV consentendo confronti con la popolazione generale.

I risultati più importanti ottenuti nel corso del 2000, per mezzo dell'incrocio tra i casi notificati al RAIDS ed i casi di tumore ottenuti dai 13 RT di popolazione attivi in Italia nel periodo 1985-1994, hanno riguardato la quantificazione dell'eccesso di rischio di linfoma nei soggetti con AIDS . E' stata valutata la frazione di linfomi non-Hodgkin (LNH) attribuibile all'HIV/AIDS e si è stimato, tramite l'uso di standardized incidence ratios (SIR), il rischio di sviluppare un LNH nei soggetti con AIDS rispetto ai non infetti. Sono, inoltre, state effettuate le ricodifiche e le analisi necessarie per il confronto tra gli istotipi, le localizzazioni e la prognosi dei LNH nelle persone con AIDS rispetto alla popolazione generale.

Nei soggetti con AIDS di età inferiore ai 50 anni, sono stati identificati 136 LNH, pari all'8% del totale dei LNH in quella fascia di età in Italia (1617 casi). Tale percentuale è risultata variare notevolmente nelle diverse aree coperte dai RT, da circa il 3% a Trieste e Ragusa a oltre il 12% a Genova, Varese, Romagna e Macerata. Si è mostrato un eccesso di LNH particolarmente elevato (SIR=394) in prossimità della diagnosi di AIDS (da 1 anno prima a 2 mesi dopo) che si riduce a 170 nel periodo successivo (da 3 mesi ad 3 anni e mezzo dopo l'AIDS). I SIR sembravano più elevati nelle femmine (428), rispetto ai maschi (280), ma praticamente uguali nelle principali categorie di trasmissione. Nei soggetti con HIV/AIDS, è stata confermata l'elevata proporzione di LNH di alto grado ed a localizzazione cerebrale rispetto alla popolazione generale. Si è infine mostrato come, nel periodo studiato, il grado istologico del LNH non sia un fattore prognostico significativo nei soggetti con HIV/AIDS che, a 2 anni dalla diagnosi, hanno mostrato una sopravvivenza di meno del 10%.

Il miglioramento della sopravvivenza dei pazienti affetti da infezione da HIV, determinato dalla profilassi e dalla più efficace terapia delle infezioni opportunistiche e dall'HAART, comporterà presumibilmente un cambiamento dell'incidenza e dello spettro dei tumori HIV associati.

Lo sviluppo del progetto prevede, quindi, l'inizio della seconda fase dell'incrocio dei soggetti con periodo di incidenza 1993-1998 presenti sia al RAIDS che in un RT. Tale periodo coincide con quello coperto dal nuovo volume di "Cancer Incidence in Five Continents" cui presumibilmente contribuiranno anche i 5 nuovi RT italiani che sono diventati attivi a meta degli anni '90 (Friuli-Venezia Giulia, Trentino, Umbria, Campania e Sassari).

Questo aggiornamento consentirà:

1. di valutare, a livello di popolazione, l'impatto dell'HAART sui tumori nei soggetti con HIV/AIDS, sulla base di oltre il 25% della popolazione italiana;
2. di confrontare l'incidenza e la presentazione dei casi di carcinoma anale, di carcinoma invasivo della cervice ed altri tumori con sospetto legame con l'HPV nei soggetti con HIV/AIDS con quelli nella popolazione generale. Sarà, inoltre, possibile quantificare il rischio relativo e la frazione attribuibile per questi tumori nei soggetti con AIDS rispetto alla popolazione italiana, anche in relazione alla disponibilità ed all'accesso di tale popolazione agli screening.

L'esecuzione sistematica e prolungata (almeno 5 anni) di questo progetto è resa possibile dalla collaborazione del Centro Operativo AIDS con il Servizio di Epidemiologia del Centro di Riferimento Oncologico di Aviano ed dell'Associazione Italiana dei Registri Tumori.

Lo studio di incrocio sistematico dei dati (linkage) descritto continuerà ad essere sviluppato con programmi informatici appositamente predisposti per rispettare le normative vigenti (privacy) e non svelare, in nessuna fase dello studio, identificativi personali.

The National research program on AIDS

(Extramural research projects)

Project

PATHOLOGY, CLINIC AND THERAPY OF AIDS

Scientific Coordinator: **Stefano VELLA**

Projects financed N° 81

ANALYSIS OF FACTORS ASSOCIATED WITH ADHERENCE IN HIV INFECTED SUBJECTS TREATED FOR THE FIRST TIME WITH PROTEASE INHIBITOR

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BACKGROUND: Inconsistent adherence to HAART regimens may lead to the development of drug resistant mutations resulting in treatment failure. This increases the probability of at risk populations becoming infected with multi–drug resistant HIV, producing a potential public health consequence. Predicting the ability to adhere to treatment is an important component to determine whether or not a patient should start HAART.

OBJECTIVES: The aim of the study is to find simple, self –administered instruments to predict adherence to HAART.

METHOD: Three self-reported questionnaires (SCL-90-R., Health Belief Model, Multidimensional Health Locus of Control) focused on beliefs about HIV illness and psychopathological aspects were administered to protease inhibitors naives patients. Psychopathological and cognitive profiles were related to adherence rates measured with direct and indirect methods.

RESULTS: To date 37 patients have been enrolled. Results obtained with the analysis of 17 subjects who underwent the 12 months follow -up visit showed that : 1) non-adherent patients have significantly higher level of casual control at Multidimensional Health Locus of Control than adherent subjects 2) there is a trend for non-adherent patients to have pathological scores on SCL90-R subscales 3) patient's beliefs about disease and therapy as evaluated by Health Belief Model do not affect adherence

CONCLUSIONS : Multidimensional Locus of Control has been shown to be useful in assessing patient's attitude to HAART treatment.

Accordo di collaborazione N.ro 30C.1

PROVIRAL HIV-DNA DECLINE AFTER THREE YEARS OF THERAPY IN EARLY PHASE OF INFECTION.

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To assess the decay of cellular proviral HIV-DNA and viral load in patients receiving HAART in very early phase of HIV infection. Eight patients were retrospectively selected out of a larger cohort of HIV infected naive individuals treated with HAART in an early phase of infection (CD4 cell counts >500/mm^3). At baseline, 5 patients had HIV-RNA <5000 copies/ml (group 1) and 3 patients had HIV-RNA >5000 copies/ml (group 2). Plasma HIV-RNA, CD4 cell count and percentage, HIV proviral DNA/10^6 CD4 cells with quantitative and qualitative methods were performed every 2 months (mo) over a three years study period.

	T 0	T12 mo	T24 mo	T34 mo
N. Patients Group 1	5	5	5	4
CD4 count (%)	685 (33,3)	960 (40,9)	1196 (42,1)	1066 (45,7)
HIV-RNA copies/ml	2460	<20	22	<20
Proviral DNA Quantitative	481	15	19	10
n.Patients <10 copies HIV-DNA/10^{6}CD4 cells	0	2	2	3
Proviral DNA Qualitative	5	5	5	
N. Patients Group 2	3	3	3	3
CD4 count (%)	781 (29,7)	867 (33,2)	1071 (30,6)	1267 (32,4)
HIV-RNA copies/ml	21987	<20	<20	<20
Proviral DNA Quantitative	641	47	94	123
n.Patients <10 copies HIV-DNA/10^{6}CD4 cells	0	0	1	1
Proviral DNA Qualitative	3	3	3	

Over time 6 patients had plasma viremia <20 copies. Two patients, 1 for each group, reported poor adherence, had a transient viremia (66 and 4619 copies) and their HIV-DNA increased. Proviral HIV-DNA mean decreased in the first 12 months and then remained stable with the lowest levels in patient of group 1. The number of patients with undetectable values grew from 2 to 4. A long term HAART in patients in early phase of infection produced a progressive immune restoration with undetectable levels of HIV-RNA. The treatment does not produce a complete HIV-DNA clearance from PBMC even if the highest reduction was detected in patient with the lowest viral load level at baseline.

Progetto 2; COLLABORAZIONE N°: 30C.2

DISCORDANCE BETWEEN GENOTYPIC AND PHENOTYPIC DRUG RESISTANCE PROFILES IN HIV-1 STRAINS ISOLATED FROM PERIPHERAL MONONUCLEAR CELLS.

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Genotypic and phenotypic assays provide complementary information on the drug resistance of HIV isolates. During HIV infection, in naïve antiretroviral patients, discordance between genotypic and phenotypic drug resistance analyses was detected.
The aim of the study was to analyse the relationship between genotypic and phenotypic drug resistance profile of HIV-1 strains isolated from patients during double analogue nucleoside therapy. HIV resistant strains were isolated from 20 out of 25 patients, 16 (64%) subjects carrying a virus with multiple drug resistance mutations. Discordance between genotypic and phenotypic profile for at least one drug was detected in 16 out of 25 strains. A genotypic resistant pattern with phenotypic sensitive profile was detected in 11 isolates (4 to zidovudine, 2 to lamivudine and 5 to abacavir). On the other hand a genotypic sensitive pattern with phenotypic resistant profile was detected in 14 strains (2 to zidovudine, 1 to lamivudine, 1 to abacavir, 3 to stavudine and 7 to didanosine). After a follow up period of 8 months, an impairment of virological and immunological parameters was detected in subjects with HIV-1 isolate with phenotypic resistant and genotypic sensitive profile.
Predicting resistance phenotype from genotypic data has important limitations. Particularly, during failing therapy with analogue nucleosides, a phenotypic analysis should be performed in spite of an HIV genotypic sensitive pattern.

Progetto 2; COLLABORAZIONE N°: 30C.2

NEUTRALIZING ANTIBODIES AGAINST AUTOLOGOUS HIV-1 ISOLATES IN PATIENTS WITH VIROLOGICAL AND IMMUNOLOGICAL DISCONNECTION DURING ANTIRETROVIRAL TREATMENT

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Antiretroviral treated HIV-1 seropositive individuals can remain clinically stable for a long period of time with an increasing CD4 cell count irrespective of incomplete viral suppression. The aim of the study was to evaluate the role of neutralizing antibodies (NtAb) activity in the etiopathogenesis of the viro-immunological disconnection, defined as an increasing CD4+ cells despite a persistent detectable viral load (>4$\log_{10}$), during the antiretroviral treatment.
Thirty-three patients, treated with two analogue nucleoside reverse transcriptase inhibitors, were evaluated for CD4+ T cell count, plasma HIV-RNA levels, CCR-5 cell surface expression, β-chemokines levels and NtAb activity against autologous contemporaneous HIV-1 isolates.
A HIV NtAb titer of ≥1:25 was detected in 16 out of 33 (48%) patients, 6 (18%) individuals showed a high NtAb level (>1:125). A significant correlation was found between NtAb titers and CD4+ cell counts trends (p=0.001; r=0.546), whereas no correlation was found between NtAb titers and HIV-RNA plasma levels. Five patients, that during antiretroviral treatment showed a viro-immunological disconnection, had a NtAb titer >1:125, statistically higher respect to the NtAb titration of the remaining 28 patients with both virologic and immunologic failure (p=<0.0001). Before starting therapy, a level of >200 CD4+ cell/mm3 was detected in all but one patient with viro-immunological disconnection and in 3 out of 28 patients with virological and immunological failure.
The HIV specific humoral immune response could play a role during antiretroviral treatment to improve immunological function despite an incomplete suppression of HIV viral load.

Progetto 2; COLLABORAZIONE N°: 30C.2

FACTORS INFLUENCING CEREBROSPINAL FLUID VIROLOGICAL RESPONSE TO ANTIRETROVIRAL DRUGS IN ADVANCED HIV-1 INFECTED PATIENTS

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Objectives. In order to determine the role of virological response in the CNS compartment during antiretroviral treatment, the effectiveness of HAART in treating cerebrospinal fluid (CSF) HIV-1 infection and factors related to virological response in advanced neurologic patients were assessed.
Methods. Consecutive HIV-1 infected patients eligible for lumbar puncture assessment for diagnostic investigations were prospectively enrolled. Paired CSF and plasma samples were collected prior to initiation or changing highly active antiretroviral therapy (HAART). Variables associated with undetectable HIV-1 RNA level in CSF and different compartimentalization of infection were determined in all patients at baseline. In a patients subgroup a longitudinal observation after starting or changing HAART was performed. HIV-1 RNA concentrations were assessed by Nuclisense HIV-1 QT assay in plasma and CSF at baseline and after follow-up. Factors associated with a probability of undetectable HIV-1 RNA measurement at baseline were assessed by Fisher's exact test and multiple logistic regression. Simple linear regression analysis and Student t-test were employed to investigate correlation between CSF and plasma HIV-1 RNA levels according with several variables at baseline and to analyzing factor related to changes in CSF and plasma concentrations after therapy.
Results: Seventy-five patients were enrolled for cross-sectional analysis, 55 (73%) affected by neurological disease, with a median CD4 count of 131 (IQR 47-317) cells/µl. Twenty-eight (37%) of them were naive for antiretrovirals at time of CSF collection. The median plasma and CSF HIV-1 RNA was 5.0 (IQR 4.2-5.8) and 3.5 (IQR 1.8-4.7) $\log_{10}$ copies/ml respectively. Thirty-nine (52%) patients displayed a difference between plasma and CSF HIV-1 RNA levels of <1.5 $\log_{10}$ copies/ml, as a sign of local viral replication. A significant correlation between plasma and CSF levels at baseline was observed in experienced (r=0.483; P=0.0005) but not in naive paired samples (r=0.262; P=0.17). Neurologic picture (OR 0.10;95%CI 0.02-0.53), $\log_{10}$ copies of plasma viremia (OR 0.32; 0.17-0.59) and indinavir exposure before lumbar puncture (OR 6.62; 1.44-30.37) resulted independently associated to a baseline CSF HIV-1 RNA level <80 copies/ml at multivariate logistic regression. The longitudinal study was performed on 29 patients, and a significant reduction of CSF HIV-1 RNA between baseline and after a mean of 12 weeks of follow-up was observed (mean 1.07 $\log_{10}$ copies/ml; 95%CI 0.42-1.71; P=0.002). Plasma HIV-1 RNA change (r=0.636; P=0.0002), CSF HIV-1 RNA $\log_{10}$ copies at baseline (r=-0.720; P <0.0001), overall months of antiretroviral treatment (r=0.643; P=0.0001) and magnitude of difference between plasma and CSF HIV-1 RNA levels (r=0.509; P=0.004) were all correlated to reduction of CSF HIV-1 RNA change during treatment. A significant difference in magnitude of CSF HIV-1 RNA reduction was observed according with naive status (-2.25 vs -0.23 $\log_{10}$ copies; P=0.001) and with use of >3 drugs penetrating blood-brain barrier (BBB) after starting or changing therapy (-1.73 vs –0.25 $\log_{10}$ copies; P=0.01), but only naive status at baseline was associated to an increased probability of a CSF reduction more than 1.0 $\log_{10}$ during therapy at multivariate analysis (OR 32.5; 95%CI 3.12-337.8).
Conclusions: Variable response to antiretroviral therapy was observed in CSF, reflecting a different compartimentalization of CSF infection during treatment. Naive status and use of CNS penetrating drugs was required for effective viral reduction. Negative interaction with duration of antiretroviral treatment suggest long-term selection of drug-resistant CSF HIV-1 strains. -

Accordo di collaborazione N. 30C/3.

CLINICAL IMPACT OF GENOTYPING RESISTANCE TEST (GRT) IN HIV-INFECTED PATIENTS WITH VIROLOGICAL FAILURE TO HAART: IMPLICATIONS FOR AN ADVANCED STRATEGY OF SALVAGE THERAPY.

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Objectives. In order to implement strategies at antiretroviral failure, observational studies on a cohort of HAART-failed patients were performed. Primary objectives of retrospective studies were the role of multinucleoside-analogue resistance in failure, discordant viro-immunologic response and resistance-mutations as predictive factors. In a prospective approach the role of GRT-guided therapy in clinical practice was assessed.
Methods. Observational design for retrospective and longitudinal studies. Plasma HIV-1 RNA were prospectively analyzed by direct sequencing of RT and PR using the HIV-1 Genotyping kit (Perkin-Elmer Biosystems) and also by True Gene Assay (Visible Genetics) in a subgroup of patients. Statistical analysis was performed by independent and paired t-test for continuous and Fisher's exact test for discrete variables and results controlled by a logistic regression model. In longitudinal analysis time to first HIV-1 RNA undetectable (<500 c/ml) was estimated by Kaplan-Meier curves and predictive factors adjusted by Cox analysis.
Results. A) Multi-drug resistance. 216 pts were enrolled [F/M 67/149; median age 38 yrs; 25% IVDUs, 35% heterosexuals; mean CD4 count 301/μl, HIV-1 RNA 4.81 $\log_{10}$/ml]. Q151M was detected in 11/216 (5.1%) pts and not associated with gender, age, HIV route, previous AIDS, HIV-1 RNA and CD4 count before therapy and at genotype, duration of ART, number of previous failure. A significant association was detected between Q151M and time from last virologic failure (any HIV-1 RNA rebound or never undetectable) to genotype (OR 1.61 for each 6-month;95%CI 1.21-2.14). We failed to find any correlation between Q151M and ZDV, ddI, d4T, 3TC or NNRTIs use, even though it was associated with longer time of ddI treatment (P <0.001) with a 1.12-fold risk (95%CI 1.05-1.20; P<0.001) increase for each month of ddI exposure. At logistic regression period of ddI treatment (OR 1.94 for each 6-month;95%CI 1.31-2.89) and of NRTIs exposure during last failure (OR 1.57;95%CI 1.10-2.27) were associated to an increased risk of Q151M genotype. Q151M was directly associated with multinucleoside analogue-resistance mutation group [V75I (P<0.001); F77L (P=0.001); F116Y (P<0.001); A62V (P=0.04)] and inversely associated with AZT-related mutations [M41L (P=0.01); L210W (P<0.01); T215Y (P=0.001). B) Resistance-conferring mutations and discordant viroimmunologic response to HAART. In 315 retrospectively observed pts [median age 38 yrs; male gender 243 (69%); IVDU 95 (27), MSM 84 (24%), heterosexuals 120 (34%); previous AIDS 139 (40%); mean CD4 and HIV-1 RNA at genotyping 300/μl and 4.63 $\log_{10}$ c/ml] an analysis of factors predictive of increasing CD4 count despite persistant virological failure was performed. A CD4 count increase of >100 cell/μl from baseline to genotyping regardless of virological failure was observed in 159 (50%) patients. At logistic regression, in patients with >6 months of virological failure, CD4 at baseline (OR 0.80;95%CI 0.66-0.96; P<0.05), months on HAART (OR 1.0;95%CI 1.0-1.07; P<0.05) and rtY181C (OR 0.28;95%CI 0.12-0.64; P<0.01) were predictive of achieving of failing an immunorecovery regardless to virological failure. Patients with rtY181C mutants had higher mean values of log10 HIV-1 RNA

(4.86 vs 4.59; P<0.01) and lower values of CD4 at genotying (231 vs 313 cells/μl; P<0.01). C) Longitudinal evaluation of GRT-guided salvage therapy: 175 enrolled patients [median age 39 yrs; F/M 50/125; IVDU 26%; heterosex 36%) were observed for a median of 24 weeks (IQR 12-36]. Average CD4 and HIV-1 RNA at genotyping were 303/μl and 4.8 log_{10}/ml respectively. Pts had a median of 2.0 failure (>1 failure 63%) and were treated for a median of 29 months of HAART (IQR 19-36). Pts were failing on regimens containing AZT (20%), ddI (32%), d4T (70%), 3TC (57%), NVP (26%), EFV (6%), IDV (31%), SQV (15%), NFV (38%). A median of 5.0 mutations in RT as well as in protease was observed. After 12 weeks mean change of HIV-1 RNA and CD4 count were -1.0 (95%CI 0.79-1.20) and +55/μl, respectively. The 6-month probability of achieving HIV-1 RNA <500 c/ml after GRT-guided therapy was 0.32. Cox analysis identified as predictive of increasing or decreasing probability of HIV-1 RNA <500 c/ml female gender (2.67;95%CI 1.57-4.52), HIV-1 RNA at genotyping (0.44 for each log_{10};95% 0.30-0.66), rtL210W (0.33;95%CI 0.16-0.68), prM36I (0.48;95%CI 0.24-0.98), prV82A (0.40;95%CI 0.19-0.84), ABV (2.59;95%CI 1.50-4.45) and EFV (2.00;95%CI 1.15-3.49) use after GRT.
Conclusions. Prolonged exposure to NRTIs in presence of detectable viremia during HAART failure, especially if ddI was included in the scheme, may be associated with the emergence of multinucleoside resistant genotype. Early genotype-guided change of drugs is advisable in virological failure in order to prevent high level resistance. HAART-failed patients harboring NNRTI-associated Y181C mutation may have higher risk of CD4 depletion without discordant viroimmunologic response, with relevant implications for clinical management. A relationship of discordant response with replication fitness of mutant strains should be considered. In patients with failure to HAART, GRT-guided therapy seems a reliable effective strategy. HIV-1 RNA level at genotyping, pattern of mutation and addition of new drugs may increase response rates.

Accordo di collaborazione N. 30C/3.

SELF-REPORTED SYMPTOMS AND MEDICATION SIDE EFFECTS INFLUENCE ADHERENCE TO HAART IN PERSONS WITH HIV INFECTION

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Background: Adherence to highly active antiretroviral therapy (HAART) is crucially important to achieve successful and prolonged viral load suppression. Socio-demographic factors, social support, behavioral characteristics, psychological co-morbidity, self-efficacy for medication taking and convenience of antiretroviral treatment were found to be associated with adherence to HAART in several studies Although the association of symptoms with adherence behavior seems intuitive, only few authors addressed this issue specifically.
Objective: To identify variables predictive of sub-optimal adherence to HAART in an Italian population and, in particular, to assess whether self-reported symptoms or drug-related side effects are related to medication adherence.
Methods: Prospective, multicenter, cohort study within the observational ICoNA cohort. Inclusion criteria: HIV infection, antiretroviral therapy with PI or NNRTI for at least 1 month; exclusion criteria: hospitalization at enrollment, ADC >2 Price stage, inability to fill the questionnaire. Self-administered 16-item anonymous questionnaire, previously validated on HIV+ Italian patients. At the same time CD4 cell count, HIV-RNA, viral genotype and PI/NNRTI predose plasma levels were planned. Questionnaire and blood sample are repeated every 6 months. Socio-demographic, epidemiologic and clinical characteristics were collected.
Results: From May 1999 to March 2000, 385 patients (358 eligible) have been enrolled in 23 centers: 28% females; 32% IDU, 23% MSM, 35% heterosexual; 20% CDC group C; mean CD4 577/μl; mean HIV-RNA 4.49 $\log_{10}$/ml; median months on current HAART scheme were 12. The overall item missing rate was very low, ranging from 0.8 to 4.7%. A total of 79 participants (22%) reported having missed at least one dose of ART in the last three days. Overall 132 persons (37%) could not correctly recall the name, color and timing of the drugs they were currently on and 88 (25%) reported sometimes running out of pills between clinic visits. Patients reporting sub-optimal adherence in the last three days were less likely to have a favorable virologic outcome (HIV RNA <500 copies/ml) at the time of completing the questionnaire (OR 0.51; 95%CI 0.31-0.85; P=0.01). In univariate analysis non-adherent persons were more likely to be younger (P=0.001), to report active drug use (P=0.03) and to be unemployed (P<0.001). Compared with persons who reported a good adherence, those who reported to have missed at least one dose in the last three days were more likely to not recall name, color and timing of the drugs (P=0.01), to run out of pills between clinic visits (P=0.001) and to be receiving lamivudine (P=0.02) or nelfinavir (P=0.03) containing HAART regimens. Mean Mental Health status score was significantly lower among participants who reported to have missed at least one dose in the last three days when compared to those to were adherent (55.9$\pm$20.8 versus 63.8$\pm$19.9; P=0.002). The most frequently recorded reasons for missing drugs were having to take too many pills (52%), being worried about future side effects (42%) and being away from home (36%). Frequency of the different symptoms or medication side effects in non-adherent participants ranged from 3.6 to 30% and was highest for anxiety (30%), fatigue (24%), abnormal fat accumulation (19%), diarrhoea (17%), nausea (16%), anorexia (11%), insomnia (10%). Persons with sub-optimal adherence in the last three days also had a

significantly higher mean overall symptom score (12.3±9.2 vs 8.1±6.6; P<0.001) and mean medication side effect score (2.9±2.7 vs 1.9±1.9; P<0.001) when compared to adherent partecipants. According to multivariate analysis, younger age (OR 2.51, 95% CI 1.34-4.69; P<0.001), unemployment (OR 3.28, 95%CI 1.73-6.23; P<0.001), incorrectly recalling name, color and timing of drugs (OR 2.23, 95%CI 1.21-4.12; P=0.01), running out pills between visits (OR 2.48, 95%CI 1.31-4.71; P=0.005), being too busy (OR 2.48, 95%CI 1.26-4.88; P=0.008) and receiving a lamivudine containing HAART regimen (OR 2.62, 95%CI 1.25-5.49; P=0.01) were all independently associated with a poorer adherence behavior in the last three days. Complaining about nausea (OR 4.31, 95%CI 1.55-12.0; P=0.005) and anxiety (OR 2.35, 95%CI 1.15-4.80; P=0.02) were also significantly associated with non-adherence.
Conclusions: Patient-reported symptoms and medication side effects, beside patient characteristics, medication-related variables, reasons for non-adherence, are significantly associated with non-adherence to HAART. Improved efforts to assess and to control HIV- and HAART-related symptoms by the caring health-care staff are necessary to optimize adherence to HAART and treatment outcome. Also in the design of multi-faceted intervention strategies to improve HAART adherence, symptom control could represent an easy and relatively inexpensive target within the health care provider-patient setting.

Accordo di Collaborazione N. 30C/4

TIME ON ANTIRETROVIRAL THERAPY, EDUCATION LEVEL, EMPLOYMENT, AND PRESENCE OF A SOCIAL CARE WORKER WERE ASSOCIATED WITH CONCORDANCE BETWEEN PHYSICIANS' ESTIMATE AND PATIENTS SELF-REPORTED ADHERENCE.

Murri R, Ammassari A, Pezzotti P, Trotta MP, Abrescia N, Alberici F, Antonucci G, Caramello P, Carosi G, Delia S, Esposito R, Izzo MC, Manconi PE, Mazzotta F, Narciso P, Noto P, Ortona L, Pauluzzi S, Piazza M, Pizzigallo E, Ravasio L, Scalzini A, Scasso A, Soscia F, Tirelli U, Vullo V, Rezza G, Ippolito G, d'Arminio Monforte A, and Antinori A for the AdICONA Study Group.

Background: Adherence to highly active antiretroviral therapy (HAART) is crucially important to achieve successful and prolonged viral load suppression. Measurement of adherence is often difficult since there is no gold standard. In clinical practice adherence is often estimated by physicians.
Objective: To evaluate the predictive value of adherence of physician's estimate; to assess variables associated with agreement on adherence estimate between patient and physician.
Methods: Prospective, multicenter, cohort study within the observational ICoNA cohort. Inclusion criteria: HIV infection, antiretroviral therapy with PI or NNRTI for at least 1 month; exclusion criteria: hospitalization at enrollment, ADC >2 Price stage, inability to fill the questionnaire. Self-administered 16-item anonymous questionnaire, previously validated on HIV+ Italian patients. Physicians were asked to evaluate patient adherence based on a 3-point scale (optimal, sub-optimal, poor). At the same time CD4 cell count, HIV-RNA, viral genotype and PI/NNRTI predose plasma levels were planned. Questionnaire and blood sample are repeated every 6 months. Socio-demographic, epidemiologic and clinical characteristics were collected.
Outcome of the analysis was considered the patient's estimate of adherence ("not have forgotten at least one dose of antiretrovirals during the last week"). Physician's estimate was grouped into optimal adherence versus sup-optimal or poor adherence. Kappa statistic as well as sensitivity, specifity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. A logistic regression analysis was done considering concordance on both adherence and non-adherence between patient and physician as the dependent variable.
Results: From May 1999 to March 2000, 385 patients (358 eligible) have been enrolled in 23 centers: 28% females; 32% IDU, 23% MSM, 35% heterosexual; 20% CDC group C; mean CD4 577/μl; mean HIV-RNA 4.49 $\log_{10}$/ml; median months on current HAART scheme were 12. The overall item missing rate was very low, ranging from 0.8 to 4.7%. A total of 114 participants (32%) reported having missed at least one dose of ART in the last week. For 320 patients a related physician's estimate was available.
One-hundred forty-three out of 320 physicians (45%) agreed on patient's self-reported adherence and 66/320 (21%) physicians agreed on patient's self-reported non-adherence. Kappa statistic resulted 0.27. Sensitivity, specificity, PPV, and NPV of physician's estimate on patient's adherence were 64.7%, 66.6%, 81.2%, and 45.8%, respectively. Of the patients for whom the physicians made the wrong prediction 33 were predicted to be compliant when in fact they were not, and 78 were predicted to be nonadherent when they actually reported to be adherent (McNemar's test: $p<0.0001$).
On bivariate analysis, variables associated with concordance between patient and physician were not having an AIDS diagnosis, time of previous antiretroviral therapy, presence of a social care worker or a psychologist in the Center, high education level, and employment, belonging to a Universitary or Northern-Italy Clinical Center.
On multivariate analysis, time of previous antiretroviral therapy (OR 1.56; 95% CI 1.04-2.34; $p=0.03$), education less than 8 years of formal schooling (OR 0.43; 95% CI 0.25-0.72; $p=0.001$), unemployment (OR 0.45; 95% CI 0.25-0.82; $p=0.009$), and presence of a social care worker in the Clinical Center (OR 2.02; 95% CI 1.18-3.45; $p=0.01$) were independently and significantly correlated to concordance between patient and physician even when controlled for clinical center.
Conclusions: Overall, physicians's agreement on patients' self-reported adherence is poor. Physicians tend to better estimate adherence while predictive value of non-adherence is very low. Physicians should be cautious in assessing adherence based only on their own evaluation. Longer time on antiretroviral therapy, high education level, employment, and presence of a social care worker in the Clinical Center were associated with concordance between physicians and patients on adherence level.

Accordo di Collaborazione N. 30C/4

CELLULAR RESISTANCE TO ANTI-HIV DRUGS.

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Long-term treatment of HIV-1 infected patients with antiretroviral agents may result in failure of therapy, due to the emergence of resistant virus mutants with decreased susceptibility to therapeutic agents. Genotypic changes correlate well with phenotypic susceptibilities; however the loss of sensitivity to a drug is sometimes difficult to explain exclusively by genetic changes. Then, recent studies arise the question whether cellular factors, in addition to viral mutations, may account for the failure of antiretroviral therapy. In this framework, a project aimed to gain new insights into the cellular factors involved in the resistance to nucleoside analogues (NA) and to protease inhibitor (PI) is being performed by our group.
Here we described some results we have obtained in the last year. Specifically two main issues have been addressed:

1. The cellular resistance to d4T and 3TC;
2. The influence of P-glycoprotein (Pgp) expression on antiviral activity of PI.

1. In order to study the cellular resistance to nucleoside analogues, cellular lines resistant to antiviral activity of 3TC (CEM3TC) and d4T (CEMd4T) were obtained. Kinetics experiments performed in such cellular lines to determine the intracellular accumulation of NA indicate that, at each point tested (1, 2, 4 hours) there is a reduction ($p<0.005$) of the drug concentration inside the cells. In order to identify the mechanism/s underlying this phenomenon, enzymatic assays were performed to evaluate the activity of thymidine kinase (TK) and cytidine kinase (CK) in CEMd4T and in CEM3TC, respectively. The results indicate that in the d4T resistant line the TK activity is significantly reduced (65 %) compared to the TK activity displayed by parental line (CEM). On the contrary, in CEM3TC cells, the enzymatic activity of CK is similar to control cells, suggesting that mechanisms different from the enzymatic defect may account for the acquisition of cellular resistance. Indeed, efflux study performed on CEM3TC, indicated that these cells have an increased capability to expel the drug compared to CEM. In order to evaluate whether an ABC protein can be involved in the efflux of 3TC, the expression of Pgp, MRP1 and MRP4 in CEM and CEM3TC has been evaluated. Western blot analysis indicated an overexpression of immunoreactive MRP4 from cells resistant to 3TC. On the contrary the level of MRP1 immunoreactivity and the levels of mRNA for Pgp were unchanged in the resistant line. These data suggest that the phenomenon of resistance to 3TC could be due to the everexpression of MRP4. Further study are in progress to verify whether the overexpression of MRP4 in cells confers a resistant phenotype.
2. Several studies have demonstrated that PI are substrates of the multidrug transporter Pgp. Since this glycoprotein plays a role in limiting the efficacy of many drugs, the effect of Pgp on antiviral activity of PI was analyzed. To this regard the cellular line CEMVBL100, expressing high levels of Pgp was used. The data obtained indicate that Saquinavir and Indinavir display a significantly reduced antiviral activity in CEMVBL100 cells compared to parental line. Specifically 5 nM Saquinavir or Indinavir reduced HIV yield in the parental line by 70 and 80 %, respectively. In contrast the same compounds in CEMVBL100 at the same concentration inhibited HIV yield by 40 and 20%. Importantly, both drugs partially

recover the ability to inhibit replication of HIV-1 in the presence of inhibitors of Pgp function, thus indicating that Pgp expression may affect the antiviral activity of PI. Since the expression of Pgp it has also been reported in CD4+ T-lymphocytes, the effectiveness of this compounds on these cells may be significantly reduced. Studies are in progress to verify this hypothesis.

The data reported here indicate that not only viral factors but also cellular factors may contribute to the loss of sensitivity to an antiretroviral drug. The cellular resistance may contribute directly to the failure of antiviral therapy by the generation of subtherapeutic levels of antiviral compound and/or their active forms. Indirectly such subtherapeutic dosage of substance might allow the replication of virus in the presence of the drug permitting a faster emergence and development of a resistant virus population. Again studies are in progress to verify such hypothesis.

Publications in the framework of the project:
O.Turriziani, P. Di Marco, F. Dianzani, G. Antonelli. May the drug transporter P glycoprotein affect the antiviral activity of human immunodeficiency virus type 1 proteinase inhibitors? Antimicrobial Agents and Chemotherapy 44, 473-474; 2000.
O.Turriziani, G. Antonelli, F. Dianzani. Cellular factors involved in the induction of resistance of HIV to antiretroviral agents. International Journal of Antimicrobial Agents. 16, 353-356; 2000.

Accordo collaborazione N. 30C.5

GENOTYPIC RESISTANCE REVERSAL AFTER THERAPY WITHDRAWAL AND DEVELOPMENT OF A GENO-PHENOTYPIC ASSAY TO MEASURE THE PHENOTYPIC DRUG-RESISTANCE

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Little data indicated that multiple drug-treated patients (Pts) may experience the reversal of drug-related mutation after a therapy discontinuation of variable length. To study the rate and effects of genotypic reversal we analysed a group of Pts (i) who experienced 3TC administration and discontinuation and a group of Pts (ii) with virological failure after long treatment history with all class of antiretrovirals (ARV). Further, we developed a geno-phenotypic assay (iii) to measure the in vivo drug susceptibility to ARV that allow to correlate genotypic to phenotypic data in Pts with virological failure.

i) The kinetic of emergence of 3TC-associated 184V mutation is rapid in Pts treated with sub optimal 3TC containing regimens. We studied 11 Pts of whom genotypic results were available before and after 3TC use in combination regimens. The sequence of the RT region was obtained both on HIV-1 RNA and DNA species by direct sequence. When the reversal of the 184V was observed in both RNA and DNA by direct sequence, 10 clones from HIV-1 proviral DNA of each Pt were sequenced to rule out that the reversal could not represent the total viral population. Among the 11 Pts who showed the reversal of 184V in RT sequences from plasma 7 were studied in detail. All had a wild type (WT) genotype at position 184 in HIV-1 RNA, while six out of 7 displayed a 184M in DNA major species as detected by direct sequence. The genetic analysis of 10 proviral clones obtained in Pts with 184 reversal revealed the presence of a Met at position 184. The reversal of 3TC-induced M184V appears to be a frequent event in Pts who discontinue 3TC treatment. HIV-1 archive proviral DNA does not contain 184V variants. These results suggest that the use of 3TC in new regimens after the clearance in HIV-1 archives may have significant benefit.

ii) NRTI, NNRTI and PI mutation reversal was analyzed in 7 Pts, 3 of whom discontinued all ARV while 4 interrupted PI drugs only. For the latter Pts only the PR region was considered. Pt 1 had two therapy interruptions of 6 and 2 months. Pt 2 was analysed in both RNA and DNA. Pt 4, 5, 6 and 7 had the pol genotype at 6,3,16 and 7 months, respectively. All Pts experienced comparable length of treatment with each class of drugs before treatment interruption. The results are shown in the table.

Pts	Codon / Wild type	10 L	20 K	36 M	46 M	54 I	71 A	82 V	84 I	90 L	41 M	67 D	69 T	70 K	74 L	98 A	103 K	181 Y	184 M	190 G	210 L	215 T	219 K
1	On th*	I	-	-	I	L	V	-	V	M	L	N	-	R	-	G	N	C	M/V	A	-	F	Q
	1st Off th*	I	-	-	M/I	I/L	V	-	V	M	L	N	-	K/R	-	G	N	C	-	A	-	F	Q
	On th	I	-	-	I	L	V	-	V	M	L	N	-	R	-	G	N	C	M/V	A	-	F	Q
	2nd Off th	I	-	-	M/I	-	A/V	-	V/I	M	L	D/N	-	K/R	-	A/G	N	Y/C	-	G/A	-	F	Q
2°	On th RNA	-	R	I	-	-	V	A	-	M	L	-	-	-	-	-	-	-	V	-	-	Y	-
	On th DNA	-	K/R	M/I	-	-	A/V	A/V	-	M	L	-	-	-	-	-	-	-	M/V	-	-	Y	-
	Off th RNA	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	Y	-
	Off th DNA	-	K/R	M/I	-	-	A/V	A/V	-	M	L	-	-	-	-	-	-	-	-	-	-	Y	-
3	On th	I	-	-	I	-	T	-	V	M	-	-	-	-	V	-	N	-	-	-	-	Y	-
	Off th	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4°	Off th	-	-	-	-	-	-	-	-	-	L	-	-	-	V	-	N	C	V	-	W	Y	-
5	Off th	I	R	I	-	V	V	A	-	M	L	-	-	-	-	-	-	-	-	-	-	Y	-
6°	Off th	-	-	-	I	V	-	A	-	-	L	N	D	-	-	-	-	-	V	A	W	Y	-
7°	Off th	I	-	-	I	-	V	T	-	M	-	N	-	R	-	-	N	-	V	-	-	-	Q

* On and off therapy; ° Pts who discontinued PI drugs only

WT HIV-1 outcompeted resistant mutants in all Pts although different degree of reversal was present at comparable time-points. Different patterns and kinetics of mutation reversal were observed in individual Pts regardless of the duration of previous therapy and the length of drug wash-out. Moreover, the PR region seems to be more flexible that RT region. No difference in regard of primary or secondary mutations reversal was seen in both PR or RT regions. These findings indicate that an incomplete reversal may take place in some Pts leading to the prompt reappearance of resistant escape mutants after the reintroduction of ARV. This assumption is further supported by the analysis of HIV-1 archive in one Pt. Data obtained in Pt 2 suggest that ARV-related mutation persist in DNA compartment and reappear after the introduction of a new ARV regimens. Further, these findings underline that genotypic results should be supported by the phenotypic analysis of susceptibility of individual Pts to new ARV.

iii) To develop a fast and quantitative recombinant strategy for evaluating the HIV-1 phenotype to PI we designed a nonreplicative HIV-1 molecular vector (named pΔproΔenv) able of expressing exogenous PR-encoding sequences. The PR sequences were amplified from either viral isolates or plasma samples (both from 21 HIV-1 infected subjects, 19 of whom failing different anti-HIV-1 combination treatments) and cloned in the pΔproΔenv backbone. The HIV-1 recombinant phenotype to PI was determined directly after transfection of viral chimeric clones by measuring PR activity and calculating a percent sensitivity index (SI%; the ratio between the results from each clone and those from a PI-sensitive reference strain). The SI% values obtained from the recombinant clones paralleled the IC_{50} results of the HIV-1isolates and indicated different degrees of resistance and cross-resistance to PI compatible with the respective genotype. An inverse correlation between SI% values and the presence of primary mutations for resistance to PI ($p=0.0038$ and $p=0.0414$, for IDV and RTV, respectively) and a difference in SI% between samples harboring increasing number of mutations (IDV, $p=0.022$; RTV, $p=0.0466$) were observed. These data prove the reliability of the novel strategy for a fast (5 days) quantitative evaluation of HIV-1 phenotype to PI.

No. dell'Accordo di Collaborazione: 30C.6

EVALUATION OF TELOMERASE ACTIVITY AND USE OF αVβ3 INTEGRIN ANTAGONISTS IN AIDS-ASSOCIATED KAPOSI'S SARCOMA MANAGEMENT.

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AIDS-associated Kaposi's sarcoma (AIDS-KS) arises as an angioproliferative disease but may evolve into an aggressive cancer, characterized by the proliferation and invasion of spindle cells of endothelial origin (KS cells, KSC). KS progression is triggered by inflammatory and angiogenic cytokines, human herpesvirus 8 and the Tat protein of HIV-1 [reviewed in Barillari G and Ensoli B. Clinical Microbiol. Rev. ASM, in press].
We found that these KS progression factors upregulate in KSC the expression of telomerase, a ribonucleoprotein which allows cells to replicate indefinitively. In addition, we reported on the mechanism of action of HIV-1 Tat, the angiogenic basic fibroblast growth factor (bFGF) and the αvβ3 integrin receptor in AIDS-KS pathogenesis. In particular, we found that the highly basic residues of Tat, by competing for heparin-binding sites, retrieve extracellular-bound bFGF into a soluble form which promotes endothelial cell growth and upregulates the expression of αvβ3; this integrin, in turn, binds Tat RGD region and, by this interaction, mediates Tat-promoted locomotion of KSC and activated endothelial cells and provides these vascular cell types with the adhesion signal they require in order to grow [Barillari G et al. Blood 94: 1-11, 1999].
Other research groups have shown that Tat binds and phosphorylates VEGFR-2, the receptor mediating most of vascular endothelial growth factor (VEGF) angiogenic effects [Albini A et al. Nature Med. 2: 1371-1375, 1996; Mitola S et al. J. Virol. 74: 344-353, 2000]. Since also VEGFR-2 activation requires the triggering of αvβ3 [Soldi R et al. EMBO J. 18: 882-892, 1999], antagonists of this integrin could be usefully employed to contrast Tat, bFGF and VEGF angiogenic effects involved in KS development and progression.
In agreement with this hypothesis, here we show that αvβ3 integrin antagonists such as cyclic RGD peptides or anti-αvβ3 monoclonal antibodies specifically block the development of angioproliferative, KS-like lesions induced in mice by the injection of combined Tat and bFGF [Barillari G et al. J. Imm. 163: 123-128, 1999 and submitted]
Altogether, these results suggest that the evaluation of telomerase activity could monitor AIDS-KS clinical evolution and that competitors of Tat-αvβ3 interaction, combined with anti-inflammatory and anti-angiogenic drugs, could be employed as new tools for AIDS-KS therapy.

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CASE-CONTROL STUDY OF INDINAVIR PHARMACOKINETICS IN PATIENTS UNDERGOING CHEMOTHERAPY FOR AIDS RELATED NEOPLASIA.

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Background: The study had the purpose of characterizing indinavir (IDV) pharmacokinetics in patients with AIDS undergoing chemotherapy for non-Hodgkin lymphoma HIV-related.
Methods: Seven patients were enrolled at the Oncology Center of Aviano. They had been on treatment with IDV (800 mg q8h) in combination with two nucleoside reverse transcriptase inhibitors (NRTIs) for at least 3 months They were eligible for CHOP chemotherapy (CHOP protocol: four cycles of chemotherapy alternated by three weeks of interval; adriamycin 75 mg/m2, cyclophosphamide 1200 mg/m2, vincristin 1.4 mg/m2). Demographic parameters were: median age (range) 40 (34-59) years, weight 68.4 (48-87) kg, gender:7 male. Baseline values of CD4+ cells and viral load were 296 (72-760) cells/µl and 3.57 (1.69-5.08) log copies/ml, respectively.
Blood samples for IDV PK were drawn in two occasions for each patients: PI without CHOP (controls) before the beginning of the first cycle of chemotherapy or, at least two weeks after a cycle; PI+ CHOP the same day of CHOP administration.
Indinavir plasma concentrations were determined by HPLC at time 0 (predose), 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours following a morning dose; the calibration curve ranged between 0.025-20 mg/L.
The following parameters were compared between study days: AUC, Cmax and Tmax, CO (concentration at time 0) and C8 (concentration at 8 hr following administration) (two tail paired t test).
Results: Results were the followings, shown as mean value (SD):

	IP without CHOP	IP + CHOP	p
AUC (mgxh/L)	14.9 (9.5)	20.5 (9.0)	0.03
Cmax (mg/L)	6.94 (4.63)	9.95 (3.89)	NS
Tmax (h)	1.3 (0.9)	1.0 (0.5)	NS
C0 (mg/L)	0.25 (0.28)	0.37 (0.20)	NS
C8 (mg/L)	0.39 (0.45)	0.17 (0.15)	NS

Conclusions: We observed a statistically significant difference between the AUC of IDV+CHOP compared to the AUC of IDV without CHOP.
The AUC of IDV+CHOP was comparable to the AUC reported in literature, while the IDV AUC without CHOP was lower (35%).
Also, three patients had IDV C8 < 0.1 mg/L (IC50, wild type) without CHOP while none of the patients had IDV C8 < 0.1 mg/L with CHOP.
Based on the results of our small study, a modification of IDV dose during the co-administration of CHOP chemotherapy is not required. The observation of a lower–than-average AUC of IDV in this patient population with non-Hodgkin lymphoma (IDV without CHOP) needs further verification.

N°. dell'Accordo di Collaborazione : 30 C8, 1999

PREDICTING FACTORS OF VIROLOGIC RESPONSE TO NELFINAVIR IN HIV+ CHILDREN.

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Background: We studied a number of factors, including nelfinavir (NFV) plasma trough concentration, in their capability of predicting virogic response to AVR treatment.
Methods: Ten HIV+ children receiving NFV (mean dose 24.4 mg/kg TID, range 17.4-31.9) in combination with two nucleoside reverse transcriptase inhibitors (NRTIs) were studied. Demographic parameters were: mean age (range) 7.3 (2.2-13.9) years, weight 23.6 (9.26-43.0) kg, gender: 5 female and 5 male. Baseline values of %CD4+ cells and viral load were 21.0 (3.7-40.7) and 5.05 log copies/ml (2.96-6.15), respectively. Seven children were PI naive, the others were ritonavir experienced; eight patients, modified both NRTIs at initiation of NFV treatment.
Peak (3 hours after administration) and trough (predose) plasma concentrations of NFV were determined, by HPLC, in one occasion at steady-state after a morning dose. The calibration curve ranged between 0.05-15 mg/L. Immunologic and virologic response to treatment was quantified as the increase of baseline value of %CD4+ cells observed at 3 and 6 months of therapy and the difference between the baseline value of viral load and the value observed at 3 and 6 months of therapy (ΔVL3 and ΔVL6), respectively. The relationship between the parameters of response to therapy and demographic parameters (age, weight, baseline viral load value, trough and peak NFV concentrations, PI experience) was studied by linear regression and Mann Whitney U test. Also, patients were subdivided in two groups depending on the trough concentration (Cmin) using 1 mg/L as a the cut-off value.
Results: The correlation between ΔVL3 NFV Cmin was statistically significant (p=0.01). Also, ΔVL3 observed in patients (n=6) with Cmin >1mg/L was higher than in patients (n=4) with Cmin <1mg/L (2.20 vs 0.83 log copies/ml, p=0.004).
NFV Cmin mean value was 1.34 (0.13-2.53) mg/L.
Discussion: Nelfinavir trough concentration was a significant factor predicting response to therapy at 3 months of follow-up. The adjustment of NFV dosage regimen based on NFV plasma concentrations may be of benefit in the HIV+ pediatric population.

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MITOCHONDRIAL AND CYTOSOLIC DEOXYNUCLEOTIDASES: IMPLICATIONS FOR AIDS TREATMENT.

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Deoxynucleotidases are catabolic enzymes that remove the phosphate group from deoxynucleoside monophosphates releasing membrane - permeable deoxynucleosides that can diffuse from the cell along the concentration gradient across the plasma membrane. This reaction reverses the kinase-catalysed phosphorylation that traps deoxynucleosides inside the cell feeding the pool of DNA precursors (dNTPs). The two classes of enzymes create futile (substrate) cycles that regulate membrane traffic of deoxynucleosides and modulate the sizes of dNTP pools. The kinase reaction is the anabolic arm of the cycle and is the first step in the activation of antiviral nucleoside analogs used in AIDS treatment. The nucleotidase reaction may -in principle- hinder or enhance the activation of the analogs. The former effect is expected when the phosphorylated analog is a good substrate for the nucleotidase, the latter when the enzyme more efficiently dephosphorylates the physiological nucleotides. In this case the final ratio between the antiviral nucleotide and the competing physiological counterpart will become more favourable from the pharmacological point of view.
We have cloned the cytosolic deoxynucleotidase (dNT1), the only nucleotidase described to prefer deoxy- over ribo-nucleotide substrates. In cells transfected with an inducible expression vector carrying the dNT1 cDNA we demonstrated that this enzyme is involved in substrate cycles regulating the dTTP pool. In a comparison of the dephosphorylation of the natural substrate dTMP with two phosphorylated prodrugs, d4TMP and 3AZTMP, the substrate efficiency (Vmax/Km)) of d4TMP was less than 1/10 that of dTMP, whereas AZTMP was a slightly better substrate than dTMP. This suggests that in intact cells d4TMP remains available for a longer time than AZTMP and can be more efficiently phosphorylated to the active antiviral triphosphate. Indeed, isotope experiments with intact cells showed that this was the case.
Based on the sequence of the dNT1 cDNA we identified a homologous sequence in the data base that had the hallmarks of a mitochondrial enzyme. We cloned the new cDNA and demonstrated that the coded protein is imported into mitochondria both in vitro and in intact cells. During import the N-terminal mitochondrial leader sequence is cleaved and the processed protein is a deoxynucleotidase (dNT2) with similar but distinct enzymic properties relative to the dNT1. The discovery of this new enzyme suggests that also in mitochondria dNTP pools are regulated by substrate cycles. Two mitochondrial deoxynucleoside kinases were known previously, now the presence of dNT2 completes the picture and offers a tool to counteract the mitochondrial toxicity of nucleoside analogs. By using analogs whose monophosphates are better substrates for dNT2 than for dNT1 it may be possible to avoid accumulation of toxic triphosphates inside mitochondria while favouring the cellular activation of the prodrug to target viral DNA synthesis in the nucleus. We are now starting experiments with several analog monophosphates to compare their affinity for the two purified enzymes.

Grant n. 30C.9

ANGIOPOIETIN-1 ACTIVATES HUMAN MICROVASCULAR ENDOTHELIAL CELLS ORIGINATED FROM EPIDEMIC KAPOSI'S SACOMA LESIONS.

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Angiopoietins (Ang1 and Ang2) constitute a family of endothelial growth factors that are ligands for the tyrosine kinase receptor Tie2, expressed in endothelial cells (EC) and up-regulated in tumor microvessels. Ang1 promotes *in vitro* EC sprouting, survival and migration, whereas Ang2 blocks the activation of Tie2 induced by Ang1. . Disruption of the function of either Tie2 or Ang1 in mice resulted in lethal defects in the developing vasculature such as simplification of the vascular branching pattern and failure to recruit accessory cells, indicating that thse molecules participate in maturation phase of angiogenesis. Ang1 and its receptor Tie-2 are strongly expressed in Kaposi's sarcoma (KS) lesions (Brown DL Am J Pathol 2000 156:2179, 2000) and in KS cells in vitro (E Audero, in preparation). On the light of this observation and of the role of angiogenesis in the progression of KS, in this study we evaluated the biological effect of recombinant Ang1 produced in our lab on microvascular EC isolated from KS epidemic cutaneous lesions (EC-KS) of three patients and compared with those of normal skin. EC were isolated from collagenase and dispase treated samples by magnetic beads coated with mAb anti-CD31 and then separated by FACS-sorter with the same mAb. EC-KS expressed higher amount of Tie-2 mRNA and a discrete amount of the receptor was constitutively phosphorylated. This was abrogated by the presence in the culture medium of a neutralizing Ab anti- Ang1 suggesting that an autocrine loop is operative in EC-KS. The ability of EC-KS to differentiate in tube-like structure when plated on Matrigel or collagen was higher than normal EC. The presence of the Ab anti-Ang1 reduced the spontaneous differentiation, supporting the fact that the autocrine loop was operative. However, KS-EC were able to respond to exogenous Ang1 probably for the high amount of Tie-2 expressed. We observed that the adhesion of KS-EC to fibronectin and collagen is increased by the cell pre-treatment with Ang1 and these effects are dependent of the activation of Rho GTPase. Unstimulated KS-EC produce MCP-1 which increases after challenged with Ang1 . Of interest, normal EC did not share these activities, suggesting that the KS microenvironment alters the normal biological response of EC to Ang1. These effects are time and dose-dependent and require the gene transcription. In conclusion here we demonstrate that the sensitivity of KS-EC to Ang1 is different from that normal EC and in particular this growth factor may participate in the formation of leukocyte infiltration by the specific production of MCP-1

N^{O}. DELL'ACCORDO DI COLLABORAZIONE: 30C10

SINGLE ECTOPIC EXPRESSION OF mT ONCOGENE ALLOWS HUMAN MICROVASCULAR ENDOTHELIAL CELLS TO ACQUIRE TRANSIENT CAPACITY TO RECRUIT MESENCHYMAL CELLS: A MODEL TO STUDY THE PROGRESSION OF KAPOSI'S SARCOMA

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The mT oncogene of murine polyomavirus (PymT) rapidly transforms and immortalizes murine embryonic endothelial cells, leading the formation of vascular tumors in newborn mice, by recruitment of host, non-transformed endothelial cells (EC). This model has been previously proposed to study the mechanisms of mesenchimal cell recruitment, which characterizes the development of Kaposi's sarcoma. We have recently improved this model by infecting human primary microvascular EC with a retroviral vector carrying PymT. EC infected by pLX-PymT (PymT-EC) retain endothelial specific markers, reach the confluence without signs of overgrowth. Their proliferating rate is increased as well as the response to vascular endothelial growth factor-A (VEGF-A). The balance between urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) is modified, being the former highly expressed and PAI-1 inhibited. Furthermore, VEGF-A participates in an autocrine loop resulting in a reduction of apoptotic index. PymT-EC at early passages induce vascular tumors in nu/nu mice by recruitment of murine EC, as shown by the presence of EC expressing human or murine CD31. The ability of PymT-EC at later passages to induce tumors was reduced. In both cases, their regression was observed over a period of 3 weeks. Of note, sometimes Kaposi's sarcoma can regress spontaneously, but in later stages of development often associated to HIV-1 infection, a cell clone may assume neoplastic features, subsequent to genotypic alterations, causing the hyperplastic lesions of Kaposi's sarcoma to transform into real sarcomas .

These in vitro and in vivo effects of PymT are specific for EC, since human primary fibroblasts do not change their phenotype after pLX-PymT infection. In contrast with murine model characterized by an immortalized phenotype, the effects induced by PymT in human EC are transient. After X-XII passages, they stop to proliferate, assume a senescent phenotype, the amount of middleT protein lowered and lose the ability to activate $pp60^{src}$ and to induce tumors. The correction of unbalance between uPA and PAI-1 or the interruption of VEGF-A-mediated autocrine loop by specific antibodies reduced the in vivo recruitment of EC and the tumor development suggesting that the correction of unbalances of mediator networks may be curative for Kaposi's sarcoma. Our results indicate that PymT oncogene is capable to modify the phenotype of human EC and these features are independent to cells immortalization. Therefore, in contrast to murine EC model where PymT oncogene transform in a single step way, we can hypothesize that PymT could play a role a co-factor in the human vascular oncogenesis. Furthermore, these data improve the previous murine model and offer a new tool to study some pathogenetic events in Kaposi's sarcoma progression.

N°. dell'Accordo di Collaborazione: 30C.10

ROLE OF PAF SYNTHESIS IN THE ANGIOGENIC ACTIVITY OF HIV-1 TAT

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On endothelial cells, Tat mimics the effect of the vascular endothelial growth factor (VEGF) by activating the angiogenic program after interaction with the Flk-1/KDR receptor. Tat has also been shown to synergize with basic fibroblast growth factor (bFGF) in inducing endothelial cell growth and promoting neoangiogenesis. The angiogenic properties of Tat may be relevant for the pathogenesis of Kaposi's sarcoma, an angioproliferative disease very frequent and aggressive if associated with HIV-1 infection. We recently observed that motility induced by HIV-1 Tat on Kaposi's sarcoma cells requires the synthesis of platelet activating factor (PAF) [Am. J. Pathol.. 155: 1731-1739, 1999]. PAF belongs to the structurally related family of acetylated alkyl phosphoglycerides produced upon activation by a broad range of cells including endothelial cells and Kaposi's sarcoma cells. It acts through a specific receptor belonging to the family of seven spanning membrane domain receptors. Recently we found that PAF mediates the angiogenic activity of certain cytokines, including the angiogenic effect of VEGF [Arterioscler. Thromb. Vasc. Biol. 20: 80-88, 2000]. The aim of the present study was to evaluate whether the angiogenic property of Tat may depend on PAF synthesis. The results obtained indicate that human umbilical cord vein-derived endothelial cells (HUVEC) stimulated with HIV-1 Tat synthesized platelet-activating factor (PAF) in a dose- and time-dependent manner. Moreover, in vitro experiments were performed to evaluate whether migration of HUVEC induced by Tat was dependent on the synthesis of PAF. It was found that the cell motility induced by Tat was inhibited by WEB 2170, a specific PAF receptor antagonist. In vivo, in a murine model, in which Matrigel was injected subcutaneously the neoangiogenesis induced by Tat was associated with PAF synthesis within the Matrigel with a kinetic that parallel the recruitment of endothelial cells. Moreover, WEB 2170 significantly inhibited the angiogenesis induced by PAF. PAF both mediated the Tat-induced endothelial cell motility and amplified Tat-induced expression within Matrigel of several angiogenic factors and chemokines including bFGF, macrophage inflammatory protein 2, VEGF itself and its specific receptor flk-1. These results suggest that the synthesis of PAF by endothelial cells mediates, at least in part, the angiogenic activity of Tat by promoting the endothelial cell migration.

N°. dell'Accordo di Collaborazione 30C.11.

SISTEMI RECETTORIALI COINVOLTI NELLA PATOGENESI DEL SARCOMA DI KAPOSI E NELL'INFEZIONE DA HIV NELLE CELLULE NON LINFOIDI

Although HIV-1 Tat protein has been directly implicated in the pathogenesis of AIDS-related Kaposi's sarcoma (KS), its effects on KS spindle-shaped cells and endothelial cells are largely unexplored. Since the susceptibility to apoptosis is relevant for tumor development and therapeutical response, we investigated the effect of Tat on KS and endothelial cell survival from apoptosis.
The effect of Tat was evaluated on three lines of KS cells (KS imm, KS cap, KS L3) exposed to the chemotherapic agent vincristine, that is currently used for the treatment of this tumor, and on human umbilical vein-derived endothelial cells (HUVECs) induced to undergo apoptosis by serum withdrawal.
Apoptosis was assessed by enzymatic assay, microscopical examination of chromatin and cytoskeleton, evaluation of plasma-membrane integrity and subdiploid DNA content, TUNEL assay and caspase-3 activity.
The preliminary results showed that Tat exerted protective activity on the three KS cell lines and on endothelial cells in a dose-dependent manner. Such effect seemed to be independent from modulation of Bcl-2 or Bax expression. By contrast, Tat up-regulated Bcl-X_L expression and induced a relevant decrease of caspase-3 activity in vincristine-treated KS cells.
In conclusion, these results suggest that HIV-1-Tat protein may favor KS development and progression by sustaining endothelial and transformed cell survival.

N^{o}. dell'Accordo di Collaborazione 30C.11.

ROLE OF HIV-1 AND HHV-8 IN THE DEVELOPMENT OF KAPOSI'S SARCOMA AND BODY CAVITY BASED LYMPHOMAS DURING AIDS.

THE TAT PROTEIN OF HIV-1 AND HHV-8 INFECTION, THE LATTER ASSOCIATED TO MESOTHELIAL ACTIVATION, ARE THE PATHOGENIC FACTORS THAT DISTINGUISH KS AND BODY BASED LYMPOMAS IN AIDS PATIENTS FROM THE OTHER FORMS OF KS AND NON-HODGKIN B CELL LYMPHOMAS IN IMMUNOCOMPETENT SUBJECTS. THE PROJECT IS DEVELOPED IN TWO PARTS.

(a) ROLE OF HIV-1 TAT PROTEIN IN THE PATHOGENESIS OF KS

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The goal is to study the role of Tat and IC (IL-1, TNFα, INFγ) on the development, frequency of occurrence and regression of the lesions in VBK/tat transgenic mice, and to determine the effect of anti-Tat antibodies, drugs and vectors on lesion formation and regression. In VBK/tat transgenic mice dorsal simil KS-lesion develop spontaneously in 30-40% of male animals around the 3rd month of age. These lesions are generally not very severe (0.5-2) and regress spontaneously in 20-30 days.
Two-months old male transgenic mice are routinely screened by PCR for the presence of the tat gene, and then Tat-positive male mice are observed daily for lesion appearance. So far results indicate that IC, injected in the lesion both at an early (≤0.5) or at a more advanced phase of development (>1), induce a more aggressive phenotype (4-6), and lesions regress in a longer time (70-90 days) as compared to untreated mice. When mice are simultaneously injected with IC and a rabbit anti-Tat serum in lesions of grade ≤0.5, they develop less aggressive lesions (0.5-1.5) that are completely healed in 30-40 days. In contrast, when mice are simultaneously injected with IC and anti-Tat antibodies in lesions of grade ≥ 1, lesions show a very aggressive phenotype (4-6) as control animals given IC alone. Control mice injected with IC and normal rabbit serum are under study. All mice are observed for 120-140 days. Statistical analysis will be carried out. Eventually lesion' biopsies, obtained at different time points, and autoptic samples will be analyzed histologically for factors involved in lesion formation and regression (bFGF, VEGF, Tat, integrin receptors, p53 and bcl-2).

(B) ROLE OF HHV-8 INFECTION, ASSOCIATED TO MESOTHELIAL ACTIVATION, IN THE PATHOGENESIS OF BODY CAVITY BASED LYMPHOMA (BCBL)

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Primary effusion lymphomas (PELs or BCBLs) are B cell lymphomas representing 3% of all non-Hodgkin's lymphomas observed in AIDS patients and are invariably associated with HHV-8 infection. BCBL cells exhibit a very specific homing for serous membranes of body cavities to which they adhere, sometimes covering them completely. The mechanisms underlying homing and adhesion of BCBL cells to mesothelial cells of serous membranes are essentially unknown. In this part of the project, we propose to investigate the role of HHV-8 latency and lytic genes in the process of homing and adhesion of BCBL cells to mesothelia.
B1. Analysis of the effects of HHV-8 latent and lytic genes on the phenotype of B cell lines. Burkitt lymphoma HHV-8-negative cell line, BJAB, have been transduced with a recombinant retrovirus expressing HHV-8 latent genes, ORF K13, ORF72 and ORF73, under the

transcriptional control of their natural promoter. Expression of the viral genes have been analyzed by RT-PCR and Northern-blot. Moreover we isolated by suppression subtractive hybridization a HHV-8 lytic transcript, orf K3-ORF70, and produced a retroviral construct expressing this lytic function. BJAB cells were then transduced and characterized for K3-ORF70 expression as described above. BJAB cells were chosen because in preliminary studies we have shown that they adhere to activated mesothelial cells with a significantly higher efficiency (60-70%) as compared to different BCBL derived cell lines (5-15%). Thus, experiments were carried to determine whether HHV-8 gene expression may explain the peculiar phenotype of BCBLs, in particular the lack of invasiveness. B2. Study of the mechanism of adhesion to serous membranes. HHV-8 transduced cells have been used in adhesion assays on cultures of human mesothelial cells maintained under normal conditions or activated by proinflammatory cytokines (TNF α, IL-1 β and γ-IFN). The same analysis has been performed employing BCBL-1 cells, activated with TPA or not treated. Statistical analysis of the results is ongoing. Similar experiments will be carried out with BJAB cells transduced with HHV-8 lytic genes with paracrine effects (v-MIP, v-IL6).

Accordo di collaborazione N° 30C.12

PROLIFERATION IN HHV-8 POSITIVE PRIMARY EFFUSION LYMPHOMAS IS ASSOCIATED WITH EXPRESSION OF HHV-8 CYCLIN BUT INDEPENDENT OF P27/KIP1

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Primary effusion lymphoma (PEL) develops in immunodeficient patients, selectively localizes to the serous body cavities and harbors infection by human herpesvirus type-8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV). HHV-8 encodes a viral (v-)-cyclin homologous to cellular D-type cyclins, a class of positive cell cycle regulators that are physiologically modulated by the p27/KIP1 cell cycle inhibitor. The aims of the present study were i) to establish the expression pattern of p27/KIP1 in PEL, and ii) to address the relationship between p27/KIP1 expression, proliferation index, and expression of v-cyclin in PEL.

To this aim, 19 PEL samples (including 9 clinical samples and 10 PEL cell lines) were analyzed by immunocyto-histochemistry, and Western blot for p27/KIP1, Ki-67 and v-cyclin. v-cyclin was also tested by in situ hybridization. Ten lymphomatous effusions secondary to a tissue-based lymphoma, 86 AIDS-related systemic non-Hodgkin's lymphomas (AIDS-NHL), including all major subtypes, and 20 AIDS-related primary central nervous system lymphomas (AIDS-PCNSL) were also analyzed.

Expression of p27/KIP1 was detected in all (n=19) PEL samples and in most AIDS-related immunoblastic lymphomas (AIDS-IBL), either systemic or primarily localized to the central nervous system. All PEL displayed a high proliferation index as assessed by Ki-67 staining and expressed v-cyclin. In contrast to PEL and AIDS-IBL, lymphomatous effusions secondary to a tissue-based lymphoma as well as the remaining systemic AIDS-NHL and AIDS-PCNSL generally failed to express p27/KIP1.

These data show that PEL and AIDS-IBL consistently express p27/KIP1 protein despite the high proliferative rate of the lymphoma clone, suggesting that p27/KIP1 may be unable to drive cell cycle arrest in PEL and AIDS-IBL cells. The co-existence of p27/KIP1 expression and high proliferative index is a selective feature of PEL among lymphomas involving the serous body cavities, since lymphomatous effusions secondary to a tissue-based lymphoma generally display the inverse relationship between p27/KIP1 positivity and growth fraction observed in normal lymphoid tissues and in most AIDS-NHL included in this study. Furthermore, expression of p27/KIP1 in PEL associates with expression of HHV-8 v-cyclin. The fact that HHV-8 v-cyclin is resistant to p27/KIP1-modulated inhibition may explain, at least in part, the co-existence of p27/KIP1 expression and high proliferative index observed in PEL

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SALVAGE TREATMENT WITH HYDROXYUREA IN HIV INFECTED PATIENTS FAILING PROTEASE INHIBITOR CONTAINING REGIMENS.

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Up to 50 % of patients treated with highly active antiretroviral therapy (HAART) does not reach a full virological control or an immunological improvement. Therefore therapies are needed to treat such patients failing a HAART. Hydroxyurea (HU) has given interesting results both in acute and in chronic HIV infection, but data about its use in antiretroviral failure is lacking. The objective of the study is to evaluate the efficacy of a salvage treatment that includes HU.

The study is nested within the Master-2-experienced study, that aims at evaluating different therapeutical skemes in patients failing a protease inhibitor containing antiretroviral regimen for the first time. A failure is defined as: 1- persistent viral load above 500 copies/ml after 6-8 months of therapy or 2- a rebound over 1-2,000 copies/ml after a value below detection level. Patients enrolled in this study will first perform a genotypic assay, and subjects with resistance will be excluded. The patients will then be randomized on a 50% basis to be treated either with a standard rescue regimen or with a HU contaning regimen (1:1). The patient in the HU arm will be randomized on a 1:1:1 basis to receive either: 1- d4T+ddI+HU; 2- d4T+ddI+EFV or NVP; 3- d4T+ddI+HU+EFV or NVP. Pharmacokinetic studies of d4T and ddI will be performed. At 6 months an interim analysis will be performed.
Primary end points of the study will be: 1- clinical progression to AIDS; 2- AIDS related death; 3- time to viroimmunological failure; 4-grade IV toxicity.

New data about salvage therapy prompted us to deeply revise the protocol and to resubmit it to the ethical committee.

As of December 31st, 2000, the first patients have been enrolled. The study aim to enroll 120 patients within 2001.

N°. dell'Accordo di Collaborazione. 30C.14

INDUCTION/MAINTENANCE ANTIRETROVIRAL THERAPY INCLUDING EFAVIRENZ: THE HAM (HIGHLY ACTIVE MAINTENANCE) STUDY

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Summary
The long term need for complex anti-HIV polychemiotherapy is a major constraint for patients' complete adherence to drugs prescription. Clinical trials that have addressed the feasibility of an induction/maintenance approach (Adam, Trilege, ACGT 343) have provided discouraging results, which may be due to: 1) limited duration of the induction phase; 2) complexity of the maintenance phase; 3) presence of drug resistant strains in the enrolled patients.

Aim of this study is to evaluate tolerability and efficacy of a potent, 5-drug regimen (Induction phase) followed by a simplified 3-drug regimen (maintenance phase).

This is a prospective, controlled, open-label, randomised study. The study design call for the recruitment of 60 naive HIV positive patients with CD4+ > 100 (and HIV-RNA >10,000 if CD4+ > 500) and without evidence of viral resistance to any of the drugs in use. Enrolled patients will be randomised to the following arms:

- Arm A: AZT + 3TC + IDV + RTV + EFV (n. 20 pts)
- Arm B: AZT + 3TC + EFV (n. 20 pts)
- Arm C: AZT + 3TC + IDV + RTV (n. 20 pts)

Patients in arm A will be shifted to AZT + 3TC + EFV after 6 months if HIV-RNA is undetectable (lower than 50 c/ml).
Viro-immunological efficacy of the therapeutic regimens will be based on HIV-RNA and CD4+ cell counts, scheduled at quarterly intervals. Recruitment will take place over a period of 6 months and patients will be followed-up for 24 months (for patients in arm A the follow-up period will start at the beginning of the maintenance therapy).
Before inclusion and at month 6 a genotypic test on plasmatic RNA and lymphocitic DNA will be performed to exclude the presence of viral resistance that may bias the results of the study. Genotyping will additionally be performed on isolates of all patients with virological failure.
The endpoints of the study are: 1) incidence of shifting due to virological failure (HIV-RNA > 1,000 after reaching the undetectable level; 2) the duration of treatment in the assigned arm before shifting become necessary; 3) Incidence of shifting due to reduced adherence.
Seventeen Infectious Diseases Clinics from all over Italy participate in this study. After obtaining ethical clearance from the appropriate bodies, recruitment of patients started in November 2000. Seven patients have been enrolled at 31.12.2000 (12% of the total expected) and randomised to arm A (n = 1), Arm B (n = 3) or arm C (n = 3).

N°. dell'Accordo di Collaborazione: 30C.15.

ISOLATION OF KS DERIVED CAPILLARY ENDOTHELIUM: IDENTIFICATION OF TWO ENDOTHELIAL CELL SUBSETS WHICH EXPRESS VEGFR-3, A MARKER OF KS SPINDLE CELLS.

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We have successfully isolated and cultured Kaposi's Sarcoma (KS) derived capillary endothelial cells from skin specimens of 4 classic (C-KSdCEC) and 1 AIDS associated KS (AIDS-KSdCEC). When compared to normal dermal capillary endothelium (HDCEC), C-KSdCEC have a very low growth rate (50% less than HDCEC), on the contrary the AIDS-KSdCEC shows a remarkable growth capability (3 to 4 times more than HDCEC). The addition of conditioned medium derived from culture of CD4+ lymphocytes infected with HTLV-1/HTLV-2 virus (RTCM), significantly enhances the proliferation of AIDS-KSdCEC stimulated with bFGF, whereas C-KSdCEC and HDCEC are not. When compared to C-KSdCEC, the AIDS-KSdCEC have a higher expression of E-selectin, Endoglin (CD105) and Ve-cadherin. Due to their high growth rate, AIDS-KSdCEC can be cloned by using human monocytes as feeder cells. We have isolated two different endothelial subsets (AIDS-KSdCECA1 and A2) that can be rapidly expanded in culture. These two cloned cell populations require less stringent growth medium condition and are strongly stimulated by RTCM + bFGF treatment. The phenotypic characterization shows that both cloned cell lines while still express endothelial cell markers (CD31, CD34, Ve-cadherin and bind ULEX lectin) and the adhesion molecule E-selectin, they also express the VEGFR-3 (VEGF receptor 3/Flt4) which is not present on the parental AIDS-KSdCEC. This report highlights two important observations: a) the AIDS-KSdCEC shows to retain some phenotypic and functional features of the KS cells and b) the AIDS-KSdCEC population contains subsets of endothelial cells which express VEGFR-3 that is suggested to be a specific marker of the KS spindle cells and lymphatic vessel endothelium. We therefore conclude that the endothelial cells infiltrating the pre-neoplastic KS lesions, might be involved in the origin of the tumor spindle cells in HIV-1 infected individuals.

N^{o} 30C.16. dell'Accordo di Collaborazione.

INTRAFAMILIAL TRANSMISSION OF HUMAN HERPESVIRUS 8.

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To investigate intrafamilial transmission of human herpesvirus 8 (HHV-8), we determined the presence and titer of HHV-8 antibodies in a HIV-1-seropositive family nucleus. Sequential plasma samples from a woman, her two sexual partners and her two children were analyzed for HHV-8 antibodies to a structural protein encoded by ORF65, and to a latency-associated nuclear antigen (LANA).

Ten year follow-up (1989-1999) of the woman showed increasing and high titers of ORF65 antibodies before diagnosis of a very aggressive Kaposi's sarcoma (KS) and during disease progression. The woman's first partner (from 1984 to 1990), who was affected by a stable KS, was found to have low titers of both ORF65 and LANA antibodies before and during KS. Their daughter, analyzed at 16 months of age, and their son, at 17 months, 3 and 4 years of age, were found to have LANA antibodies. The woman's second partner (1990-1995) was persistently HHV-8-seronegative.

These findings suggest that heterosexual transmission of HHV-8 via female-to-male is not a preferential route for HHV-8 dissemination. The presence of LANA antibodies in the 4 year old son confirmed the possibility of additional modalities of intrafamilial transmission of HHV-8 infection. ORF65 antibody titers were found to correlate with tumor burden and to be a possible marker of disease evolution.

Accordo di Collaborazione 30C.17

HIV-1 POL ANALYSIS OF PAIRED CEREBROSPINAL FLUID AND PLASMA SPECIMENS: CORRELATION BETWEEN PAIRWISE NUCLEOTIDE DISTANCE AND HIV-ASSOCIATED DEMENTIA.

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Background and objectives. Viral load studies of paired cerebrospinal fluid (CSF) and plasma specimens indicate that HIV replication can be variably segregated in these two compartments. Objective of this study was to evaluate the extent of variation between CSF and plasma HIV-1 pol sequences and its association with HIV-induced CNS disease.
Patients and Methods. Paired CSF and plasma specimens from 48 neurologically symptomatic HIV-infected patients, including 23 antiretroviral naive, 19 RTI-experienced and 6 RTI and PI-experienced patients, were retrospectively examined. Viral load was assessed by RT-PCR (Roche Amplicor Monitor) and reverse transcriptase and protease sequences were obtained by direct sequencing (Applied Biosystems 7700). Sequencing data were analysed for presence of resistance mutations; nucleotide distance between CSF and plasma sequences was calculated by using the Kimura 2-parameter model.
Results. RT resistance mutations were found in CSF and/or plasma of 26 patients, respectively, including three antiretroviral naive patients. A different CSF/plasma pattern was observed in 10/48 patients (22%), with mutations present in CSF but not in plasma in four (8%). Protease resistance mutations were observed in 24/29 patients, in all but one case consisting of accessory mutations at codons 10, 36, 63 or 77. No correlation was found between the CSF/plasma nucleotide distance and type of CNS disease, CSF cell counts, CSF or plasma viral load, CSF to plasma VL ratio or duration of HIV infection. Neither was any of these factors associated with the presence of a different CSF/plasma resistance pattern. However, when only the antiretroviral naive patients were analysed, higher CSF/plasma distance values were observed in patients with dementia than in those without (RT: median 0.0192 vs. 0.0110, p=0.008; protease: median 0.0384 vs. 0.0205, p=0.019).
Conclusions. In patients with HIV dementia, and in absence of antiretroviral therapy, relatively distant pol sequences may be observed between CSF and plasma, possibly supporting an autonomous HIV replication in the CNS.

Accordo di Collaborazione N° 30C18

LONG-TERM VIROLOGICAL RESPONSE TO HAART IN CEREBROSPINAL FLUID: CORRELATION WITH PLASMA VIRAL LOAD AND GENOTYPIC RESISTANCE.

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Background and objectives. The central nervous system (CNS) is a potential reservoir for HIV infection in patients receiving highly active antiretroviral therapy (HAART). The brain barriers might prevent adequate drug penetration into the CNS and low drug levels predispose to local development of drug resistance. Objective of this study was to study the long-term virological response and genotypic resistance in cerebrospinal fluid (CSF) of HAART-treated patients.
Patients and Methods. HIV load was measured in paired CSF and plasma specimens from 115 neurologically symptomatic patients receiving HAART for a period of <1-38 months. In addition, in a subgroup of 11 patients treated for 9-30 months from whom baseline CSF and plasma specimens were also available, genotypic resistance was also assessed by direct sequencing of RT and protease (PR) genes.
Results. Patients receiving HAART for two months or longer (calculated after 2, 3, 6, 9, 12 or >12 months) had always CSF HIV-RNA levels significantly lower than those treated for one month or less. In contrast, plasma levels were no longer significantly lower in patients treated for ε 9 months than in those treated for δ one month. Accordingly, HIV was undetectable in CSF of 34/42 patients (81%), but in plasma of only 15/38 patients (39%) after 9 months of treatment. Among the 11 patients with both baseline and follow-up analyses, HIV-1 RNA was detected in CSF of 4 (36%)(median 4.05 log10 copies/ml) and in plasma of 5 (45%)(median 4.30 log10 copies/ml) at long-term therapy follow-up. However, virological failure in CSF was observed exclusively in patients failing to respond in plasma. At baseline, CSF RT and/or PR resistance mutations were found in 5 of 7 CSF responder patients and in 3 of 4 patients failing to respond in CSF. New RT or PR resistance mutations were detected only in plasma or in both CSF and plasma of all of the CSF non responder patients at the time of virological failure.
Conclusions. At long-term follow-up, the proportion of patients failing to respond in CSF was lower than that of patients failing to respond in plasma. In addition, CSF virological failure was frequently associated with virological failure in plasma and onset of new drug resistance mutations in both CSF and plasma compartments, thus minimising the possible role of the CNS as viral reservoir.

Accordo di Collaborazione N° 30C18

TREATMENT WITH INHIBITORS OF THE HIV PROTEASE SELECTS RESISTANT STRAINS WITH VARIABLE DEGREE OF PROCESSIVITY AND DRUG DEPENDENCE

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The clinical outcome of HIV infection has markedly improved after the introduction of viral protease inhibitors (PI) in combination therapy regimens; however, selection of drug resistant viral variants and subsequent virologic failure of treatment is becoming increasingly common. In these conditions, the wide cross-reactivity among all of the available PI usually leaves not much room for an effective substitution, thus raising the dilemma whether or not continuing PI treatment. At present, insufficient information is available about the replicative features and pathogenic potential of PI-resistant viral strains, although recent data suggest that they frequently show lower fitness in vitro than that exhibited by wild-type HIV strains.

In the present study, we investigated the recombinant viral phenotype and growth characteristics associated to HIV protease sequences from PI-failing subjects. Additionally, HIV proteases from subjects with virologic failure, but stable clinical benefit from PI treatment were also evaluated. A novel recombinant and non-replicative phenotypic assay recently developed and optimized in our laboratory (Menzo S. et al., AIDS 14, 1-10, 2000) was used to identify protease-resistant genes. The genes were subsequently cloned in a replicative viral backbone to test growth dynamics and fitness in cell culture assays.

Among the 20 selected resistant HIV proteases, 6 variants were identified whose activity in vitro resulted consistently higher (up to 2-fold) in the presence of PI than in their absence. The majority of resistant proteases (70%) showed decreased processivity and conferred slower viral replication, in the absence of drug, compared to wild-type. On the other hand, the resistant proteases fully retained normal processivity in 30% of cases (in one case, activity was even enhanced). Correlation of the drug-driven viral evolution to clinical benefit has been further investigated in the subset of "discordant" subjects.

The data highlight the biology of PI-resistant HIV protease sequences and their role in infection activity. Overall, these results suggest that (1) the evolution process of the protease gene in the presence of strong selective pressure of PI can lead to the emergence of viral variants bearing drug-dependent proteases, and that (2) reduced viral fitness is not an obligate step and is highly variable among isolates. Since signature genotypes for drug dependence and reduced fitness were not yet identified, the use of specialized phenotypic testing, capable of showing both protease resistance and processivity of this enzyme is crucial for the correct management of subjects failing PI treatment, identifying those who will benefit from persistent treatment. Phenotypic testing will also add valuable data for the development of new effective antiretroviral compounds.

No. dell'Accordo di Collaborazione: 30C.19

A DYCOTHOMY BETWEEN GRANULE DEPENDENT-(PERFORIN and GRANZYMES) AND INDEPENDENT-(TNFa) MECHANISMS OF LYSIS IN HIV-INFECTED, HAART-TREATED INDIVIDUALS.

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Background: Immune reconstitution in HIV-infected patients undergoing HAART is incomplete and HIV-specific T helper and cytotoxic T cell (CTL) responses have been repeatedly shown to be defective. CTL are activated by type 1 cytokines (IL-2, IL-12, IFNg) and can kill targets via granule dependent-(perforin and granzymes) and/or independent-(TNFa) mechanisms. We examined if these mechanisms are defective in HAART-treated patients.
Methods: Intracellular concentration of perforin, granzymes (basal and IL-2 stimulated) and TNFa (basal and PMA+ionomycic) was measured by FACS in PBMC and in CD8+ T cells of 38 chronically HIV-infected individuals (> 6 years of infection) divided in two groups: HAART-naive (HN)(n=14) and HAART-treated (HT)(n=24). PHA-stimulated intracellular IL-2 and IFNg were measured as well. All patients had CD4 counts >500; HIV plasma viremia was undetectable in 20/24 HT and in 11/14 HN individuals and ranged between 1200 and 25,000 copies in the other patients.
Results: A similar percentage of CD8+ T cells expressed TNFa in basal (HN= 0.16; HT= 0.26) and stimulated conditions (HN= 5.7; HT= 3.5); perforin positive CD8+ T cells (11.22 vs. 6.49 p=. 02) and PBMC (21.02 vs. 13.61 p=.01) as well as granzyme positive CD8+ T cells (20.87 vs. 10.36 p=. 03) and PBMC (24.12 vs. 17.5 p=.08) were significantly reduced in HT compared to HN individuals. Intracellular concentration of perforin was reduced (degranulation) in IL-2-stimulated cells. IFNg- expressing CD4+ and CD8+ T lymphocytes were reduced in HT compared to HN patients.
Conclusions: Defective CTL function observed in HAART-treated individuals are at least partially secondary a quantitative impairment of intracellular perforin and granzymes. Granule-independent (TNFa) mechanisms of lysis do not seem to be defective in these individuals, but they might not be sufficient to assure proper target lysis.

N°. dell'Accordo di Collaborazione 30C.20

HIV-1 INTERACTIONS WITH THE DIFFERENT RENAL CELLS CAN INDUCE DISTINCT PATHOGENIC MECHANISMS CAPABLE TO EXPLAIN THE DEVELOPMENT OF HIV-ASSOCIATED NEPHROPATHY IN VIVO

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HIV-associated nephropathy (HIVAN) is a relevant complication of HIV infection manifested by heavy proteinuria and progressive renal failure with severe prognosis. If untreated, HIVAN may result in end-stage renal disease in few months. Pathologically, HIVAN is a pan-nephropathy characterized by a collapsing form of glomerulosclerosis associated with severe tubulo-interstitial disease (microcystic dilatation and apoptotic changes of tubuli). Glomerular lesions display segmental or global tuft collapse with striking visceral epithelial cell alterations, which include podocyte hyperplasia and dysregulation of mature podocyte phenotypic markers. A relevant increase in the number of cases of chronic nephropathy in HIV-infected individuals occurred in the past years, but the pathogenesis of HIVAN remains undetermined. Current information suggests this disease is etiologically related to HIV infection, but the mechanisms of HIV-induced renal injury are still unknown. In order to evaluate the direct role of virus infection, we have analyzed in vitro the effects of HIV-1 on renal glomerular and tubular cells. Primary cultures of proximal tubular epithelial cells (PTEC), glomerular mesangial cells (MC) and glomerular epithelial cells (GEC or podocytes) were obtained from surgically removed kidneys and characterized according to published criteria. Established lines of differentiated PTEC, MC and GEC were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus. We recently reported that tubular epithelial cells and glomerular mesangial cells express virus-specific receptors (CD4, CXCR4, CCR5, CCR3) and sustain viral productive replication with pathological effects mimicking the changes involved in HIVAN development. Indeed, HIV-1 replication causes the death of PTEC by apoptosis triggering caspase activation and Fas upregulation, but not FasL expression (Conaldi et al., J. Clin. Invest. 102: 2049, 1998). This evidence does demonstrate that the virus can activate apoptotic pathways also in non-lymphoid cells. In MC we found that HIV-1 can establish persistent infection inducing the synthesis of inflammatory cytokines (IL-6, IL-8, TNF-α), and PDGF and TGF-β, crucial mediators of glomerulosclerosis (Conaldi et al., AIDS: 14: 2045, 2000).

In spite of the recently enlightened role of GEC in HIVAN, in our experiments podocytes resulted to be non-permissive to HIV replication, possibly because they do not bear virus receptors (CD4 and chemokine receptors). In this report we demonstrate that stimulation with HIV-1 Tat can induce changes of podocytes similar to those detected in HIV-associated glomerulopathy. In fact, the treatment with exogenous Tat stimulated a relevant increase of GEC proliferation. The detection of Ki-67 Ag, a nuclear antigen expressed by proliferating cells, demonstrated a striking increase of the number of cycling cells in podocyte cultures treated with Tat. Cell growth assays with peptides containing the basic [49-57] and RGD [66-86] regions indicated that both Tat domains are required for this phenomenon, but the role of the basic region appears to be prominent. Enhancement of GEC proliferation was promoted also by immobilized Tat, suggesting that its effect is mediated by the interaction of Tat with cell-surface molecules expressed by podocytes. Blocking experiments with antibodies against $\alpha 3\beta 1/\alpha v\beta 5/\beta 1$ integrins and VEGF receptors excluded the involvement of these molecules in

Tat-induced GEC changes, as reported for other cell types. Since the treatment with β-d-xyloside, a proteoglycan synthesis inhibitor, caused a significant reduction of Tat-stimulated proliferation, the interaction of Tat with proteoglycans on cell surface may play a role in the biological activity of the viral protein on podocytes. bFGF is involved in all the experimental models in which Tat stimulates cell proliferation and has been implicated in renal pathology. We found that podocytes produced low levels of bFGF and that Tat promoted a relevant augmentation of bFGF content in GEC cultures, both for increased synthesis and retrieval of the bound fraction of this growth factor. bFGF removal from conditioned media of Tat-treated GEC resulted in a reduced increase of podocyte proliferation, suggesting that this factor can be involved, at least partially, in Tat-induced hyperproliferation. In this study we did also find that Tat caused loss of the expression of synaptopodin and WT-1 Ag, specific podocyte markers, and induced alteration of GEC cytoarchitecture with change of cell shape and modification of the distribution of actin stress fibers. Finally, we revealed that Tat impaired the permselectivity of podocyte monolayers to albumin.

In conclusion, our results demonstrate that the interaction of Tat with podocytes does indeed induce the pathological features of GEC (hyperproliferation, dysregulation of phenotype, alteration of cytoarchitecture, loss of protein permselectivity) characterizing the glomerular pathology of HIVAN in vivo.

Accordo di Collaborazione N° 30C.21

ROLE OF HIV-1 REPLICATION AND HIV-INDUCED PATHOGENIC MECHANISMS IN RENAL CELLS IN THE DEVELOPMENT OF HIV-ASSOCIATED NEPHROPATHY

Accordo di Collaborazione N° 30C.21
Responsabile Scientifico: Prof. Pier Giulio Conaldi

PRELIMINARY RESULTS

- Proximal tubular epithelial cells

1) HIV infection induces the synthesis of C_2 ceramide by PTEC. Our previous results indicate that Fas-FasL pathway is not involved in HIV-induced apoptosis of PTEC. The sphingomyelin pathway is a ubiquitous signaling system initiated by hydrolysis of the plasma membrane phospholipid sphingomyelin to generate ceramide, which triggers the apoptotic cascade. Experimental evidence suggest that this pathway may play a role in apoptosis of tubular epithelial cells. In order to characterize the molecular mechanisms of HIV-induced killing of PTEC we tested the effect of virus infection on the production of C_2 ceramide, a cell-soluble mediator of apoptosis that could also explain, in our model, the killing of bystander uninfected PTEC. HPLC-spectrofluorimetric analysis of lipidic extracts obtained by PTEC monolayers at different times post infection demonstrated a progressive increase of C_2 ceramide content. Whilst it was 2.1±0.2 $\mu g/10^6$ cells in the uninfected PTEC cultures, C_2 ceramide levels were 4 $\mu g/10^6$ cells at day 2 p.i., 4.3 at day 3 p.i., 5.8 at day 4 p.i., 10.1 at day 5 p.i. (time of full-blown manifestation of apoptotic changes). On the contrary, the content of C_6 and C_8 ceramide did not result to be affected by HIV infection. The role of Tat in HIV-mediated induction of ceramide synthesis is under investigation. We are going to test our model inducing programmed cell death of PTEC by addition of exogenous C_2 ceramide and blocking HIV-induced apoptosis by inhibition of sphingomyelinase. Interestingly, in preliminary experiments with various corticosteroids a reduction of the death of the infected PTEC has been obtained with dexamethasone, which is known to reduce the production of ceramide. Probing this evidence might explain the beneficial effect of corticosteroids in HIVAN and represent a model for therapeutic strategies.

2) HIV infection induces the expression of Bax by PTEC. Bcl-2 family members play both pro-apoptotic and anti-apoptotic activities: particularly, the ratio of Bcl-2/Bax protein levels has a key role in these events. By immunofluorescence and western blot analysis we found that Bcl-2 is not expressed both in uninfected and infected PTEC. On the contrary, by western blotting we detected Bax in HIV-infected PTEC from day 3 p.i., time of the beginning of apoptotic changes of the tubular cells. Based on these results, we are going to evaluate the influence of HIV infection on the expression of other proteins of Bcl-2 family, such as Bcl-$X_{s/l}$ and Bad. During apoptosis Bax translocates to mitochondria: thus, we are going to evaluate the effects of HIV infection on mitochondrial membrane polarization and cytochrome c release by flow-cytometric assays and western blot analysis. Moreover, we are performing experiments of apoptosis blocking by using different anti-oxidants (ebselen, glutathione, N-acetylcysteine). Preliminary data obtained with 25 nM ebselen, a glutathione peroxidase mimic, indicate a significant reduction of HIV-induced apoptosis. The molecular mechanisms of oxidative stress in this phenomenon and the therapeutic implications of these result are under investigation.

- Glomerular mesangial cells

3) HIV-1 glycoprotein-antibody complexes induce cytokine production by MC. Induction of cytokine secretion is frequently caused by the interactions of HIV-1 glycoproteins with lymphoid and non-lymphoid cells. Thus, we investigated the effect of gp120 and gp41 challenge on MC. Since these cells are activated by immune complexes and, according to our results, are CD4 positive and susceptible to HIV-1 productive infection, we evaluated the effect of IgG antibody-Env Ags complexes directly formed on MC membranes. In our study neither gp120 and gp41 alone were able to stimulate MC synthesis of cytokines but low levels of IL-6 and TNF-α. On the contrary, the addition of Env-specific antibodies after treatment with HIV-1 glycoproteins did promptly cause the production of IL-6 and TNF-α by MC. Under these conditions, the response of MC appeared to be rapid but transient, with peak levels occurring 24 hrs after challenge. The exposure of MC to antiviral antibodies alone did not induce cytokine synthesis.

- Glomerular epithelial cells

4) HGF stimulates podocyte proliferation and Tat increases the expression of c-met. Tat has a basic domain similar to that of several binding angiogenic factors including FGF, VEGF and HGF. Several reports show that HGF has a mitogenic, motogenic and morphogenic activity for renal cells, particularly epithelial cells. HGF effects on tubular epithelial cells are known. In a collaborative study with the Department of Nephrology, University of Pavia, we detected higher levels of HGF in patients with glomerulopathies characterized by GEC hyperplasia (manuscript in preparation). Our experiments in vitro demonstrated that HGF can stimulate podocyte proliferation in a dose-dependent manner (1-100 ng/mL) and its proliferative activity can be blocked by a specific neutralizing antibody. By immunocytochemistry and western blot analysis we revealed that podocytes bear c-met, the receptor of HGF. Treatment of GEC cultures with 10 ng/mL of Tat resulted to increase c-met expression. The effect of Tat (and/or Tat peptides, especially basic domain) on the synthesis of HGF by podocytes and the possibility that Tat does directly interact with HGF receptor are under investigation.

5) Tat reduces the expression of IGFBPs in podocytes. Insulin-like growth factor-I (IGF-I) is secreted by both endocrine and paracrine pathways and exerts a strong effect on cell proliferation and differentiation in several tissues. Its activity is modulated by specific binding proteins (IGFBP-1 to 6). IGFBP-3 and -4 were reported to inhibit the biological function of IGF-I by reducing the free IGF-I amount available for binding to its specific receptor. Based on these observations, we evaluated the effect of Tat on IGF-I system in podocytes. We incubated podocytes with Tat for 48 h and measured IGF-I and IGFBPs in the supernatants and in the cells, respectively. By RIA, we found a reduction of IGF-I concentrations from 19.5±0.05 (control) to 10.8±0.6 μg/L ($p<0.001$) after treatment with Tat. By immunocytochemistry, IGFBP1-6 were detected in normal podocytes. The addition of Tat strongly affected IGFBP expression with the exception of BP-6. The percentage of positive cells fell from 80% to 20%, 40% and 20% for BP-1, -2 and -5. Remarkably, the number of positive cells for BP-3 and –4 decreased from 80% and 70% to 0% in Tat-treated GEC. These data indicate that Tat affects IGF-I production by podocytes, but its inhibition of IGFBP expression (mainly IGFBP-3/4) is much stronger. Thus, Tat induces an increase of free IGF-I, which may contribute to the proliferative effect of the viral protein in GEC cultures.

6) Effect of Tat on the expression of nephrin by podocytes. HIVAN is characterized by heavy proteinuria of nephrotic range. Nephrotic syndrome is a clinical disorder associated with several primary and secondary glomerulonephritis, that may be caused by different pathogenetic mechanisms. Several studies have addressed the mechanisms involved in the loss of perm-selectivity of glomerular capillary walls. Recently, the identification of nephrin has stressed the role of podocytes in maintaining glomerular permeability. Nephrin is a 1242 amino acid residue transmembrane protein of the immunoglobulin superfamily, specifically expressed at the slit diaphragm located between the glomerular podocyte foot processes. Several lines of evidence indicate that this protein may have a relevant role in the filtration mechanism of glomeruli and more generally in the pathogenesis of proteinuria. These data raise the possibility that an acquired alteration of nephrin distribution at the level of slit diaphragm may account for proteinuria. In collaboration with the Laboratory of Renal Immunopathology of University of Torino we studied the expression of nephrin in biopsies of patients with primary acquired nephrotic syndrome. We found that the immunohistochemical staining pattern of nephrin was severely altered in these cases ("Nephrin redistribution on podocytes is a potential pathomechanism for proteinuria in patients with primary acquired nephrotic syndrome", submitted to Am. J. Pathol). In vitro experiments using GEC cultures suggest that the reduction of nephrin expression observed in glomeruli of patients with acquired primary nephrotic syndrome may be the consequence of podocyte injury triggered by different stimuli, such as antibodies, immune complexes, terminal components of complement and cytokines. A common activity of these stimuli is the alteration of cytoskeleton organization, which may induce the redistribution and "shedding" of nephrin from the surface of GEC. According to our results on Tat-induced changes of podocyte cytoarchitecture, we are going to investigate the effects of Tat on nephrin expression and distribution and to correlate these data with the function of perm-selectivity of podocytes. Our results will allow to evaluate the possible involvement of nephrin alterations in podocytes in the pathogenesis of the heavy proteinuria of the patients with HIVAN.

ANALYSIS OF CJ-TREC+ AND SJ-TREC+ LYMPHOCYTES IN PERIPHERAL BLOOD FROM HIV+ PATIENTS.

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The study of the production of virgin cells has a consistent importance during HIV infection. Several molecules have been proposed as markers of antigen-experience or unexperience, but nowadays the most sensitive approach is the quantification of the so called "Recent Thymic Emigrant" lymphocytes, that can be studied by analyzing the fragments of excision of T cell receptor (TCR) δ region. These fragments are cut during the intrathymic rearrangement of the TCR α-chain but remain into the cell as DNA circles. Indeed, the genes for the δ chain of the TCR are distributed within the genomic region that codifies for the α chain, and are removed in two steps (the first giving the signal joint, sj-TREC, the second the coding joint, cj-TREC) during the recombination of V-α with J-α. The removal of genes for the δ chain from the α region does not imply their elimination, as their DNA remains into the nucleus as a circle, and gives origin to the so called TREC (TCR Rearrangement Excision Circles), that are not able to replicate. As a consequence, when a cell undergoes a division, TREC are passed only to one of the two daughter cells; during the following cell cycles, TREC are then diluted into the lymphocyte population that origin from the first cell.

To quantify cells that possess sj-TREC or cj-TREC we set up an original method, that makes use of double competitive-quantitative PCR with a DNA competitor for cj-TREC we have prepared, which has been cloned into the plasmid pFasL we had produced to determine the number of copies of genomic DNA codifying for CD95L (Pinti M. et al., FEBS Lett., 458: 209-214, 1999). That plasmid allows the simultaneous determination of the number of copies of TREC present in a determinate amount of DNA, and then normalize the result for the number of genomic DNA copies in order to precisely calculate the number of copies of TREC per cell. An identical approach has been used to prepare the DNA competitor for sj-TREC.

Data obtained in healthy donors indicate that in children <5 years old about 20% of T lymphocytes have cj-TREC, while such percentage significantly decreases with age. Indeed, in adults 20-40 years old, the percentage we found was 0.21-3.59. In most cases, sj-TREC was not detectable in healthy adults, while in children its ratio with cj-TREC was about 1/3-1/4, as expected. Lymphocytes from individuals with severe, chronic HIV infection were first studied before the beginning of HAART. A statistically lower percentage of cj-TREC+ cells was found, ranging from 0.05 to 1.56. Interestingly, cell from rapid progressors had a lower amount of TREC. Studies are actually in course either on different lymphocyte populations (CD4+, CD8+), and in cells collected from the same HIV+ individuals during HAART, in order to ascertain the role of thymus in the recovery of CD4+ T cell number that usually follows such therapy.

N°. dell'Accordo di Collaborazione: 30C.22

IMMUNOLOGICAL RECONSTITUTION WITH INTERLEUKIN-2 AND G-CSF OF HIV-POSITIVE, ASYMTOMATIC PATIENTS TREATED WITH COMBINED ANTIRETROVIRAL THERAPY. IMMUNOLOGICAL AND VIROLOGICAL STUDY. REINTRODUCTION OF HAART AFTER DRUG HOLIDAYS.

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Therapeutic approach with Highly Active Antiretroviral Therapy (HAART) can lead to suppression of HIV–1 plasma viremia to undetectable levels. However, adherence to complex drug regimens with the occurrence of several side effects can be problematic, and patients may temporarily discontinue HAART.
The aim of our study was to evaluate the safety of antiretroviral therapy interruption, and the immunological changes following the reintroduction of HAART. In addition, we are going to evaluate whether boosting HIV-1 specific immune response should be considered a novel strategy for HIV-1 patients on HAART.
We enrolled 14 patient with HIV-1 chronic infection (12 female) that suspended HAART for severe lipodystrophy (12), hypertransaminase (1) pregnancy (1). All patients gave written informed consent approved by the Ethical Committee. All patients have been on HAART for > 1 year with undetectable HIV RNA copies for 6 months prior to study entry. We evaluated clinical immunologic and virologic parameters, at the suspension of HAART (t0), after 1 month from the suspension (t1), to the resumption of therapy (t2) and after 15 (t3) and 30 days (t4). 7 patients completed the study , 4 patients are completing it, while 3 patients have not still restarted HAART according to the international guidelines on HIV therapy. In 7 patients that have completed the cycle we observed: median t0 CD4+ 625 (SD±132), t1 CD4+ 401 (SD±117), t2 CD4+ 380 (SD±89), t3 CD4+ 411 (SD+263), t4 CD4+ 563 (SD±225). Plasma viremia median value (NASBA) was: t0 HIVRNA: 80 (SD±538), t1 HIVRNA 42,000 (SD± 61,172), t2 HIVRNA 42,000 (SD±439,457), t3 HIVRNA 1700 (SD±21,989), t4 HIVRNA 470 (SD±275). In the 2 patients still out of therapy, we have observed: median t0 CD4+ 878 (SD±323), t1 CD4+ 838 (SD±191). After 4 months from the suspension (t1c) CD4+ was 841 (SD±82). Median of HIVRNA was: T0 80 (SD±0), t1 210 (SD±33,981). After 4 months of suspension (t1c) HIVRNA was 300 (SD±12592). Only a modest reversal of the body shape abnormalities was referred by the patients after HAART withdrawal. In 4 patient a mild cervicoaxillary lymphadenopathy has been observed.
Discontinuation of HAART was not associated with clinical events or deleterious effects in this group of patients after 1 and 4 month of suspension. The interuption of HAART did not seem to modify the fat distribution in patients with lipodystrophy. Viral load did not rebound in 2 patients that showed a marked immuno recovery. The reintroduction of HAART shows viral kinetics and a pattern of immune reconstitution similar to that of naive patients. Further analysis on HIV specific T-helper cell response are under investigation.

Programma nazionale di ricerca sull'AIDS-1999

Accordo di collaborazione scientifica n. 30C/23

ADEQUATE PI LEVELS ARE ASSOCIATED TO HIGHLY MUTATED PR AND RT GENES IN HAART-FAILED POORLY ADHERENT PATIENTS.

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Background: Among the most relevant factors correlated with failure to highly active antiretroviral therapy (HAART) adherence to drug regimen, drugs plasma levels and genotypic mutations have been identified. However their combined role has not been clearly assessed.
Objectives: To determine the role of protease inhibitors (PI) trough levels combined with self-reported poor adherence in a cohort of HAART-failed heavily pre-treated pts undergone genotypic assessment of drug resistance.
Methods: HIV+ HAART-failed pts consecutively followed at a single center. PI plasma trough levels detemined by HPLC and stratified for optimal concentration levels (IDV 100 ng/ml; SQV 100 ng/ml; RTV 1450 ng/ml; NFV 700 ng/ml). Sequencing of RT and PR genes performed on plasma HIV-RNA by TruGene sequencing assay. Adherence analyzed by self-administered previously validated questionnaire.
Results: 111 pts enrolled (M 71%; IVDU 30%;MSM 30%; heteros 31%; CDC group C 37%), median CD4 263/mmc and HIV-RNA 4.28 log10c/ml. 57% of pts had > than 1 HAART failure. Median concentrations of PI (IQR): 150 (64-545) for IDV, 1303 (754-2205) RTV, 76 (34-431) SQV, 1495 (293-2047) NFV. PI levels were optimal for 14 IDV pts (31%), 9 RTV (36%), 21 NFV (60%) and 12 SQV (37%). No baseline viroimmunologic caracteristics were associated with different PI levels. In pts who reported to have forgotten at least 1 dose during the last week (n=46;41%), optimal PI levels were significantly associated with higher mean number of overall RT/PR primary (4.7 vs 2.7;P=0.003) and secondary mutations (5.0 vs 2.8;P=0.001), as well as primary (1.9 vs 1.0; P=0.01) and secondary (3.9 vs 2.2;P=0.004) PR mutations. Non-adherent pts with adequate PI levels had higher probability of having >7 overall RT/PR mutations (OR 30.0; 95%CI 4.2-210.6; P=0.0001). Optimal IDV levels were predictive of undetectable plasma viremia at 3 and 6 mts (OR 17.5;95%CI 1.2-250.3;P=0.04) after genotyping in adherent pts only.
Conclusions: In HAART-failed pts single measurement of PI trough levels only combined with self-reported adherence assessment may allow to identify poorly adherent pts in whom adequate drug levels cannot prevent the appearance of drug resistance mutations. Optimal IDV levels were predictive of virological response in a genotype-guided management of HAART failure.

Accordo di Collaborazione N. 30C.24.

A PROSPECTIVE, RANDOMIZED STUDY ON THE USEFULNESS OF GENOTYPIC RESISTANCE TESTING AND THE ASSESSMENT OF PATIENT-REPORTED ADHERENCE IN UNSELECTED PATIENTS FAILING POTENT HIV THERAPY (ARGENTA): FINAL 6-MONTH RESULTS.

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Background: the relative importance of resistance-guided treatment decisions and of patient adherence assessment on virologic and immunologic outcome is not fully established.
Methods: consecutive patients failing HAART, with HIV-RNA (VL) >2,000 c/mL (bDNA, limit 50 c/mL), were prospectively randomized (1:1) to receive standard of care (SOC) or additional genotypic resistance information (G) (Visible Genetics). Treatment decisions were panel-discussed in both arms. Adherence was measured by a self-administered questionnaire. T-test was used to compare continuous and exact test for categorical variables. Logistic regression was used to analyse associations of variables with virologic endpoints.
Results: 174 patients were randomized (89 to SOC, 85 to G). At baseline, median CD4=264, VL 4.3 log, 25% had failed $\geq$3 HAART regimens, 41% experienced 3 drug classes, median resistance mutations =7 (range 0-17), these were unbalanced between arms (SOC=7, G=8, p=0.03). 123 pts (71%) filled adherence questionnaire; 43% of these, reported last missed dose last week (<95% estimated adherence = non-ADH; $\geq$95% adherence = ADH). By intent-to-treat (174/174 pts), pts with VL <500 c/mL at 3 mo were 12% in SOC and 27% in G (p=0.02); at 6 mo 17% in SOC and 21% in G (p=ns). Among pts with baseline VL<4 log c/mL, those with VL<500 c/mL at 3 mo were 11% in SOC and 48% in G (P=0.0008); at 6 mo 21% in SOC and 41% in G (P=0.10). At 3 mo, pts with VL<500 were 7% in non-ADH with SOC, 19% in non-ADH with G, 25% in ADH with SOC and 32% in ADH with G. CD4 changes at 3 and 6 mo did not differ between randomization groups. Mean CD4 changes at 3 mo were +50/μL in ADH and -12 in non-ADH (p<0.01), at 6-mo +62 in ADH and -13 in non-ADH (p<0.01). Independently predictive of VL<500 c/mL at 3 mo were being assigned to G (OR 2.6;1.1-6.0), a pre-randomization history of VL<500 (OR 2.9; 1.2-6.8), failing the 1st or 2nd HAART (OR 3.2; 1.0-10.0) and <95% adherence (OR 0.4;0.1-0.9); at 6 mo independently predictive of VL<500 c/mL were baseline VL (OR 0.4;0.2-1.0 for each log more), failing the 1st-2nd HAART (OR 5.6;1.2-25.9) and a pre-randomization history of VL<500 (OR 3.0; 1.2-7.5).
Conclusion: genotype-guided treatment decisions benefit also heavily pre-exposed pts but in a time-limited fashion. Patient-reported adherence strongly influences virologic responses and its assessment is more useful than genotyping in predicting CD4 responses. Maximal virologic benefit from genotype-guidance is obtained in adherent pts, in those with lower VL at failure and when enough residual treatment options are available.

Accordo di collaborazione scientifica n.30C.24.

CHANGES IN THYMIC FUNCTION AND IN CD4 T CELL PROLIFERATION IN IL-2 TREATED HIV+ PATIENTS

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Riassunto. Despite its potent antiviral activity, highly active antiretroviral therapy (HAART) only exerts a marginal effect on CD4+ T-cell regeneration in HIV-infected subjects. Combination therapies aimed at boosting T-cell activity and maturation may provide an important contribution to the restoration of immune function. Here we report the results obtained by a two-year follow up of a cohort of HIV-infected patients treated with a combination of HAART and interleukin-2 (IL2). In these patients, besides a series of quantitative virological and immunological parameters, we investigated T-cell regeneration by an immunophenotypic assay monitoring CD4+ naïve T cells and by analysis of thymic function through the quantification, by a competitive quantitative polymerase chain rection assay, of the excision DNA products of T-cell receptor rearrangement (TRECs) in lymphocytes. Furthermore, we also assessed CD4 T cell proliferation by the flow cytometric measurement of intracellular Ki67 positivity. As compared to HAART alone, we found that the IL2 combination therapy was equally effective in reducing the levels of viremia and marginally more effective in decreasing proviral DNA load. Strikingly, the IL2 combination produced a marked increase in the number of CD4+ T-cells bearing a naïve phenotype (CD45RA+, CD62L+), which was apparent for over 96 weeks after therapy. To assess whether these cells were the product of improved T-cell generation, we exploited a competitive quantitative molecular assay to quantify TRECs in peripheral blood lymphocytes. Surprisingly, we found that, as compared to pretreatment values , the levels of these molecules were unchanged in these patients (t=0 300 molecules/10^6molecules in HAART and 650 mol in the HAART/IL-2, t=96 weeks 400 mol in HAART and 630 in HAART/IL-2).When patients treated with IL-2 were further analyzed, we found a sharp increase of the percentage of Ki67+ CD4 lymphocytes within the first 24 weeks of therapy, while proliferation was negatively affected in the HAART group. These findings indicate that improved thymic function does not account for the early rise of CD4 naïve cells in HIV+ patients treated with IL2, and suggest that alternative mechanisms of T-cell maturation and differentiation are responsible for this event. As compared to HAART alone, IL-2 produced a rapid increase of Ki67+ CD4 lymphocytes, suggesting that the expansion of this subset during therapy may be related to a selective CD4 proliferation.Collectively, these results may offer the potential to correctly plan immune activating regimens.

N°. dell'Accordo di Collaborazione 30C.25.

IMMUNORECONSTITUTION AND VIRAL DYNAMICS IN HIV-1 INFECTED INFANTS UNDER HIGHLY ACTIVE ANTIRETROVIRAL THERAPY.

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Immunoreconstitution in HIV-1-infected adults under highly active antiretroviral therapy (HAART) is biphasic with an initial rapid rise in CD4+ memory cells accompanied by a slow repopulation with newly produced naive CD4+ cells. In HIV-1-infected infants, immunoreconstitution may differ due to the thymus' greater potential for regeneration. This study evaluated immunoreconstitution and viral dynamics in 18 HIV-1 infected children (median age 8.5 years, range 2.4 - 14.4 years), all naive for protease inhibitors, receiving HAART, and followed for at least 12 months. Plasma HIV-1 RNA and HIV-1 cell associated DNA were quantified by reverse transcriptase-PCR assay and real-time PCR assay, respectively; output of recent emigrant thymic cells was quantified by measuring the excisional DNA products of TCR-gene rearrangement (TREC), using a real-time PCR assay.
Plasma HIV-1 RNA dropped to undetectable levels (<200 copies/ml) within 3 months of therapy in 12 infants, and remained persistently high in 6 infants. After 12 months of therapy, virological responders showed a median decrease of 0.5 $\log_{10}$ in HIV-1 DNA load. CD4+ cell count increased in all virological responders, and in the 6 children with virological failure. Among virological responders, the slopes of TREC level increase correlated significantly with the slopes of CD4+ cell increase, considering both the entire follow-up period ($p< 0.05$), and the first 3 months of therapy ($p< 0.05$). Of interest, in the 6 viremic immunological responders, no correlation emerged between the slopes of TREC level increase and slopes of CD4+ cell increase, and the ratios of these two parameters were higher than those observed in virological responders.
Our results indicate that repopulation by recent emigrant thymic cells is an early event in the immunoreconstitution occurring in children having a virological response under HAART, and suggest that a higher thymic cell output sustained the immunological improvement in children with virological failure.

Accordo di Collaborazione N. 30C.26

A POLYMORPHISM IN THE CCR5 PROMOTER REGION INFLUENCES DISEASE PROGRESSION IN PERINATALLY HIV-1-INFECTED CHILDREN

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The role of CCR5 promoter polymorphisms on the natural history of disease was studied in 73 human immunodeficiency virus type 1 (HIV-1)-infected children. The $CCR5_{59338\text{-}59537}$ promoter haplotype, the CCR5-59029A/G polymorphism, the CCR5Δ32 and the CCR2-64I alterations were investigated. Excluding carriers of CCR5Δ32 or CCR2-64I, Kaplan-Meier analysis disclosed that children with $P1/P1_{[59353C,\ 59356C,\ 59402A]}$ genotype progressed faster to disease than children with other haplotypes (p=0.016); when CCR2-64I carriers were included, this effect had borderline significance (p=0.065), and was lost when CCR5Δ32 carriers were also considered (p=0.387). Moreover, the P1/P1 effect was strongest early after infection, when progression to disease was mainly associated with CCR5 coreceptor-using viruses. These results indicate that P1/P1 genotype is predictive of rapid progression in HIV-1-infected children lacking CCR5Δ32 or CCR5-64I alleles. Furthermore, the observation of a linkage disequilibrium between P1 and 59029A might explain the previously reported association between 59029A homozygosity and rapid disease progression.

Accordo di Collaborazione N. 30C.26

ANALYSIS OF NEUROTOXIC ACTIVITIES AND PRODUCTION OF PUTATIVE NEUROTOXIC MEDIATORS BY LYMPHOMUNONUCLEAR CELLS FROM HIV-1 INFECTED INDIVIDUALS DURING HAART

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N°. dell'Accordo di Collaborazione: 30C. 27; 1999

HIV-1 induced neurological dysfunction has been directly related to CNS invasion by the virus which is principally, if not exclusively, supported by blood-derived lymphocytes and monocytes/macrophages. We have previously determined that mononuclear cells from HIV-1 infected individuals spontaneously release soluble mediators which inhibit the growth and survival of developing neurons as well as the viability of quiescent neuronal cells by inducing apoptotic cell death and that oncostatin M (onc-M) is a major mediator of these effects, although low TGFβ concentrations were capable of enhancing oncM-mediated neuronal alterations. Thus, oncM may play a prominent role in the setting of the neuronal cell damage observed in HIV-1 infected individuals although it may function in concert with other cytokines in inducing neuronal demise. These observations are consistent with previous studies and suggest that both HIV-1 infection and functional activation can induce lymphomononuclear cells to express neurotoxic potentials which relay upon the production of bioactive substances that, in turn, induce neuronal growth perturbations and apoptotic cell death. However, only a fraction (14 out of 35; 40%) of the patients that showed immune-mediated neurotoxic activity (32 out of 35; 91%) had clinical or subclinical evidence of CNS alterations at study enrollment. This may simply reflect a dissociation between the presence of CNS histopathological alterations, undetected by neuroimaging studies, and clinical or subclinical evidence of CNS injury which has been often described in HIV-1 infected individuals. Alternatively, these data suggest that in order to exert neurodamaging effects, the immune-mediated "neurotoxic potential" requires additional steps and/or components such as CNS influx of HIV-1 infected or activated cells which, in turn, depend upon the integrity of the blood-brain barrier and the production of soluble chemoattractants. The recent availability of highly effective antiretroviral regimens based on multiple drug combinations, indicated that the sustained decline of viral load in plasma and tissues can result in an increase of T-cell counts in peripheral blood which is often associated with an improvement of the clinical and immunological status. However, whether and how HAART can impact the deleterious effects of HIV-1 infection in the brain is, at present, unclear. To verify this, the neurotoxic activities against primary neuronal cells and the production of putative neurotoxic mediators such as oncM by lymphomunonuclear cells were investigated in thirteen HIV-1 positive patients during a twelve-months follow up of HAART regimen. The results indicated a progressive and significant increase of the neurotoxic activities spontaneously expressed by lymphomononuclear cells in vitro. Such increased levels of neurotoxicity were evident at any time point during HAART ($p<005$ after 6 months of therapy; $p<0.01$ after 12 month of HAART) and were accompanied by significant increments of spontaneous oncM production by the same cells. Indeed, different class of soluble mediators appear altered by HAART. Similarly to oncM, both IL2 and IL4 production were increased by HAART, while production of IFNγ was further reduced during therapy in association to a significant downregulation of the production of β-chemokines (RANTES, MIP1-α and MIP1-β). These results suggest that HAART might not entirely control the expression of soluble mediators capable of exerting neurotoxic activities, although a progressive normalization of systemic immune activation as well as the reduced production of soluble chemoattractants may contribute in controlling the influx of activated/infected lymphomonocytes in the CNS thus beneficially complementing the antiviral efficacy of HAART.

NEURONAL INJURY INDUCED BY ONCOSTATIN M: MECHANISM OF ACTION AND MODULATION BY HIV-1 GP120 AND TAT

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By utilizing primary cultures of fetal human neurons, we determined that oncostatin M (oncM) produced by HIV-1 infected or activated immune cells exerts anti-proliferative and pro-apoptotic effects on both developing and post-mitotic neurons and that $TGF\beta_1$ can act in concert with oncM and synergistically increase the inhibitory effects induced by oncM. The mechanism(s) by which oncM can alter neuronal viability and growth have been investigated both at the level of receptor-mediated interactions, by using distinct gp130 ligands as well as neutralizing or agonist Ab and by exploring potential interferences with the production of neurotrophic growth factors, assessed by northern blot analysis and blocking experiments. The results indicated that the effects of oncM on primary neuronal cells may not depend upon the expression of the gp 130 or LIFRα molecules and that an additional receptor component is required on target cells, since other gp 130 ligands such as IL6, LIF, CNTF or IL11 as well as agonist anti-gp130 Ab, exert a protective effect on neuronal survival while oncM is capable of reducing neuronal viability in the same experimental setting. Northern blot analysis of total RNA from oncM-treated neurons revealed a consistent inhibition of the expression of bFGF transcripts. This suggests that oncM can downregulate the production of neurotrophic growth factors such as bFGF that play key roles in supporting neural cell survival, growth and functional maturation. Indeed, blocking of endogenous bFGF production or utilization by antisense oligomers or anti-bFGF neutralizing antibodies, respectively, induces a strong inhibition of neuroblast proliferation. In addition, blocking of bFGF may also provide pro-apoptotic signals, though potency and kinetic differ from that observed in the presence of oncM. Finally, the addition of exogenous bFGF to oncM-treated neuronal cells can rescue them from the growth inhibition induced by oncM. These results suggest that multiple and possibly distinct pathways, which involve interactions with specific receptor subunits and altered expression of endogenous neurotrophic growth factors are responsible for the anti-proliferative and pro-apoptotic effects exerted by oncM on CNS cell targets. In subsequent studies, we investigated whether viral proteins such as gp120 and Tat which have been suggested as potential neurotoxins and are released by infected cell in a biologically active form, might contribute to the deleterious effects induced by oncM. The results of these studies, though preliminary, indicated that both gp120 and Tat are devoided of any neurotoxic activity when directly incubated with primary neuronal cells, at least in the model systems examined, nor they showed any combinatorial effect when tested on primary neurons in the presence of different oncM concentrations. However, both proteins were capable of inducing the expression of neurotoxic activities when pre-additioned to uninfected cells of monocyte/macrophage lineage. These effects were associated to an increased production of oncM by the same cells. These results indicate that the levels of viral replication in the brain, with the consequent increased production of both regulatory and structural proteins can contribute to neuronal damage both directly and indirectly, by altering the production of soluble mediators which ultimately control neuronal survival and growth.

THE BFRF1 GENE PRODUCT OF EPSTEIN-BARR VIRUS: INTRACELLULAR LOCALIZATION OF THE PROTEIN IN EBV PRODUCING AND NONPRODUCING CELL LINES, AND ITS POTENTIAL USE AS A SEROLOGIC MARKER OF VIRAL INFECTION.

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We have recently identified a novel protein encoded by the BFRF1 gene of the Epstein-Barr virus, which is antigenic "in vivo" and expressed early in the viral replicative cycle (Farina et al, J. Virol., 2000). The only herpesvirus homologue thus far described, the UL34 protein of herpes simplex virus type I, has been very recently shown to be targeted to the nuclear membrane following viral entry and required for the envelopment of newly produced virions, suggesting that UL34 might exert multiple functions both at early and late steps during infection. To begin to address the issue of the possible functional role(s) of BFRF1, we analyzed in more detail its subcellular localization in EBV producing and nonproducing cell lines using immunoblotting and cellular fractionation, immunofluorescence and immunoelectron microscopy. In TPA-induced Raji and B95-8 cells, the protein was preferentially distributed over the cell nuclear membrane, whereas upon transfection of BFRF1 in the EBV negative Burkitt's lymphoma cell line DG75, it localized predominantly over the plasma membrane and in the cytoplasm. The membrane localization was abolished when the cells were transfected with a C terminal deletion mutant of BFRF1 lacking the transmembrane domain. The above observations indicate that the nuclear membrane localization is independent by the presence of budding virions, but required the expression of EBV genes, and suggest that additional proteins, expressed early during viral lytic infection, might be necessary to target the protein to the nuclear membrane. Experiments are ongoing to attempt to identify these proteins and their possible interactions with BFRF1.
In addition, to verify the possible usefulness of BFRF1 as a diagnostic or prognostic marker of EBV infection "in vivo", we performed a survey for antibodies to BFRF1 in subjects affected by different EBV related and unrelated diseases. Prevalence of antibodies to BFRF1 was highest in patients affected by nasopharyngeal carcinoma (77.5%) and Burkitt's lymphoma (46.7%), whereas only 2/219 (0.9%) seropositive individuals were present in a heterogeneous group encompassing subjiects affected by infectious mononucleosis, multiple myeloma, hemophilia, breast cancer and healthy blood donors. Patients affected by lymphomas other than BL, mostly HIV positive, showed an elevated prevalence of anti-BFRF1 antibodies (9.4%). Thus, since 46 out of the 48 seropositive individuals were affected by an EBV associated neoplastic disease, our data suggest that BFRF1 might be considered an additional marker to study pathologies that could be related to EBV reactivation.

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MODULATION OF THE TRANSLOCATED C-MYC ONCOGENE BY PNA-NLS SPECIFIC FOR THE Eμ ENHANCER IN BL CELL LINES.

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In Burkitt's Lymphoma (BL) a reciprocal translocation t(8;14) juxtaposes the second and third c-myc exons, to the IgH loci. BL cells are characterized by c-myc hyperexpression which causes an increased proliferative capacity concomitant with a special propensity to undergo apoptosis. The translocated c-myc oncogene expression is under the control of the Eμ enhancer, a potent Ig transcriptional controlling element.
Peptide Nucleic Acids (PNA) are synthetic structural homologues of DNA or RNA. They can tightly and selectively bind to complementary strand of DNA and, if allowed to enter the cell, they can block the expression of the target gene. Unmodified, uncharged PNA can not access viable cells nuclei since they enter the cells but remain entrapped into cytoplasmic vesicles.
In a previous study, we described a PNA complementary to a unique sequence located at the beginning of the second exon of the c-myc oncogene that was covalently linked in the N-terminal position to the Nuclear Localization Signal peptide of the SV40 virus (PNA-myc-NLS). When BL cells were exposed to PNAmyc-NLS in vitro, the anti-gene construct was localized predominantly in the cell nuclei and a rapid consequent downregulation of c-myc expression occurred. Under these conditions, both completion of a productive cell cycle and apoptosis were inhibited.
The PNAmyc-NLS, however, could not be extended to pre-clinical. Since prevents the expression of the translocated c-myc as well as of the normal gene, it cannot be used for clinical purposes.
In order to achieve a selective modulation only of the translocated and hyperexpressed c-myc allele we designed a new PNA-NLS directed to a non coding region within the sequence of the Ig Eμ enhancer (PNAEμ-NLS). A PNAEμ-NLS, was chosen that specifically hybridized to an Eμ sequence representing the binding site for two major regulatory factors responsible for the hyperexpression of the translocated c-myc
We have demonstrated the specific hybridization of PNAEμ-NLS to the desired Eμ sequence by Electro Mobility Shift Assay (EMSA). The exposure of BL cell lines to PNAEμ-NLS in vitro resulted in downregulation of c-myc and IgH expression. No effect on c-myc expression was detected on lymphoblastoid cell lines without t(8;14), as determined by run off transcription assay, Western blot, and RT-PCR. Finally PNAEμ-NLS treated BL cells displayed a largely impaired growth capacity and concomitantly there was a substantial reduction in the proportion of cells in the S or G_2M phases of the cell cycle.
We believe that the new modified PNAs (PNA-NLS) could provide an innovative and effective tool for the in "vivo" antisense and anti-gene therapy. effective tool for the in "vivo" antisense and anti-gene therapy.

N° dell' Accordo di Collaborazione 30C.29

ANALYSIS OF STEPWISE GENETIC CHANGES IN AN AIDS-RELATED BURKITT'S LYMPHOMA.

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Patients with AIDS have a high frequency of Burkitt's Lymphoma (BL). In this setting, BL resembles sporadic BL (sBL) except that the EBV genome is found more frequently (in up, to 50% of cases, depending on different reports).
BL cells are characterized by chromosomal translocation, of the c-myc oncogene, which moves from its normal location on chromosome 8 to chromosome 14 or, less frequently, to chromosome 2 or 22. Because of this translocation, c-myc becomes juxtaposed with the regulatory regions if Ig genes and is generally over-expressed.
C-myc translocation appears to be insufficient to cause fully neoplastic transformation of the cells in vivo. Other cytogenetic changes commonly found in BL include mutations of c-myc and p53 and changes in the 5'-untraslated region of BCL-6.
We previously showed that primary samples obtained from the bone marrow and the peripheral blood of this BL as well as the cell lines and cell clones derived from them displayed 2 different EBV-fused termini. The 2 groups of malignant cells, however, had the same VDJ and c-myc re-arrangements. Based on this observation we suggested that EBV infection was a late event in lymphomagenesis.
In this study we investigated the timing and the features of other genetic changes that characterized the malignant cells such as immunoglobulin variable (IgV) region genes, c-myc re-arrangements and sequence and p53 status. The Ig V gene sequences were identical for the 2 groups of clones with different EBV-fused termini. The Ig variable heavy (VH) gene sequence displayed a substantial accumulation of point mutations (but no intraclonal diversification), whereas the productive Ig V lambda (V_λ) gene sequences was virtually unmutated. Studies on the Ig V kappa (V_k) locus suggested a receptor revision event (with a switch from k to λ chain production) prior to EBV infection. Likewise, it was determined that the mutation observed in both p53 alleles and in the re-arranged c-myc gene occurred before EBV infection. Based on these findings, we present a model for the various steps of lymphomagenesis. It is proposed that the stimulation by an antigen or a super antigen initially favored the clonal expansion and accumulation of other cytogenetic changes, including those involved in receptor editing. These events occurred prior to or during the germinal center phase of B-cell maturation. Thereafter, possibly upon exit of the cells from the GC, EBV infection occurred, further promoting lymphomagenesis.

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MECHANISM OF HIV-1 COAT PROTEIN, GP120, INDUCED APOPTOSIS IN THE BRAIN OF RAT

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Cognitive impairment, postural disorders and tremor are among the most common symptoms encountered in patients suffering from AIDS dementia complex (ADC), a neurological syndrome described in some 80% of the patients suffering from AIDS (see Price & Perry, 1994). Neuropathological features of the brain described at post mortem are myelin pallor, appearance of multinucleated giant cells, infiltration by blood-derived macrophages, astroglial cell reaction and brain cortical neuronal cell loss (see Price & Perry, 1994). The syndrome has been attributed to infection of the brain caused by the human immunodeficiency virus type 1 (HIV-1) because it is observed in patients free from opportunistic infections or concomitant cancer in the brain, though neuroinvasive strains of the HIV virus infect macrophages, microglial cells and multinucleated giant cells but not neurones (see Price & Perry, 1994). Processing of the virus by cells of the mielomonocitic lineage yields host and viral products known to initiate a complex network of events which may lead neurones to death and development of ADC. In particular, the HIV-1 coat protein gp120 has been proposed as a likely aetiologic agent of the described neuronal loss because it causes death of neurones in culture. Using the TUNEL technique we have shown the occurrence of DNA fragmentation in brain cortical tissue sections of adult rats receiving injections of the viral protein into one lateral cerebral ventricle (i.c.v.; see Bagetta et al., 1999) suggesting the apoptotic nature of neuronal death; the latter deduction has been confirmed at the ultrastructural level (see Bagetta et al., 1999). Recent immunohistochemical and western blotting data do implicate the pro-inflammatory cytokine interleukin 1 beta (IL-1β) in the mechanisms of apoptosis caused by gp120 (Bagetta et al., 1999). Interestingly, recent experimental evidence demonstrates that gp120 induces the expression of COX-2 (Bagetta et al., 1998; Maccarrone et al., 2000), an inducible enzyme whose expression is enhanced by IL-1β. The latter effect is paralleled by a significant accumulation in the neocortex of PGE_2 (Maccarrone et al., 2000) a product of arachidonic acid metabolism which may elevate synaptic glutamate to cause excitotoxic neuronal death (Bezzi et al., 1998). In fact, pre-treatment with a COX-2 inhibitor, e.g. NS398, with competitive and non-competitive NMDA receptor antagonists or with U-74389G, a free radical scavenger of the 21-aminosteroid family, reduced gp120-induced apoptosis (Corasaniti et al., 2000).

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ABERRANT DNA PROMOTER HYPERMETHYLATION OF THE DEATH-ASSOCIATED PROTEIN KINASE AND O^6-METHYLGUANINE-DNA-METHYLTRANSFERASE GENES IS A NOVEL AND FREQUENT MECHANISM OF AIDS-RELATED LYMPHOMAGENESIS

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Non-Hodgkin's lymphomas (NHL) represent a frequent complication of HIV infection and a major source of morbidity and mortality among AIDS patients. In addition to AIDS, the incidence of lymphoproliferative disorders is also increased in individuals with congenital or iatrogenically induced immunodeficiency. The molecular pathogenesis of immunodeficiency-associated lymphoproliferative disorders is a complex process involving viral infection, activation of proto-oncogenes, and inactivation of tumor suppressor genes. Aberrant methylation of CpG islands within promoter regions, causing inappropriate gene silencing, is an acquired epigenetic alteration that serves as an alternative to gene defects in tumor suppressor inactivation in many human cancers. Promoter hypermethylation has been recently shown to be a major mechanism of inactivation of the Death-Associated Protein Kinase (DAP-Kinase), O^6-Methylguanine-DNA-Methyltransferase (MGMT) and p73 genes. DAP-Kinase is a serine/threonine kinase required for apoptosis induced by interferon-γ, TNF-α and FAS. MGMT is responsible for removal of O^6-methylguanine adducts produced by alkylating agents and its loss of expression favors lymphomagenesis in $MGMT^{-/-}$ knockout mice. p73, a recently identified member of the p53 gene family, is involved in the regulation of cell cycle and apoptosis. We have analyzed a panel of 84 AIDS-associated NHL (AIDS-NHL) and 28 post-transplant lymphoproliferative disorders (PTLD) for the presence of aberrant hypermethylation of DAP-Kinase, MGMT and p73 using methylation specific-PCR. Overall, aberrant methylation of DAP-Kinase occurred at sustained frequencies throughout the spectrum of AIDS-NHL, including 79% AIDS-related diffuse large cell lymphoma, 88% AIDS-related Burkitt's lymphoma, 88% AIDS-related Burkitt-like lymphoma, 84% AIDS-related primary effusion lymphoma and 35% AIDS-related primary central nervous system lymphoma. Western blot studies demonstrated absent DAP-kinase expression in cases harboring aberrant promoter hypermethylation. Hypermethylation of MGMT occurred in 65% AIDS-related primary effusion lymphoma and 38% AIDS-related diffuse large cell lymphoma, whereas was restricted to 25% AIDS-related Burkitt's lymphoma and 12% AIDS-related Burkitt-like lymphoma. Hypermethylation of MGMT was apparently absent in AIDS-related primary central nervous system lymphoma. Immunohistochemistry studies demonstrated absent MGMT expression in cases harboring aberrant promoter hypermethylation of the gene. Among PTLD, hypermethylation of DAP-Kinase occurred in 64% B-cell PTLD, but apparently in none of the PTLD with T-cell phenotype, whereas hypermetylation of MGMT occurred in 67% PTLD independent of the disease phenotype. Hypermethylation of p73 was restricted to 10% AIDS-related diffuse large cell lymphoma and primary effusion lymphoma, whereas was absent in other lymphoma types. The implications of these observations are multifold. First, our results provide the first evidence that aberrant methylation is a mechanism frequently involved in the inactivation of tumor suppressor genes in immunodeficiency-associated lymphoproliferative disorders. Second, the high frequency of abnormal methylation of DAP-Kinase indicates an important role for this molecular lesion in the development and/or progression of these disorders and suggests that DAP-Kinase inactivation may synergize with EBV and/or HHV-8 antigens in deregulating apoptosis in these malignancies. Third, the frequency of MGMT methylation is heterogeneous in different subsets of AIDS-associated lymphoproliferative disorders, corroborating the existence of multiple pathogenetic pathways in these tumors. Finally, because MGMT status may influence tumor clone resistance to the cytotoxic effects of alkylating chemoterapeutic agents, the methylation status of MGMT may potentially provide a novel prognostic marker for these disorders.

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ROLE OF CCR5-+/□32, CCR2-+/64I AND SDF1-+/3'A POLYMORPHISMS IN DETERMINING THE LONG-TERM NONPROGRESSIVE CONDITION OF HIV-1 DISEASE.

Balotta C., Corvasce S., Mazzucchelli R., Gianelli E., Santambrogio S. and Galli M. for the r-HoPeS study*.

RATIONALE: The role of certain CCR5, CCR2 and SDF-1 polymorphisms as protective factors in the natural history of HIV-1 disease has been suggested in several investigations. The most studies performed up to date correlated genetic polymorphisms with clinical end-points. Few data are available regarding the correlation between genetic background of these genes and levels of HIV-1 replication.
Aim of this study was to address the role of CCR5-Δ32, CCR2-64I and SDF1-3'A mutant alleles in determining and maintaining the long-term nonprogressive course of HIV-1 disease by (i) analysing the prevalence of the different genotypes of CCR5, CCR2 and SDF-1 genes in LTNPs compared to subjects with a typical course of disease (TPs); (ii) studying the correlation between genetic data and molecular indexes of viral replication in a sub-set of LTNPs; (iii) comparing the prevalence of CCR5, CCR2 and SDF-1 different genotypes in three sub-groups of LTNPs, defined as: hl-LTNPs (stably high CD4 cell counts, low HIV-1 RNA plasma levels), hh-LTNPs (stably high CD4 cell counts, HIV-1 RNA plasma levels>5000 copies/mL) and LPs (late progressors).
METHODS. We studied 77 Long-Term Nonprogressors (LTNPs), 112 Typical Progressors (TPs) and 117 blood donors (HCs), as controls. CCR5, CCR2 and SDF-1 genotyping was performed using PCR, RG-PCR-RFLP and PCR-RFLP, respectively. Levels of HIV-1 RNA in plasma, and unspliced (US) and multiply-spliced (MS) specific transcripts in PBMCs were measured by a competitive RT-PCR. Parametrical and non-parametrical statistic was used to perform the correlation analyses.
RESULTS. CCR5 gene analysis revealed a lower frequency of Δ32 allele (.060) than the one found in Northern European countries (.137 in Swedish). The prevalence of CCR5-Δ32 heterozygous condition was significantly higher in LTNPs (22.1%) compared to TPs (8.9%) (p=.030), while the proportion of subjects with different CCR2 and SDF-1 genotypes was similar among these clinical categories. In a sub-group of LTNPs (n=51), we correlated CCR5, CCR2 and SDF-1 different genotypes with molecular indexes of HIV-1 replication. LTNP subjects harbouring Δ32 or 64I mutant alleles showed similar levels of HIV-1 plasma viraemia and both US and MS transcripts compared to those with wild-type CCR5 or CCR2 alleles, respectively. In contrast to what observed in CCR5 and CCR2 genes, wild-type SDF-1 subjects had lower parameters of HIV-1 activity compared to those with SDF1-3'A allele. All between-group comparisons were statistically significant (p= .0021, .016 and .0031, for HIV-1 RNA, US and MS levels, respectively). The prevalence of CCR2-64I allele showed a decreasing trend among hl-LTNPs (30.8%), hh-LTNPs (22.2%) and LPs (10.3%), while the prevalence of CCR5-Δ32 allele was higher in the two hl- and ll-LTNP sub-groups (30.8% and 33.3%), compared to LPs (10.3%). Interestingly, SDF1-3'A heterozygous status was present in 88.9% hh-LTNPs (p=.034, multinomial logistic analysis).
DISCUSSION. The frequency of CCR5-Δ32 allele is lower in our population compared to Northern European countries, confirming the presence of a North to South decreasing gradient across Eurasia. In addition, we found a prevalence of CCR5-Δ32 heterozygous condition significantly higher in LTNPs compared to TPs. Nevertheless, the comparison between molecular indexes of viral replication in wild-type and heterozygous CCR5 LTNPs revealed no role of this allele in determining LTNP condition. A recent study suggested that the recessive 3'A mutation of SDF-1 gene was able to delay AIDS onset by interfering with the emergence of HIV-1 SI variants, typically during the late stages of the disease. In agreement with other reports, in this study we showed that SDF-1 wild-type rather than heterozygous LTNPs display lower indexes of HIV-1 replication. The coexistence of LTNP persistent condition with high levels of HIV-1 plasma viraemia in SDF1-3'A heterozygous subjects suggest the presence in these patients of another unknown strong factor of protection.
*r-HoPeS participants are: Cadeo GP.,Carosi GP.,Chiodo F.,Concia E.,Di Perri G.,Gritti F., Milazzo F.,Monolo G., Moroni M., Raise E., Suter F.

N°. dell'Accordo di Collaborazione: 30C.32

STRATEGIES TO IMPROVE CTL RESPONSES DIRECTED TO EBV ANTIGENS EXPRESSED IN TUMORS

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Cytotoxic T lymphocytes recognize short peptides derived from the proteolytic degradation of intracellular antigens and are presented at the surface of viral-infected cells in association with MHC class I molecules. Viral epitopes can be substituted by synthetic oligopeptides in the in vitro assays and can be used in vivo to specifically stimulate CTL responses or to amplify in vitro specific CTL that can be transferred to patients in the management of different diseases. The Epstein-Barr virus (EBV) is widely spread in human population and it persists in healthy hosts as life-long asymptomatic infection. EBV has been implicated in the pathogenesis of human malignancies including the Burkitt's lymphoma, nasopharingeal carcinoma, Hodgking's disease and immunoblastic lymphomas arising in immunosuppressed patients. It has been clearly shown, both in vitro and in vivo, that the proliferation of viral infected cells is controlled by CTL responses directed to the immunogenic EBV-antigens. This control is very efficient in healthy subjects, in contrast T cells from patients affected by immunosuppression are unable to suppress the outgrowth of autologous EBV-infected B cells that proliferate causing EBV-positive lymphomas.
New therapeutical protocols based on the passive transfer of EBV-specific autologous CTL that have been amplified in vitro or the treatment with peptides corresponding to immunogenic epitopes may offer the possibility to control the in vivo outgrowth of EBV-transformed B cells. Since the efficacy of these new therapeutical approaches depends on the stimulation performed to expand EBV-specific CTL we concentrated our research program on the development of new methods suitable for specific and full CTL reactivation and on the characterisation of synthetic peptides with high stimulatory capacity. In this respect we have characterized analogues of immunogenic epitopes expressed in tumors in terms of immunogenicity, affinity for HLA class I molecules, stability of HLA/peptide complexes, sensitivity of HLA/peptide complexes to pH treatment, stability of the peptides in biological fluids. The final goal was the identification of new peptides able to efficiently stimulate CTL responses specific for the wild-type epitope. An interesting aspect of CTL cultures we have obtain is the enrichment of subdominant specificities. It has been shown that subdominant epitopes induce protection in vivo and that, in contrast to immunodominant epitopes, they are less prone to mutate and can represent the target of choice for CTL activity. In addition we characterized proteasome inhibitors able to modulate the expression of EBV-derived epitopes in viral infected cells and in EBV-carrying tumors.

N°. dell'Accordo di Collaborazione. 30C.33

ANALYSIS OF HIV DRUG-RESISTANT QUASISPECIES IN PLASMA, PERIPHERAL BLOOD MONONUCLEAR CELLS AND VIRAL ISOLATES FROM TREATMENT-NAIVE AND HAART-EXPERIENCED PATIENTS

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The pattern of HIV-1 reverse transcriptase and protease mutations conferring resistance to antiretroviral drugs was studied in 5 treatment-naive patients and 5 HIV-infected patients receiving HAART [2 reverse transcriptase inhibitors + 1 protease inhibitor] for ≥ 1 year. Direct sequencing was performed on plasma HIV RNA, HIV DNA from peripheral blood mononuclear cells (PBMCs) and RNA from viral isolates. In addition, reverse transcriptase and protease PCR products from PBMCs HIV DNA, plasma HIV RNA and viral isolate RNA were cloned in a plasmid to study the quasispecies distribution of drug-resistance associated mutations. Direct sequencing of HIV DNA from PBMCs and HIV RNA from plasma and viral isolates did not show the presence of drug resistance associated mutations in both reverse transcriptase and protease of HIV from all 5 treatment-naive patients. On the contrary, mutation analysis obtained by cloning plasma HIV RNA and PBMCs DNA showed the presence of drug-resistance related mutations at a low frequency in both HIV enzymes of 4 out of 5 treatment-naive patients. On the other hand, direct sequencing of plasma HIV RNA showed the presence of several reverse transcriptase and protease mutations in all 5 treated patients. Mutation analysis performed by cloning PBMCs HIV DNA, and HIV RNA from plasma and viral isolates, revealed additional reverse transcriptase and protease mutations compared to direct sequencing of the relevant biological samples. All of the additional changes were observed in a minority of clones. In conclusion, our data suggest that less frequent drug-resistant viral variants not detected by direct sequencing of PBMCs, plasma samples or viral isolates are present in both treatment-naive and treatment-experienced HIV patients. These findings may have important implications in the understanding of the selection process of drug-resistant variants under drug pressure.

Accordo di Collaborazione 30C.34

HIGHER SHORT-TERM VIROLOGIC EFFICACY OF THREE-CLASS VERSUS TWO-CLASS HIGHLY ACTIVE ANTIRETROVIRAL SALVAGE THERAPY IN HIV-INFECTED PATIENTS.

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Highly active antiretroviral therapies (HAART) often fail to control viral replication due to a lack of patient compliance, a lack of tolerance to the antiretroviral drugs or the emergence of drug-resistant HIV strains. In this study, the virological response to rescue HAART regimens based upon combinations of drugs belonging to two classes of HIV inhibitors [nucleoside analogs reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs)] versus the virological response to a rescue treatment utilizing a combination of antiretroviral agents belonging to three classes [non nucleoside analogs reverse transcriptase inhibitors (NNRTIs), NRTIs and PIs] was retrospectively analyzed in 63 highly pretreated HIV-infected individuals in whom NRTI and PI therapies failed. Median exposure time for NRTIs was 4.1 years (range, 1-6 years), while median exposure time for PIs was 12 months (range, 2-24 months). Twenty-eight patients (group A) received two-class therapy: n=15 patients received 2NRTIs+ 1PI (d4T+ddI/3TC/ddC+NFV), n=13 patients received 2NRTIs + 2PIs (3TC+d4T+AZT+SQV+NFV), whereas 35 patients (group B) received three-class therapy consisting of a combination of 1NRTI+ 1NNRTI+ 1PI (d4T+ EFV+ NFV). After 3 months of treatment, a significantly greater proportion of patients in group B (23/35, 65.7%) than in group A (8/28, 28.5%) showed a $\geq$ $1\log_{10}$ decrease in the plasma HIV RNA level (P = 0.0034). However, after 9-12 months, 12 of 23 (52.1%) group B responders showed viral load rebound. The results were partially explained by the finding that, at baseline, the great majority (21/27, 77.7%) of group A patients showed mutations conferring resistance to all drugs administered, whereas in group B patients' susceptibility to at least two drug classes was retained. However, after 9-12 months of therapy, most (18/20, 90%) of the short-term responders in group B showed emergence of additional drug-resistant HIV strains. The key role of drug resistance in reducing the virological response to therapy was documented by the association between the emergence of HIV strains with resistance to EFV and NFV and rebound of HIV plasma levels. In conclusion, better short-term virological results were obtained with group B patients, possibly because of a more favourable resistance profile at baseline. However, the emergence of additional mutations hampered long-term response.

Accordo di Collaborazione 30C.34

PREVENTION OF CELLULAR FIV INFECTION USING RETROVIRUS-MEDIATED GENE TRANSFER OF A RIBOZYME WITH CYTOPLASMIC LOCALIZATION

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Reserch Project N. 30C.35

The aim of this research project is to provide proof of principle that gene therapy of HIV can be successful. For this purpose, we are addressing the issue of preventing and curing FIV infection in the cat model by means of a ribozyme expressed at high levels and with specific intracellular localization and delivered by retroviral vectors. The rationale behind this approach stands on the finding that cellular immunization can confer resistance to a population of lymphocytes by protecting cells from infection and preventing viral spread. As viral replication is active even during the latency period between infection and disease, such a population could acquire a sufficient selective advantage to confer to the infected organism some immune function, possibly preventing full-blown immunodeficiency.

We constructed a hammerhead ribozyme targeted against the primer binding site (PBS), an RNA sequence present in all retroviral transcripts and known to be accessible to RNA interaction as it pairs with the tRNA (lys) that act as a primer in viral retro-transcription. This sequence is highly conserved in all FIV as well as HIV isolates.

To achieve high level of expression and cytoplasmic delivery of the ribozyme, we cloned it into a modified adenovirus Va1 transcriptional cassette. Va1 RNA fusion constructs are transcribed by RNA polymerase III, known to be highly active for short transcripts, and possess specific cytoplasmatic localization. Once transduced into the cells, the VA1-ribozyme (Va1-Rz) cassette was able to sensibly reduce the activity of a reporter gene expressed from an HIV vector, in contrast to similar controls in which the ribozyme pairing sequence was scrambled, or had antisense orientation.

Next we prepared feline cell lines (derived form CrFK, which supports FIV replication) expressing the Va1-Rz cassettes and challenged them with active FIV virus. The results showed that the active ribozyme was capable of completely inhibiting viral replication, whereas the controls were not.

The next step was to construct a retroviral vector, based on MoMLV and carrying the Va1 cassette in the 3' LTR, thus producing a double copy vector, which is capable to express the Va1-Rz from both LTRs and as a long LTR driven transcript in transduced cells. These vectors were produced in a transient expression system based on human HEK 293 cells, pseudotyped with the VSV-G envelope, and used to transduce both feline CrFK and human Jurkat T cells.

At present we are studying expression and efficacy of the ribozyme in these cells.

A PHASE II/III RANDOMIZED, OPEN-LABEL STUDY OF COMBINATION ANTIRETROVIRAL REGIMENS AND TREATMENT – SWITCHING STRATEGIES IN ANTIRETROVIRAL NAIVE CHILDREN ≥ 30 DAYS ≤ 16 YEARS OF AGE

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PENTAPACT1 is a trial to be co-ordinated by PENTA and by the Peadiatric AIDS Clinical Trial Group (PACTG) and aims to evaluate the long-term efficacy (in terms of HIV-RNA) of four different HAART combinations in previously untreated children as well as two different strategies of switching therapy. As the questions to be addressed (which combination therapy to start with and when to switch therapy) are of the utmost importance for the management of HIV-infected children and as there is a restricted number of naive HIV-infected children in Europe and the U.S.A., a collaborative study has been deemed necessary. The interest in research and the credibility acquired by PENTA in the years have led the U.S. Regulatory Authorities to recognise automatically the PENTA centres for the first time without any previous approval by NIH or OPRR for each clinical centre, which conversely had been necessary for the previous studies carried out in co-operation with ACTG.

Primary Objectives

1. To compare two different nucleoside reverse transcriptase inhibitor (NRTI) combinations as initial therapy, followed by second-line therapy if failure occurs, determined by a long-term virologic endpoint. The two NRTI combinations to be compared are: [lamivudine+abacavir (3TC + ABV)] versus [didanosine+stavudine (ddI + d4T)].
2. To compare the above NRTI combinations plus a protease inhibitor (PI), or a non-nucleoside reverse transcriptase inhibitor as initial therapy, followed by second-line therapy if failure occurs, determined by a long-term virologic endpoint.
3. To compare two different viral load criteria for switching from first-line to second-line therapy.

Virologic Endpoint Definition

The virologic endpoint is the change in HIV-1 RNA viral load between baseline and four years post randomisation. It is likely that by four years post randomisation, nearly all children will have switched from first to second-line therapy.

Secondary Objectives

1. To evaluate and compare the safety and tolerability of each drug/combination (including first- and second-line therapies).
2. To compare the long-term clinical and immunologic outcomes (based on the initial randomised factors).
3. To compare the proportions of children who have undergone one or two regimen switches (by the initial randomised factors).
4. To compare the proportions of children who have undergone one, two, or more than two regimen switches (by the initial randomised factors).
5. To compare time from randomisation to virologic failure (≥ 400 copies/mL at week 24) of the first-line therapy analysed by initial randomisation to either (3TC + ABC) or (ddI + d4T) and initial randomisation to either NFV or EFV.
6. To compare time from randomisation to virologic failure of the deadline therapy (RNA ≥ 30,000 copies/mL) analysed by the initial randomised factors.
7. To compare the proportions of children with plasma HIV-1 RNA < 400 copies/mL at 24 weeks (analyzed by initial randomisation to either (3TC + ABC) or (ddI + d4T) and initial randomisation to either NFV or EFV).
8. To compare the proportion of children with plasma HIV-1 RNA < 400 copies/mL at 4 years (based on the initial randomized factors).
9. To describe resistance patterns at four years (by the initial randomised Groups).

10. To compare the impact of therapeutic drug monitoring (TDM) versus no TDM carried out at one month after starting first- and second-line therapies. The appropriate adjustment of drug doses will be done, determined by a long-term virologic endpoint. This will be conducted as a sub-study.

Study Design
This is an international multicenter Phase II/III, randomised, open label, and factorial (2x2x2) trial.

Sample Size
256 children, 50 % from United States and 50 % from Europe, 32 children in each of the 8 Groups.

Population
HIV-1 infected, antiretroviral naïve or who have received prior antiretroviral therapy for less than 56 days.

Stratification
Children will be randomised to eight Groups (see below) stratified by age (<3 years versus ≥3 years), origin (PACTG sites or PENTA sites) and exposure versus no exposure to antiretroviral therapy perinatally.

Regimen
The study includes a total of eight randomized Groups:
Group 1(A): 3TC + ABC + PI (switch to second-line treatment when HIV-1 RNA is ≥1000 copies/mL*)
Group 1(B): 3TC + ABC + PI (switch to second-line treatment when HIV-1 RNA is ≥ 30,000*)
Group 2(A): 3TC + ABC + NNRTI (switch to second-line treatment when HIV-1 RNA is ≥1000*)
Group 2(B): 3TC + ABC + NNRTI (switch to second-line treatment when HIV-1 RNA is ≥30,000*)
Group 3(A): ddI + D4T + PI (switch to second-line treatment when HIV-1 RNA is ≥1000*)
Group 3(B): ddI + D4T + PI (switch to second-line treatment when HIV-1 RNA is ≥30,000*)
Group 4(A): ddI + D4T + NNRTI (switch to second-line treatment when HIV-1 RNA is ≥1000*)
Group 4(B): ddI + D4T + NNRTI (switch to second-line treatment when HIV-1 RNA is ≥30,000*)
*at two consecutive visits at or after week 24.

At present PENTA had NIH/OPRR approval for the conduction of the study. Th protocol has been submitted to the relevant Ethics committee in Europe and the enrollment will start early march.

Penta references.

PENTA Publications 1998 - 2000

Results from Trials

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Accordo di collaborazione scientifica n. 30C.36

HIV-1 *TAT* INTERFERES WITH REGULATORY ELEMENTS LOCATED UPSTREAM OF HPV16 "CORE" ENHANCER

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Background: We have previously shown that HIV-1 Tat protein transactivates human papillomavirus type 16 (HPV) regulatory regions (LCR) (Tornesello M.L. *et al*., 1993) and increases HPV-16 E6 and E7 transforming activity (Buonaguro F.M. *et al.,* 1994). More recently we have reported that the transactivation by HIV-1 Tat protein of HPV-16 long control region (LCR), as well as of SV40 enhancer, is cell-type specific being observed only in epithelial cells.

Methods and Results: In order to identify the Tat responsive sequences within the HPV-16 LCR functional assays have been carried out into several cell lines (HeLa, SiHa, HT3, NTERA-2), with or without Tat stimulation, using 6 LCR subfragments to drive the CAT reporter gene. Our results show that both the 5'-end (LCR-1, nt 7307-7446, and LCR-2, nt 7425-7572) and 3'-end (LCR-4, nt 7679-7829, LCR-5, nt 7761-21, and LCR-6, nt 7851-94) of the enhancer region contain "Silencer" elements. The central region (LCR-3; nt 7545-7562), following the removal of such "silencer" shows a basic constitutive expression activity 4-fold higher than the whole LCR . The construct LCR2-3 (nt 7425-7562) shows a 50% decrease of CAT activity compared to the LCR-3 construct, Tat protein completely restores this activity but has no effect on any other LCR subfragment (Tornesello M.L. *et al*., 1999).

Conclusions: These results suggest that Tat protein interferes with negative regulatory elements, present within the LCR-2 region, and inhibits the repression of the core enhancer, present in the LCR-3 region..

Accordo di collaborazione N. 30C.37

PHYLOGENETIC AND PHENETIC ANALYSIS OF ORF 26/72 AND K1 GENE OF HHV-8 VARIANTS IN KS PATIENTS.

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Background: To identify HHV-8 variants and their pathogenic role in KS lesions from HIV-positive as well as HIV-negative patients, recruited in Europe (Greece and Italy), America (USA and Brazil) and East Africa (Uganda and Kenya)
Methods: HHV-8 sequences have been detected by PCR amplification and characterized by phylogenetic and phenetic analysis of ORF26 (Minor Capsid Protein), ORF72 (Cyclin D homolog) and K1 (a potential transforming gene). *Multiple sequence alignments* have been performed with the Lasergene Software (DNASTAR Inc., Madison, WI-USA). *Phylogenetic analysis* has been performed with the software package TREECON (Univ. of Antwerp, Belgium). *Phenetic analysis* and synonymous mutation rates have been calculated by the software PAML (Univ. of California, Berkeley).
Results: Genetic analyses of full length ORF26 show an overall variability of 2.1% among 63 HHV-8 isolates, with a variability peak of 9.0% in a 167 bp fragment (nt 349-516) characterized by low antigenic index and prevalence of synonymous (S) versus non synonymous (N) mutations (d_S/d_N=2.314). The genetic variability of ORF26 allows the phylogenetic characterization of HHV-8 variants and the identification of three major branches (A, B and C) with bootstrap values >90. K1 gene shows an overall variability of 17.8% among 14 HHV-8 isolates, with a variability peak of 80% in a 60bp fragment (nt 158-217). The high variability of K1 has been used to identify sub-clades within the 3 major branches. ORF72 is highly conserved among the analyzed samples with homology >99%, which impairs any phylogenetic analysis.
Conclusions: Phylogenetic analysis of ORF26 and K1 gene allow the identification of three major branches described in Vancouver (Buonaguro *et al.*1996) and compatible with data reported by Zong *et al.*, 1997, 1998. HHV-8 variants prevalently show an ethnic/geographical distribution without pathogenic relevance.

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ACCORDO DI COLLABORAZIONE N. 30C.37

ADHERENCE TO ANTIRETROVIRAL THERAPY IN CHILDREN WITH HIV INFECTION

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Abstract

Objective. To investigate the short and long term adherence to antiretroviral therapy in HIV-infected children and the determinants of non adherence related to children and to caregivers.

Design. Caregivers of HIV-infected children enrolled in 7 reference centers answered a structured interview.

Methods. The interview included quantitative information on adherence in the 4 days before interview and qualitative information on adherence in the last 3 months. Timing of doses was also investigated. Sociodemographic characteristics of children and caregivers were recorded.

Results. 129 children (mean age 100 months) were enrolled, of whom 94 on highly active antiretroviral therapy. 21 (16%) omitted more than 5% of total doses in 4 days and were considered non adherent. Among these, 6 children omitted more than 20% of doses. There was no difference between adherent and non adherent children with respect to age, HIV infection stage, viral load, number or types of antiretroviral drugs. Children receiving therapy by foster parents were more adherent than those receiving drugs by parents or relatives. Children aware of their HIV status tended to be less adherent than those who did not know to be infected. Dose omissions in the last 3 months were reported in 62 cases (48%) and problem in administering therapy in 49 (38%). Only 11% of caregivers reported full respect of timing of therapy.

Conclusions. Lack of adherence is a substantial problem in children and it is related to caregivers rather than to children.

Current status of the research. Caregivers are being again interviewed after a 6t to 12 month lag time from the first interview, in order to analyse the consistency of data and to test the hypothesis that non adherent children have a worse clinical and immunologic outcome.

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HIV SPECIFIC CD4 RESPONSES AND VACCINE DEVELOPMENT

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The development of an effective HIV vaccine is highly desirable as a prophylactic and therapeutic tool. Unfortunately the protective mechanisms are not well established, HIV is a hypervariable pathogen and antigenicity of HIV proteins must be optimized. Since antibodies and CTL are likely to be beneficial, and their induction is T helper cell dependent, we focused on CD4 helper cells as essential components of an immune response. We report on three experimental systems that make use of mice, macaques and humans to investigate helper responses, as summarized below.

1. Mice were used to determine in vivo the epitopes of gp120 that provide optimal help for a V3 specific antibody response. This is a functional definition of helper activity, that is not simply inferred according to CD4 phenotype. Mice were primed i.p with soluble overlapping peptides of gp120 and subsequently boosted with a substimulatory dose of gp120 i.v. V3 specific antibodies were tested in sera. A limited number of peptides was able to prime for V3 antibodies, suggesting that this type of approach identifies peptides that provide functional help for B cells.
2. Our previous data showed that low doses of antigens bound by specific antibodies as immune complexes (IC) are taken up by APC and presented to specific CD4 cells with high efficiency. Thus we prepared SIV gp120 - IgG IC that was administered intrarectally to Rh. macaques. Due to the presence of FcR on rectal epithelium, this resulted in enhanced immunogenicity.
3. Since in vivo experiments cannot be performed in humans, we mimicked priming of human CD4 T cells by an immunogen by generating CD4 T cell lines from non immune individuals. Different preparations of gp120 derived from different strains were used for these assays. PBMC were cultured with gp120, expanded with IL2 and monthly restimulated with autologous APC, antigen and IL2. The most recent experiments showed that it is possible to produce CD4 lines from naive donors specific for different env proteins, that can be used for accurate epitope mapping to define T helper epitopes (conserved or variable) to be incuded in tailored vaccines.

In conclusion, our focus on CD4 T helper cells specific for retroviral antigens can take advantage of different experimental systems and is in keeping with the current views that T helper cells are crucial to control progression of infection and to mount desirable immune responses upon vaccination.

Grant n. 30C.39

PHENOTYPIC ANALYSIS OF CD4+ AND CD8+ T LYMPHOCYTES DURING TREATMENT OF HIV INFECTION WITH HAART ASSOCIATED OR NOT WITH LOW DOSE INTERLEUKIN-2

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The aim of the reserch project was the evaluation of the effects of low dose interleukin-2 (IL-2) on immunological reconstitution in HAART treated HIV+ patients. 16 subjects (12 males and 4 females, age 24-45 years) have been randomized into two groups of 8 patients each to receive: A) HAART; B) HAART associated with low dose subcutaneous IL-2 (500.000 IU/sqm, 5 days a week for 6 months). Immune parameters were evaluated before treatment, at baseline, during treatment and after treatment and are reported in tables 1 and 2 as mean values.

Table 1

	Months		
	- 6	- 3	0
Group A			
CD4 (cells/µl)	295	298	264
CD8 (cells/µl)	824	808	702
CD4/CD8	0.4	0.4	0.4
Group B			
CD4 (cells/µl)	209	222	196
CD8 (cells/µl)	999	1171	974
CD4/CD8	0.2	0.2	0.2

Table 2

	Group A									
	Months									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**10**	**12**
CD4 (cells/µl)	264	280	245	278	271	287	284	291	300	253
CD4 (%)	17	17	16	16	16	17	18	18	17	16
CD4/CD95 (%)	12	11	11	13	13	13	13	12	13	12
CD4/CD45RO (%)	8	7	9	9	9	9	9	8	8	9
CD4/CD45RA (%)	7	7	6	7	7	8	7	7	8	8
CD8 (%)	46	44	45	43	44	42	42	44	43	43
CD8/CD28 (%)	15	19	16	19	17	17	14	16	15	17

	Group B									
	Months									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**10**	**12**
CD4 (cells/µl)	196	271	250	287	288	292	312	275	291	299
CD4 (%)	11	13	13	15	15	17	16	15	14	14
CD4/CD95 (%)	11	10	10	12	12	13	12	12	11	12
CD4/CD45RO (%)	7	6	6	7	7	8	8	8	7	8
CD4/CD45RA (%)	5	5	4	6	7	7	6	5	5	6
CD8 (%)	57	54	54	50	52	50	51	53	55	54
CD8/CD28 (%)	25	24	21	24	24	21	21	21	23	22

Results indicate that the association of HAART with low dose IL-2 leads to a significant and sustained increase in CD4+ cells number.

Project N° 30C.39

AIDS ASSOCIATED B-NHL: MOLECULAR CHARACTERISTICS AND RESPONSE TO THERAPEUTIC MABS.

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In the past years we have studied the biological and molecular characteristics of different subtypes of AIDS associated B-NHL. In particular, we have investigated the cell surface phenotype, the expression and genomic integrity of several protooncogenes including p53, bcl-6, myc and the centroblastic specific A-myb protein in a panel of Burkitt's like and body cavity derived, AIDS associated, B-NHL. The A-myb gene was found to distinguish between the Burkitt's like lymphomas and the body cavity subtype of lymphoma thus suggesting a role for this proto-oncogene in the pathogenesis of the Burkitt type.
More recently, in an effort to provide the experimental basis for the clinical development of new therapeutic tools for these lymphomas, such as B specific monoclonal antibodies, we have set up cytotoxic assays in vitro in the presence or absence of human complement using a panel of AIDS-derived and non-AIDs derived B-NHL of different subtypes. In particular we have studied the two reagents, i.e. Rituximab (a chimeric anti-CD20 antibody) and Campath-1H (humanised anti-CD52). Interestingly, the results have shown that the ultimate lytic effect is dictated by a balance between the surface expression levels of CD20 and CD52 antigens and the expression of the complement inhibitory molecules CD55 and CD59. Furthermore this analysis has been extended to antibody dependent cellular cytotoxicity experiments performed by the addition of purified populations of NK cells and to the effect of the antibodies on cell proliferation and apoptosis, using several different parameters (Annexin V, TUNEL). These studies are of primary importance to evaluate the predictive role of such in vitro tests for the clinical response to Rituximab and Campath-1H in treated lymmhoma patients. Finally the capacity of chemotherapeutic agents, and in particular Fludarabine, together with Rituximab to kill different AIDS-derived cell lines is being investigated, since Fludarabine has been shown in follicular lymphoma cells to synergise with Rituxmab. These data on the possible therapeutic strategies for AIDS-derived B-NHL will be presented.

N°. dell'Accordo di Collaborazione. 30C.40

EFFICACY OF A LOW DOSE OF INTERMITTENT SUBCUTANEOUS INTERLEUKIN-2 (IL-2) IN ANTIVIRAL-EXPERIENCED HIV-INFECTED INDIVIDUALS WITH DETECTABLE VIRAL LOAD. TWO YEAR EXTENDED FOLLOW-UP.

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At the end of the main study, all individuals were evaluated as outpatients with determination of their peripheral $CD4^+$ T cell counts on a monthly basis, and of their viremia levels every 3 months. Patients underwent new cycles of IL-2 (3 MIU/BID for 5 days every 4 weeks) if the number of circulating $CD4^+$ cells dropped below 70% of the level reached at the end of the study, calculated as the mean of the last two determinations.
At present, 38 individuals (11, 15, and 12 from the CIV/HD, HD, and LD arms, respectively) have been followed for a median of 30 months (range: 23-34 months) after completion of the trial. At the end of IL-2 administration, the median (range) of CD4 and CD4/CD8 ratios were 974 cells/μL (519-1500), and 1.04 (0.43-2.87) in the CIV/HD arm, 825 cells/μL (490-1280) and 1.02 (0.34-1.69) in the HD arm, and 926 cells/μL (452-1320) and 1.17 (0.67-3.10) in the LD arm, respectively. In order to maintain levels of $CD4^+$ T cells either above 1,000 cells/μL or twice their individuals' baseline values up to 3 cycles of IL-2 were administered to 3 individuals (2 cycles after 12 months, and a last one after 16 months from therapy interruption) belonging to the CIV/HD arm. Similarly, 6 cycles (after 9, 12, 13, 14 and 17 months, from the end of the trial) in the HD arm, and 4 cycles in the LD arm (after 9, 10, 12, and 16 months from the end of the study, respectively) were administered for the same purpose. During this period, mean viral loads were 2.81, 3.72, and 2.60 logs in the CIV/HD, HD, and LD arms, respectively. The number of individuals with HIV-RNA below 500 copies of HIV/μL was 4 out of 11 (36%), 6 out of 15 (40%), and 6 out of 12 (50%) in the CIV/HD, HD and LD arms, respectively. During this period, 8/11 patients of the group CIV/HD, 12/15 of the group HD and 8/12 of the LD arm, underwent modifications of their HAART regimen, accordingly to the genotypic analysis of HIV resistance.
In conclusion, our study provides additional indication that IL-2 can be an important agent capable of acting synergistically with antivirals and ultimately contributing to the long-term management of HIV disease. The observation that also a relatively low daily dose of IL-2 (3 MIU/BID) is effective in stably raising $CD4^+$ T cells indicates that control of HIV disease progression can likely be achieved by different, converging approaches.

Grant n° 30C.41.

LONGITUDINAL STUDY ON THE EFFECT OF DIFFERENT ANTIRETROVIRAL REGIMENS ON HPV INFECTION AND RELATED LESIONS

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Despite advances in antiretroviral treatment and the reduction of opportunistic diseases, oncologic complications of human immunodeficiency virus type 1 infection continue to occur. Objective of this study was to evaluate the evolution of human Papillomavirus related cervical lesions in women treated with Highly Active Antiretroviral Therapy (HAART) in comparison to women untreated or receiving reverse transcriptase inhibitors only (RTI).
We performed a longitudinal study on virological, cytological and hystological markers of HPV cervical infection in 206 HIV positive women. The observation period ranged from 12 to 64 months (median 29.4 months) with a mean of 3 visits/patient. The clinical and virological data were fully analysed after a median follow up period of 15.4 months in 160 of these women (27 untreated, 60 RTI treated and 73 HAART treated). HPV viral sequences were screened using a commercially available test (Hybrid Capture System II - Diagene, MD-USA) and typed by an home-made PCR. The proportions in the different groups were compared using χ^2 test, χ^2 for trend, or Fisher's exact test when appropriate. The relative risk for acquiring or persisting high risk HPV infection and for cytological lesions progression, was estimated by logistic regression after adjusting for CD4 cell count and gynaecological treatment.
We confirm that immunocompromised hosts have a greater prevalence of HPV infection (65% by HC-II, 80.9% by PCR) and squamous intraepithelial lesions (cytology: LSIL 20.2%, HSIL 6.2%; histology: LSIL 35.3%, HSIL 10.9%), and that these prevalence significantly correlate with a low CD4+ count (p= 0.015 and p=0.022 respectively). Although HAART improves immune response, it doesn't lead to significant differences in the persistence rate of HPV infection (p=0.43), or high-grade lesions (p=0.403 for progression and 0.798 for regression), from that observed in women untreated or receiving reverse transcriptase inhibitors only (RTI). Having a CD4 count below 350 at the end of the follow up period (OR:2.4, 95%CI=-4.5, p=0.013) was the only variable with a strong association with persistence of HPV infection. When patients were classified according to both CD4 cell counts and viral load at the follow-up visit (four classes: 1=CD4 cell counts above 350 and undetectable viremia, 2= CD4 cell counts above 350 and detectable viremia, 3= CD4 cell counts below 350 and undetectable viremia,4=CD4 cell counts below 350 and detectable viremia) a positive and significant trend in the frequency of persistent lesion (from 21.7% to 46.8%, p=0.05) and of persistent or worsening lesions (from 21.7% to 51.1%, p=0.04) was observed across the four classes.
We conclude that the use of HAART doesn't influence per se the clinical course of high-risk HPV infection particularly when the immuno-virological baseline is poor. It's likely that prolonged patient survival may lead to the manifestation of slowly evolving diseases (neoplasms), thus suggesting that HIV-infected women should be carefully monitored for the emergence of high-grade SILs and cervical cancer.

N°. dell'Accordo di Collaborazione: 30C.42

MONONUCLEAR CELLS OF HIV-INFECTED PATIENTS SECRETE INCREASED LEVELS OF MATRIX METALLOPROTEINASE-9.

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In vitro studies indicate that HIV-1 infection of T-cells and monocytes leads to increased secretion of extracellular degrading matrix metalloproteinases (MMPs) and enhances the ability of these cells to traverse the basement membrane. There are no data about the production of MMPs from mononuclear cells of HIV-1 infected individuals.
Culture supernatants collected from peripheral blood mononuclear cells (PBMC) (4x105) isolated from 23 HIV-infected patients and 8 HIV-seronagative healthy donors were subjected to gelatin-zymography for the assessment of MMP-9 activity.
Among the HIV-infected patients, 16 received antiretroviral therapy (median CD4+ cells, 268/µl; median HIV-RNA, 21.111 copies/ml), while 7 patients were naive for any antiretroviral treatment (median CD4+ cells, 126/µl; median HIV-RNA, 190.833 copies/ml). Quantitative determination of MMP-9 activity was done by computerized image analysis and scanning densitometry.
The levels of MMP-9 activity were significantly elevated in 24 h culture supernatants from PBMC of HIV-infected patients (27.063$\pm$3.3; mean$\pm$SEM) in comparison to HIV-negative controls (14.181$\pm$1.7; mean+SEM). In the 16 HIV-infected patients receiving antiretroviral therapy MMP-9 levels resulted to be lower (23,36$\pm$2,36; mean$\pm$SEM) than those measured in the untreated HIV-infected patients (31,33$\pm$3,85; mean$\pm$SEM). These data indicate that circulating mononuclear cells from HIV-positive patients secrete elevated levels of MMP-9 which may contribute to increased localization of HIV-infected PBMC in tissues. Moreover, antiretroviral treatment could affect MMP-9 release from HIV+ mononuclear cells.

N^{o}. dell'Accordo di Collaborazione. 30C.43

ANTIVIRAL RESPONSE AND RECOVERY OF THE IMMUNE SYSTEM FUNCTIONS IN PATIENTS TREATED WITH A STRUCTURED INTERRUPTION THERAPY (STI) REGIMEN AFTER PRIMARY AND CHRONIC HIV INFECTION.

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Background: Eradication of HIV-1 by antiretroviral regimens seems to be an unachievable goal. Even, when highly active antiretroviral therapies (HAART) result in persistently undetectable viral load (VL), withdrawal from treatment is usually followed by a rapid viral rebound. In a patient who was given the combination of hydroxyurea (HU), didanosine (ddI), and a protease inhibitor before complete seroconversion treatment was interrupted twice before permanent interruption. After 2 years from treatment interruption HIV-1 RNA in the plasma was still under the limit of detection (< 500 copies/ml). This finding suggested that STI contribute to the HIV-1 replication control, to study this issue we organized three studies.

Study 1: In a pilot study three antiretrovirals naïve patients were treated with an HU containing-HAART after complete seroconversion. Following three months of continuous treatment patients underwent to several STI cycles. In one patient after the second interruption VL remained around 2000 copies/ml for 6 months, then therapy was restarted since VL rebounded over 5000 copies/ml. In two patients, the time to rebound (VL > 5000 copies/ml) increased during subsequent interruptions, and viremia remained < 5000 copies/ml for as long as 183 and 147 days in absence of treatment (2).

Study 2: In a randomized controlled trial conducted using SIV-infected rhesus monkeys, was evaluated whether the effects of fixed-scheduled STI would improve the benefits of HAART. After 21 weeks both STI-HAART and HAART controlled VL and increased CD4 percentage. However, the drug-related side effects that occurred during continuous HAART were not observed during STI-HAART. Interestingly, all STI-HAART animals controlled virus rebound after permanent treatment discontinuation and the virus control was associated with vigorous SIV-specific T cell immunity (1).

Study 3: The above studies suggested that VL set-point can be lowered by STI during acute infection. We wanted to verify whether this can apply during chronic infection. In a prospective, randomized, controlled trial 60 drug-naïve patients with chronic HIV infection were randomized to receive ddI-d4T-IDV or ddI-d4T-HU. After 12 weeks therapy, they were further randomized to receive 24 weeks of fixed-schedule STI (3weeks on/3weeks off therapy, 4 cycles), or HAART.
The interim analysis on 30 patients showed: baseline VL copies was suppressed by week 12 to <50 and 66 in the IDV and HU groups, respectively. VL rebounded at each therapy interruption, in most cases to values similar to baseline, regardless of the drug regimen. The rate of VL rebound did not change significantly during subsequent interruptions. Restarting therapy suppressed VL to values similar to the continuous HAART group. At week 36, VL was 182 and 70 copies/ml in the STI and continuous HAART groups, respectively. CD4 count (cells/mm^3) increased in the STI-HAART/IDV group during therapy and transiently decreased during

interruptions. At week 36, the median increase was 154 (*P*=0.036 from baseline), similar to the continuous HAART/IDV group (167). In the continuous HAART/HU group a CD4 decrease of -27 was observed at week 36.

Conclusion: Combination therapies can be interrupted and restarted several times and HIV-1 can be controlled successfully, no drug resistance seem to emerge after several STI cycles. The study performed in the PHI patients as well as in the monkeys suggest that STI can lower the viral set-point if used during acute infection; however, this cannot be achieved in chronic infected patients. There are some indications that fixed-scheduled STI can decrease the toxicity of HAART without sacrificing the efficacy, during acute and chronic infection. Further studies to address the effects of STI on drug-related toxicity are highly required in order to evaluate the role of STI in the treatment of HIV infection.

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N°. dell'Accordo di Collaborazione. 30C.44

RESPONSE TO POTENT ANTIRETROVIRAL THERAPHY AND LEVELS OF SPONTANEOUS APOPTOSIS IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HIV PATIENTS.

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The aim of this study is to investigate the significance of apoptosis in peripheral blood mononuclear cells (PBMC) from HIV-1-infected receiving potent antiretroviral treatment with two nucleoside reverse transcriptase inhibitors plus one or two protease inhibitor or one non-nucleoside reverse transcriptase-inhibitor. To this purpose, a cohort of patients was analysed prior to and 1, 2, 4, 6, 12, 16, 20 and 24 months after the beginning of therapy, in the framework of a longitudinal open study. The results obtained after 6 months of therapy in 12 evaluable patients have shown that either spontaneous and anti-Fas-induced apoptosis progressively and significantly ($p<0.001$) decreased. Levels of apoptosis correlated inversely to CD4+ cell count and directly to viral load in highly significant way. Expression of Fas was directly correlated to apoptosis while Bcl-2 expression did not show significant changes until this time-point (AIDS 2000,14:939-949). The follow-up of the patients in the successive 18 months have shown that apoptosis, even if some fluctuations occurred, was invariably maintained at low level and in some cases at levels of HIV- donors (>10%), as a response to the therapy. Moreover, during this longitudinal study we had the opportunity to analyse the apoptotic pattern in three cases of HIV-infected patients who achieved and maintained a viral suppression for significant length of time before experiencing treatment interruption. Discontinuation of therapy was associated with a marked and prompt resumption of viral load. Viral rebounds due to treatment interruption were invariably associated with a marked increase in apoptosis levels in all three patients and did not correlate with changes in CD4+ cells. After re-institution of therapy, the successful virological response was followed by a new decrease in apoptosis. These results indicate that viral-load rebound have an immediate impact on levels of spontaneous apoptosis in the PBMC of HIV-infected patients. These finding should be considered when designing and evaluating structured treatment interruption (STI) strategies.

Grant N°30C.45

PREDICTIVITY OF CELLULAR AND MOLECULAR MARKERS FOR THE EFFICACY OF MULTIDRUG ANTIVIRAL THERAPY.

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The identification of the chemokine receptors CCR5 and CXCR4 as strain-specific HIV-1 coreceptors has greatly increased our understanding of the determinants of susceptibility to infection. In fact, despite many HIV isolates have been found to be able to use additional chemokine receptors to enter target cells in vitro only CCR5 and CXCR4 seem to play a significant role in vivo. Non syncytia-inducing (NSI) strains, which are the dominant viral species found in vivo at the time of seroconversion and during the asymptomatic stage of the disease, primarily use the chemokine receptor CCR5 as cofactor entry (R5-tropic strains). However, in about half of HIV-infected individuals, syncytia-inducing (SI) strains emerge, and their appearance is generally associated with both rapid CD4+ T cell counts decline and disease progression. SI HIV strains are characterized by the usage of the chemokine receptor CXCR4 as their major cofactor entry (X4-tropic strains). The factors influencing the emergence of X4-tropic HIV strains in vivo in certain HIV-infected individuals are not well understood.
In this study, we have compared the proportions of circulating CD4+ T cells expressing the CXCR4 receptor in healthy HIV-seronegative subjects, untreated HIV-seropositive individuals and HIV-seropositive patients treated with highly active antiretroviral therapy (HAART). Aim of this study was to investigate the mechanisms responsible for the emergence in some HIV-1-infected individuals of highly aggressive, syncytia-inducing (SI) HIV-1 strains, that have been shown to use CXCR4 as coreceptor to enter target cells. To this end, the percentages of circulating CXCR4+CD4+ T cells were evaluated by flow cytometry in 39 untreated and 61 HAART-treated HIV-1-infected individuals in comparison with 35 HIV-1 seronegative subjects. Plasma viremia was also measured and HIV primary isolates, from both untreated and HAART-treated HIV-1 infected subjects, were tested for the presence of SI strains. The results of this study showed enhanced proportions of CXCR4+CD4+ T cells in untreated patients in comparison to HAART-treated and healthy subjects (62 ± 16 vs. 53 ± 19 vs. 39 ± 8 respectivelly). Furthermore, the results of a 12 months-longitudinal study in a cohort of 11 patients undergoing HAART, showed a significant reduction of CXCR4 expression after successful therapy. Finally, a significant positive correlation among the proportions of circulating CXCR4-expressing CD4+ T cells, plasma viremia and the probability to isolate SI strains was found.
Despite no definitive conclusion can be extrapolated from these data on the existence of a causal relationship between increased CXCR4 expression and emergence of SI inducing strains, it may be suggested that, in the course of disease progression, the nature of the target cell population may shift to a different lymphocyte subset showing high CXCR4 expression under the pressure of both viral load and cytokine milieu. The extent of CXCR4 expression by the target lymphocyte subset may exert, in turn, a selective pressure for the emergence of X4-tropic viruses, thus resulting in a vicious circle that favors the progression of HIV infection towards the full-blown disease.

Numero contributo: 30C.47 AIDS 1999

CIOD REGIMEN COMBINED WITH STAVUDINE (d4T) AND DIDANOSINE (ddI) FOR AIDS-RELATED NON-HODGKIN'S LYMPHOMA PATIENTS (AIDS-NHL pts)

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BACKGROUND: To evaluate in a pilot study the feasibility and toxicity of d4T+ddI combined to CIOD (Cyclophosphamide, Idarubicin, Vincristine, Dexamethasone) regimen + G-CSF and opportunistic infections (OIs) prophylaxis for Diffuse Large Cell (DLC) AIDS-NHL. CIOD with high Idarubicin dose of 20 mg/sqm was given in pts without OIs prior or at onset NHL diagnosis and with WHO performance status <3. The outcome were: Rate of response, overall survival (OS) and the impact of chemotherapy (CHT) with or without d4T+ddI on immunological-virological parameters. PATIENTS: From October 1997 to August 2000, 11 DLC AIDS-NHL pts were enrolled in the study [men=9, female=2; median age: 40 years, heterosexual=5, intravenous drug users=4, homosexual=2; category CDC A=2, category CDC B=9; antiretroviral pretreatment=5 (median time: 43 months); stage III+IV of NHL disease=7, extranodal sites=7; CD4 mean/µL=221, mean HIV-1 RNA = 4.51 Log $_{10}$]. Five out of 11 pts received d4T+ddI from day 7 of the first CIOD until the end of CHT. RESULTS: An objective response was achieved in 10/11(91%) pts: 8(73%) complete (median duration: 24 months) and 2 partial. A NHL relapse occurred in one patient treated without d4T+ddI during CHT. The median overall survival was 22 months (3-67) with an actuarial survival at 24 months of 66%. Out of 11 pts, 4 died (NHL=3, acute leukemia=1) while 7 are alive in first response without AIDS-related events (4= CHT+d4T+ddI, 3= without antiretroviral therapy) at 3+,13+,18+,25+,45+,56+,67+ months from NHL diagnosis, respectively. No difference was observed as concern response to therapy, duration of response and survival between pts who received or not d4T+ddI during CHT. TOXICITY: Grading 4 WHO hematologic toxicity and febrile neutropenia were observed in 28/53 (53%) and in 4/53 (7.5%) CIOD courses, respectively. CMV retinitis occurred in 2/6 pts who received CHT without antiretroviral therapy. A significant difference was showed between pts treated or not with d4T+ddI during CHT as concern the frequency of severe neutropenia (5 vs 19, p 0.0012), the median nadir of hemoglobin (10.2 gr/dL vs 9.5 gr/dl, p 0.0180), of PMNC (2,542 vs 625 cells/µL, p 0.0003) and of platelets count (127,000/µL vs 78,000/µL, p 0.0003). A significant decrease of CD3, CD3CD4 and CD3CD8 mean cell/µL was oberved after 3 CIOD course and at end of CHT respect to baseline. A less reduction of these parameters was observed in pts treated with d4T+ddI during CHT respect to those without. High level of HIV-1 RNA was showed in all pts at NHL diagnosis. A significant decrease respect to baseline of mean HIV.1 viral load was observed only in pts who received d4T+ddI during CHT (3.9 vs 2,4 $\log_{10}$, p 0.04). CONCLUSION: The results of this pilot study showed that: 1- CIOD regimen containing high dose of idarubicin is feasible and active in DLC-AIDS NHL pts, 2- The combination of CIOD regimen with d4T+ddI is well tolerated, reduces hematologic and immunologic toxicity of CHT and have a beneficial impact on HIV infection.

Grant 30C.48 - Responsable of Project: Pietro Martino, MD

THE HIV-1 ENVELOPE PROTEIN gp120 STIMULATES Ca^{2+}- and CYCLOOXYGENASE-DEPENDENT GLUTAMATE RELEASE IN ASTROCYTES

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Gp120, the HIV-1 envelope protein, can be shed from the virus in the brain parenchyma where it acts as a neurotoxin, thereby contributing to the neurodegeneration underlying the AIDS dementia complex. In mixed cultures of neurons and glia gp120-induced neurotoxicity is prevented by NMDA receptor antagonists, suggesting that it may take place through an excitotoxic mechanism (Lipton S.A. et al., Neuron 7:111, 1991; Meucci O. & Miller R.J. J. Neurosci. 16: 4080, 1996). We have previously shown that gp120 induces $[Ca^{2+}]_i$ rises in astrocyte cultures (Codazzi F. et al., Eur. J. Neurosci. 7: 1333, 1995). Here we find that it also induces release of glutamate (Glu) (EC_{50} of about 200 pM). Therefore, the latter process might occur via the recently described Ca^{2+}-dependent mechanism mediated by prostaglandins (PGs) (Bezzi et al., Nature 391:281, 1998). In order to investigate this possibility, gp120 responses were studied in the presence of: a) the intracellular Ca^{2+}-chelator BAPTA/AM (50 μM) and b) the cyclooxygenase (COX) inhibitors, indomethacin (INDO, 1 μM) and aspirin (ASA, 5 μM). Both conditions blocked the Glu release. Moreover, gp120 (200 pM, 5 min) induced rapid generation of PGE_2, indicating that Glu release and PGs production are linked. The process finally activated by gp120 in order to induce Glu release might be regulated exocytosis. In fact, preincubation with either tetanus toxin (2 μg/ml, 20h) or bafilomycin A1 (100 nM, 1h), well known exocytosis blockers, abolished gp120-induced Glu release. This is the first demonstration that a neurotoxin, in addition to endogenous receptor ligands, activates such a release process. Furthermore, long-term incubation with gp120 (90 min) leads to a progressive extracellular Glu accumulation, due to the combination of a stimulated release and a reduced uptake. Such Glu accumulation is COX-dependent being blocked by preincubation with INDO (1 μM) and ASA (5 μM). We conclude that gp120-activated signalling in astrocytes induces significant alterations of Glu homeostasis which could be relevant to the development of HIV-1 neurotoxicity.

N°. dell'Accordo di Collaborazione: 30C.49

STUDY ON RENAL EFFECTS DUE INDINAVIR ADMINISTRATION, PATHOGENESIS AND CONNEXION WITH METABOLIC DISEASE.

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Object: the pourpose of our study may be summerized as follow: to evaluate causes of renal lesions due to indinavir (IDV); - to evaluate the possible association between lipid metabolism disorders and nephrolithiasis. The identification of risk factors associated to IDV therapy and possible prevention of renal complication will allow a reduction of renal side effects, an improvement of drug tolerability, owing to a better patient compliance.
Methods: our study includes 3 cohorts of HIV positive patients on triple therapy followed in 3 department of infectious disease. An analysis of renal side effects, epidemiological data and lipid metabolism disorders has been performed retrospectively in 340 patients and prospectively in 208 patients. 30 out of 208 patients were better evaluated with a detailed analysis of renal-glomerular function considering clinical parameters (micro/macrohematuria, crystals of indinavir in urine, proteinuria, microalbuminuria, renal colic), glomerular filtration parameters (creatinine clearance, inulina and PAI clearance, filtration fraction), proximal tubular parameters (sodium, glucose, uric acid, phosphate and bicarbonate tubular resorbption_) and distal tubular parameters (urine acidification, excrection of potassium and calcium).
Results: the results of the retrospective study have been discussed in the last report, the following data regard the prospective study and are incomplete because not all the patients have finished the follow up . 208 patients (165 M and 43 F) have been enrolled. 35% has been classified in III CDC group, 24% in IVC2 and 41% in IVC1. At baseline (t_0) mean CD4 value was 222 cell/mm3, while mean HIV RNA value in Log $_{10}$ was 4,4; at 12th month mean CD4 value was 383 and mean viral load was 2,7 log $_{10}$. 49 patients (23,5%) had side effects: 31 (14,9%) renal complications, 13 (6,2%) had body fat redistribution and 5 (2,4%) presented both alteration. 159 (76,5%) had no side effects. 17 out of 49 patients (34,7%) stopped IDV therapy for renal or lipid disorders. During the follow up no significative differences were found in mean values of urea, creatinine, uric acid, glucose , ALT, AST, while an increase in triglycerides values was seen (t_0 165 vs 229 at t_{12}). Mean time of follow-up was 11 months. 82 (39,4%) patients stopped IDV before ending the follow-up above all for therapeutic failure (10,1%), renal and lipid disorders (8,1%) and gastro-intestinal side effects (6,2%). 30 patient were better evaluated for renal function; in these there was not significant change in microalbuminuria (t_0 25,6 vs t_{12} 24,9) and also in proteinuria (t_0 179 vs t_{12} 240). Patients who presented at t_0 pathological values didn't have an improvement of these data during the study. The presence in urine of IDV crystals was found indipendent to the development of renal colic. Any patient had not nephrolithyasis (only 4 patients had renal colic). No signficant differences were found in creatinine clearance (t_0 94,7vs t_{12} 89,6) as for the clearance of inulina (t_0 142 vs t_{12} 164). Also any change were not found in PAI clearance (t_0 762 vs t_{12} 759). As PAI clearance estimates the filtration rate, this means that indinavir doesn't modify glomerural filtration. Any modification were not found in excretion litio fration (FE litio, marker of proximal tubular sodium resorbption) (t_0 5,9 vs t_{12} 5,0) and this FE litio was constantly low to indicate a state of

hypovolemia of patients. There was no case of glicosuria. The rate of excretion of uric acid remained constant (t_0 9,4 vs t_{12} 7,7) and had a range of normality during the follow up. Also no differences were found in the tubular riabsorment of phosphorum (t_0 2,7 vs t_{12} 2,7), but these values were under the range of normality. Urinary bicarbonate were found equal from baseline until 12th month (t_0 26,2 vs t_{12} 25,9). All examinations regarding distal tubular function had no signiticative modification during the follow-up.
Conclusions: the analysis of first results shows that about 1/3 of patients that had renal or lipid disorders stopped definitively the drug, even if a large number of patients interrupted for therapeutic failure or gastro-intestinal side effects. All clinical and routinary haematochemical parameters (age, sex, CDC stage, CD4 and VL count, risk factors for HIV, family history for renal and metabolic diseases, weight, BMI, urea, creatinine, uric acid , AST, ALT, urinalyses, cholesterol, triglycerides) are not correlated to the development of renal dysfuction. Besides preliminar data in patients studied with PAI and Inuline demonstrate that IDV therapy doesn't influence renal function .

Numero dell' Accordo di Collaborazione: 30C.50

IMMUNE RECONSTITUTION FOLLOWING THREE DIFFERENT HAART REGIMENS

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Objective: To investigate the kinetics of immune reconstitution and to evaluate the influence exerted by the family of antiretroviral drug used.
Design: Immunological and virological study of 20 naive HIV-patients was performed at baseline, at weeks 1, 2 and 4 and then every 8 weeks for twelve months after the initiation of therapy. Patients were treated either with three reverse transcriptase inhibitors (seven patients), two reverse transcriptase inhibitors + 1 protease inhibitor (seven patients), two protease inhibitors (six patients). Twenty HIV-negative individuals were included as controls.
Methods: Lymphocyte subsets were analyzed by flow cytometry. T-cell function was evaluated as lymphoproliferative responses to mitogen, HIV-antigens and recall antigens. HIV viral load was determined by branched-DNA technique.
Results: After 12 months of HAART all but three of the patients (one out of each group of treatment) reached HIV-1 RNA levels below 50 copies/ml. An increase in white blood cell count was observed, thus reaching levels similar to those of uninfected controls. At the end of the follow-up relevant changes in the lymphocyte subpopulations were observed, independently from drug treatment: a) an increase in CD4+ cells (from 23% to 29% p=0.04 and from 508/cmm to 633/cmm p=0.13), mainly occurred in the first 4 weeks of therapy; b) the increase in absolute numbers of both naive CD4+CD45RA+CD62L+ and central memory CD4+CD45RO+CD62L+ cells; c) a significant decrement of CD4+CXCR4+ cells; d) no changes in CD4+CCR5+ subpopulation; e) a significant decrease of CD8+ T lymphocytes (from 52% to 44% p=0.03), resulting from a reduction in CD8+CD45RO+CD62L- effector T cells greater in size than the small but significant increase of naive CD8+CD45RA+CD62L+ cells; f) a rapid decline in the expression of activation markers on CD8+ cells, i.e. CD38 and DR; g) a decrease in both CD8+CXCR4+ and CD8+CCR5+ subsets.
No changes in the proliferative response to HIV antigens, recall antigens and mitogen were seen before and after therapy.
Conclusions: Three main conclusions can be obtained from our study. First, the significant increase of memory central CD4+ T cells, together with increased numbers of naive CD4+ T cells, accounts for the increase of CD4+ lymphocytes. Second, an overall decrease of CD8+ T cells is observed, due to a decrease of effector cells greater than the concomitant increase of naive cells. Third, no differences in kinetics and degree of immune reconstitution were observed in patients undergoing the three regimens of HAART, suggesting that under the same conditions of viral suppression the pharmacological class of antiretroviral drug does not affect immune reconstitution.

Accordo di Collaborazione n° 30C.51

SECONDARY MUTATIONS IN THE PROTEASE REGION OF HIV ARE ASSOCIATED WITH VIROLOGICAL FAILURE IN ANTIRETROVIRAL-NAÏVE PATIENTS TREATED WITH PROTEASE INHIBITOR-CONTAINING HAART REGIMENS

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Background: The predictive value of mutations conferring resistance to anti-HIV drugs in drug-naïve patients remains poorly defined.
Objectives: To evaluate the possible role of mutations in the protease (PR) and reverse transcriptase (RT) regions in predicting virological failure in antiretroviral-naïve HIV-positive patients treated with highly active antiretroviral therapy (HAART).
Design: Historical clinical study.
Setting: Observational Italian cohort of antiretroviral-naive patients.
Patients: Two-hundred thirty naïve patients who began a protease inhibitor (PI)-containing HAART regimen and whose 24 week treatment virological outcomes were available.
Measurements: Genotypic testing was performed on plasma samples stored before starting therapy. Statistical analyses included multivariate logistic regression model to identify factors associated with virological failure at 24 weeks from the date of HAART initiation.
Results: 26 patients (11.3%) had mutations in the RT region, 14 of whom NRTI-related. Two patients carried a primary mutation (V82I) in the PR; 178 patients (77.4%) showed secondary PI mutations. Virological failure occurred in 60/230 (26.1%) patients; 6/26 (23.1%) with mutations in the RT region experienced failure. Virological failure occurred in 12/28 (42.9%) patients with protease mutations at codon 10, 15/40 (37.5%) at codon 36, 27/122 (22.1%) at codon 63, 5/16 (31.2%) at codon 71, 16/58 (27.6%) at codon 77 and 1/2 at codon 82. In the multivariate model, there was no statistically significant correlation between the number of RT or PR mutations and virological failure. Stepwise logistic regression identified mutations at codons 10 and 36 of the PR region as the strongest predictors of virological failure (OR: 2.09 -95%CI 1.22-3.59; p=0.008, per any additional mutation); the odds of failure in patients carrying both mutations was 4.4-fold higher than in patients carrying neither of these mutations.
Conclusions: Secondary PR mutations 10 and 36 present before antiretroviral therapy are associated with virological failure in previously antiretroviral-naïve patients treated with PI-containing regimens If confirmed in independent studies, this result may justify the increased use of HIV-genotype testing in drug-naïve patients requiring HAART.

AIDS Project n. 30C.52

INCREASING PREVALENCE OF NON-CLADE B HIV-1 STRAINS IN ITALIAN HETEROSEXUALS AND FEMALES, AS MONITORED BY THE ANALYSIS OF THE RT AND PROTEASE SEQUENCES

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OBJECTIVE: We evaluated the prevalence of HIV-1 non-clade B over time in a formerly clade B-restricted area. Protease and reverse transcriptase regions of pol gene were used for phylogenetic and recombination analysis and for clade assignment to HIV-1 A-D, F-G, J-K strains of M group.
METHODS: For 349 HIV-1 patients belonging to the Italian Cohort Naive for Antiretrovirals (I.CO.N.A.) genotypic analysis of the pol gene was performed to study the prevalence of antiretroviral-associated resistance mutations. All HIV-1 pol sequences and 32 HIV reference strains, including the reference strains for the major HIV-1 subtypes were analyzed.. The sequences that did not resulted to belong to B clade using the HIV-1 SUBTYPING TOOL program were further studied with a bootscan analysis (SimPlot) to investigate the likelihood of recombination between subtypes.
RESULTS: The phylogenetic analysis allowed the overall detection of 19/349 (5.4%) non-clade B subtypes. The proportion of patients carrying non-clade B virus distribution was 1.9% and 8.4% before and after 1997, respectively (p=.008). Among Caucasians heterosexual infection and female gender were significantly associated with the presence of non-clade B subtypes (p=.001 and p=.005, respectively). Non-clade B HIV-1 were harbured by 14.5 % of the heterosexuals who were tested HIV-1 positive after 1997, 60% of whom were females. From bootscan analysis, 4 strains were identified as F, 2 as A, 1 as C, 1 as G subtype and 11 (57.9 %) as non-clade B untypable or recombinant subtypes.
CONCLUSION: The detection of HIV-1 subtypes as well as of inter-subtype recombinants in a previously clade B-homogeneous area indicates that the HIV-1 epidemic is evolving in Italy and heterosexuals and females are at increased risk of infection with non-clade B HIV-1 subtypes. Sequences inferred from the pol gene yield to establish the subtype of circulating HIV-1 strains. As a consequence, genotyping of pol gene for testing of resistance to antiretrovirals warrants the concomitant surveillance of non-clade B subtypes.

AIDS Project n. 30C.52

RISK OPF DEVELOPINGF ADIPOSE TISSUE ALTERATIONS IN NAIVE PATIENTS ENTERED IN ART AFTER FEBRUARY 1997.

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Background: Morphologic alterations (MoA) are a frequent finding in patients on ART.
The investigations on the prevalence and incidence of each alteration, their combinations and their relationship with specific drug or drug classes have been performed prevalently in patients treated for a long time with several drug combinations and have generated partially conflicting data. On the contrary, few informations are available on the occurrence of MoA in antiretroviral drug-naive patients after entering ART.
Methods: Patients (pts) enrolled in the Italian Cohort of ART-naive pts (ICONA) were interviewed and physically examined every six months after ART initiation.
Times to MoA were compared using Kaplan-Meier and Cox regression model including covariates selected by a backward stepwise analysis with a P rem =0.1 among the following factors: age, gender, HCV status, modality of HIV transmission, weight, CD4 count and viral load (VL) at ART and time spent on the following drugs: ZDV, 3TC, d4T, ddI, IDV, RTV.
Main study endpoints were: fat loss (type 1), accumulation (type 2) and combined forms (fat loss and fat accumulation contemporary present in different body regions according to the Marrakech classification - type 3 MoA).
Results: 709 pts (196 females) who started ART within Mar 97 and Feb 00 were followed for a median time of 96 weeks. Median Pre-ART VL, CD4 and weight were 4.7 $\log_{10}$ copies (IQR 4.2-5.3), 324 cells/µL (141-479) and 70 Kg (60-77) respectively. First line ART was 2NRTI in 31.9%, 2NRTI + 1PI in 56.8%, 2NRTI+1NNRTI in 7.1%, other combinations in 4.2%. ZDV (70% first line, 74.8% ever), 3TC (56.7%-71.4%), d4T (28.3%-44.9%) and IDV (31%-44.1%) were the most frequently used drugs. MA was observed during the follow-up in 131 pts. First line ART was 2NRTI in 31.9%, 2NRTI + 1PI in 56.8%, 2NRTI+INNRTI in 7.1%, other combinations in 4.2%. ZDV (70% first line, 74.8% ever), 3TC (56.7%-71.4%), d4T (28.3%-44.9%) and IDV (31%-44.1%) were the most frequently used drugs. MA was observed during the follow-up in 131 pts. Proportion of patients with events by 112 wks of follow-up was: type 1 9.6% (95%CI 6.5-12.8), type2 11.2% (8.4-14.1), type3 5.7% (3.5-7.9), sunken cheeks 8.8% (5.3-12.4), central obesity 14.1% (10.8-17.4), adipomasty 5.8% (3.4-8.3), wasted legs 11.1% (7.4-14.1). The adjusted risk of developing type1 MoA is significantly increased in non-IVDUs, and in HCV positive patients, while the time on ZDV was protective (RH 0.59, 95%CI 0.36-0.96, p=.04 per year of treatment). Type2 was related to pre-ART body weight (RH 1.24,95% CI 1.01-1.51, p=.04 per 10 additional Kg) and, with borderline statistical significance, to female gender, time on ZDV and time on d4T. Combined MoA (type3) was related to female gender (RH 5.38, 95%CI 1.74-16.64, p=.004) and time on IDV (RH 3.32, 95%CI 1.43-7.68, p=.005) and, at a borderline statistical significance, with high pre-ART VL.
Conclusion: Our data show that MoA are a frequent finding even in patients who received ART for a relatively short time and who experienced a limited number of drugs and drug changes. Different MoA present different correlates of risk, suggesting a multifactorial pathogenesis of the so-called lipodystrophy syndrome.
Certain drugs or drug classes probably exert a toxic effect, causing particular alterations. However, some treatment related phenomena do not seem to depend on specific drug regimen.

N°. dell'Accordo di Collaborazione: 30C.53

N-ACETYLCYSTEINE INHIBITS KAPOSI'S SARCOMA GROWTH IN NUDE MICE.

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The thiol N-acetylcysteine (NAC) is a chemopreventive agent which acts though a variety of mechanisms. Its antioxidant and antigenotoxic effects, as well as its cancer preventive activity, are well established. We have previously demonstrated that NAC is able to prevent tumor progression by modulating malignant tumor cell invasion in vitro and in vivo by inhibition of matrix metalloproteases. In addition, NAC inhibits angiogenesis by preventing endothelial cell invasion without actual killing of the endothelium.

Kaposi's sarcoma is a highly angiogenic lesion, particularly frequent in HIV infected patients, that is also associated with a herpes virus HHV8. KS is becoming a leading cause of cancer death is areas of Africa that have high rates of HHV8 and endemic KS along with HIV-1 infection. Therefore identification of inexpensive, non-toxic treatments for KS is urgently needed.

Here we show that NAC reduces the migration and invasion of a Kaposi's sarcoma cell line in vitro. In addition, oral administration of NAC significantly inhibited the growth of established Kaposi's sarcoma tumors and prolonged survival. The analysis of these tumors indicated a significant reduction in foci of VEGF expression in NAC treated mice. In addition, NAC significantly reduced VEGF expression in vitro as determined by both immunocytochemistry and ELISA, but did not effect the proliferative indexes PCNA or Ki-67.

Our data suggest that NAC directly decreases VEGF expression, a key angiogenic factor in KS, and that this contributes to reduced tumor cell proliferation and reduced tumor mass in vivo.

30C.54

PATHOGENETIC IMPLICATIONS IN THE CLINICAL CHANGES DURING HAART IN AIDS-RELATED PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA AND THEIR SYSTEMIC COUNTERPART.

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Background: The role of EBV and of the genetic alterations in the AIDS-related lymphomagenesis has to be still completely defined, particularly in the era of highly active antiretroviral therapy in which the clinical changes need to be supported by pathogenetic studies.
Objectives: 1)To better characterize the molecular and phenotypic profile of AIDS-related PCNSL to help define the relationship with the systemic lymphoma counterpart; 2)To evaluate the impact of highly active antiretroviral therapy in terms of changes of prevalence of AIDS-related PCNSL and 3) of the natural history of AIDS-non Hodgkin's lymphoma (NHL).
Methods: For the biologic study the expression pattern of TCL-1, MUM1, BCL-6, CD138 and LMP1, and the usage of immunoglobulin variable genes were studied in a panel of both AIDS-related PCNSL and AIDS-NHL. For the clinical study all HIV-infected patients with PCNSL or NHL prospectively followed were included in the studies. All patients were prescribed to receive HAART during chemotherapy. An intention-to-treat approach was used for analysis of chemotherapy response and survival according to antiretroviral effect. Association between response to HAART and factors associated with clinical presentation, response to chemotherapy and toxicity was analyzed by univariate and multivariate models for categorical and continuous variables. Survival analysis was performed by Kaplan-Meier estimates and Cox proportional hazards regression model.
Results: First objective: Expression of MUM1 in combination of the expression pattern of BCL-6, CD138 and LMP1 segregates three major phenotypic patterns both in AIDS-related PCNSL and AIDS-NHL whilst TCL-1 is expressed only in systemic AIDS-IBPL. Furthermore, AIDS-related PCNSL show a preferential usage of VH4 gene segment and, in contrast to systemic AIDS-IBPL, do not present "crippling" mutations in the VH genes utilized. Second objective: In the HAART period a relevant decline of PCNSL was found (OR for 1998, 0.25; p for trend= 0.03) and confirmed as effect of progressive calendar year at multivariate analysis (OR 0.52; 95%CI 0.28-0.97). During this time period patients were more likely to receive PCR-based diagnosis (OR 2.14; 95%CI1.57-2.04), and to receive an in vivo diagnosis (OR 1.18; 95%CI 1.06-1.32) and less likely to undergo brain biopsy (OR 0.39; 95%CI 0.16-0.92). Third objective: Forty-four patients with NHL were included in this part of the study, 21 (48%) of them were in the high-risk prognostic group. A complete response to chemotherapy was achieved in 71% of the HAART-responder and in 30% of the HAART-failed/naive patients and the virologic response to HAART was the only variable associated to tumor response in the multivariate analysis (OR 6.36; 95%CI 1.56-25.81). Patients with virologic response to HAART received a higher amount of chemotherapy relative dose intensity (RDI) than patients without reponse (93% vs 66%; P=0.04). A prolonged 1-year survival was observed in patients with virologic (0.78 versus 0.18; P at log-rank 0.0001) and immunologic (0.84 vs 0.26; P at log rank 0.0004)

response to HAART. At Cox regression analysis, immunologic response to HAART (HR 0.14;95%CI 0.04-0.55), higher RDI (HR 0.24;95%CI 0.06-0.87) and complete response to chemotherapy (HR 0.16;95%CI 0.05-0.54) were all associated with a reduced risk of death.
Conclusions: Preferential usage of VH4 gene segment, lack of TCL-1over-expression and absence of "crippled" mutations in AIDS-related PCNSL suggest that brain microenvironment may play a direct and specific role in the initiation of lymphomagenesis of EBV-infected germinal center B cells which have gone to maturation in the periphery and then migrated in the brain. The specificity of AIDS-related PCNSL could explain why during the HAART era AIDS-PCNSL showed a strong decline, differently from what observed for AIDS-NHL which continue to be observed. In AIDS-NHL response to HAART was strongly associated with a better response to chemotherapy and a prolonged survival. Preliminary results suggest an improved survival also in HAART-treated PCNSL compared to historical patients. Molecular and phenotypic studies analyzing the impact of HAART on the pathogenesis of these tumors are ongoing.

Accordo di Collaborazione N. 30C.55

EVIDENCE OF A COMPARTIMENTALIZED HIV-1 REPLICATION IN THE CENTRAL NERVOUS SYSTEM DURING NEUROLOGICAL DISORDERS: THE ROLE OF CONCOMITANT MACROPHAGE ACTIVATION AND THE EFFECT OF POTENT ANTIRETROVIRAL THERAPY.

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Objectives. To analyse HIV-1 replication in plasma and CSF according to immune status, presence of CNS disorders, markers of macrophage activation, use of potent antiretroviral therapy.
Methods: cross-sectional and longitudinal analysis of matched plasma and CSF samples from HIV+ patients with or without neurological signs/symptoms. HIV-RNA was quantified by ultrasensitive RT-PCR, MCP-1 in CSF by sandwich ELISA.
Results: in the cross-sectional analysis plasma/CSF pairs from 80 HIV+ pts were examined with (42) or without (38) neurological disorders; 28% were on potent antiretroviral therapy at baseline. There was a significant correlation between log HIV-RNA levels in plasma and CSF ($r=0.40$, $p<.001$), but this was not the case in patients not on HAART. HAART was associated with significanly lower replication in both compartments. Neurologically symptomatic pts showed significantly higher MCP-1 levels ($p<.05$) and a higher CSF/plasma HIV-RNA ratio (0.76 vs 0.64, $p=.05$). In 11 cases, HIV-RNA levels were higher in CSF than in plasma (OR = 7 for those with MCP-1 > 1 ng/mL). 53 patients were longitudinally followed with at least one new CSF sample, 62% of cases were on HAART. Although HAART was associated with a significanly more profound reduction of CSF HIV-RNA and CSF/plasma ratio, the changes in CSF HIV-RNA were significantly less pronounced in patients with baseline CD4<100 (-.58 log vs -1.83 log, P=.02), neurological symptoms and baseline MCP-1>1 ng/mL (-0.79 log vs -1.58 log). HAART did not reduce MCP-1 levels.
Conclusions: HIV replication in the CNS does not correlate with systemic replication in neurologically symptomatic patients: in these cases a local production is associated with signs of macrophage activation. HAART does not reduce macrophage activation in the CNS and HIV-RNA inhibition in this compartment is less pronounced in the presence of neurological disorders.

Accordo di collaborazione n. 30C.56

COMBINED CELLULAR AND GENETIC THERAPEUTIC APPROACHES FOR ERADICATING HIV-1 PERSISTENTLY INFECTED CELLS

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Adoptive immunotherapy using T lymphocytes specifically able to recognize HIV-1 antigens represents a useful approach for eradicating persistently infected cells. The tropism of HIV-1 for T lymphocytes requires that antigen specific cells are genetically modified to resist to the lentiviral infection. Our group has previously proposed in the literature a new strategy of adoptive immunotherapy based on the ex vivo transfer of autologous CD4 T cell lines, resistant to HIV-1, which recognize specific antigens of HIV-1 and opportunistic infectious agents (Manca et al., 1997; 1999). However, the MHC-restricted nature of viral antigen recognition by the T cell receptor limits the application of adoptive immunotherapy strategies to MHC-matches individuals. A way to circumvent this problem is to generate MHC-unrestricted chimeric T-cell receptors to redirect the antigenic specificity of primary T cell populations (both $CD4^+$ and $CD8^+$) in order to recognize viral antigen of choice. To this end, we designed engineered chimeric TCR (cTCR) composed of the zeta (ζ) subunit of the CD3 T-cell receptor (the cytoplasmic domain involved in signal transduction) fused to the transmembrane region of CD8 (in collaborazione con il Prof. Siccardi). The extracellular domain consists of an antibody single chain variable region (scFv) specific for an epitope of the HIV-1 envelope glycoprotein close to CD4 binding site (scFv105). A c-myc tag has been included in the construct to allow cell surface expression by FACS analysis. The cTCR has been cloned into a modified Moloney-based retroviral vector, MFG, containing a therapeutic molecule and the neo gene as a selectable marker. We intend to transduce human T cell lines and primary $CD4^+$ and $CD8^+$ cells and to evaluate the ability of the cTCR to initiate cell activation upon binding to viral antigens. In particular, antigen-specific proliferation, cytokine production and cytolytic activity against HIV-1_{env} positive target cells will be analyzed. The next phase will be the assessment of the efficacy of our strategy. HIV-1- infected SCID mouse will be treated with antivirals and infused with recombinant TCR lymphocytes. Persistence of gene-modified T cells, tissue homing (trafficking) and antiviral activity against tissue reservoirs of HIV will be analyzed. These studies should provide further insights into the feasibility of adoptive immunotherapy for the treatment of AIDS.

Accordo di Collaborazione N. 30C.57

PRIMARY CHARACTERIZATION OF KSHV LANA2: A NEW LATENCY ASSOCIATED NUCLEAR ANTIGEN WITH LYMPHOCYTE-RESTRICTED EXPRESSION.

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KSHV or HHV8, the eighth human herpesvirus, is associated with three human proliferative diseases ranging from hyperplastic proliferation to neoplasia: Castleman's disease (CD), Kaposi's sarcoma (KS), and primary effusion lymphoma (PEL). The KSHV genome encodes a number of genes that modify host cell regulatory pathways and have been postulated to play a role in the pathogenesis of these disorders. Here we report a new latency-associated gene which is not a component of the previously described major latency transcript locus, LT1 and LT2 encompassing v-cyclin (ORF72) and latency-associated nuclear antigen (LANA)1 (ORF73). This 1704 bp spliced gene encodes a protein designated latency–associated nuclear antigen 2 (LANA2) whose transcript is abundantly and constitutively expressed in KSHV infected B cell lines. Monoclonal antibodies produced against LANA2 show the protein to be localized to nuclei of cultured KSHV infected B-cells in a finely speckled pattern which does not co-localize with LANA1. Although LANA2 is expressed in vivo in all KSHV-infected hematopoietic disorders including primary effusion lymphomas and multicentric Castleman's disease, tissue immunolocalization studies indicate that LANA2 is not expressed in KS lesions. Unlike LANA1, LANA2 does not elicit a serologic response as determined by a panel of sera from patients with KS, PEL and MCD. Although there is limited sequence homology of LANA2 to portions of the interferon regulatory factor (IRF)4 regulatory domain, there is no homolog to the DNA binding domains of this family and gel shift experiments using an interferon response element (ISRE) probe show no interaction. ISRE-luciferase reporter studies are also negative. However, in luciferase reporter assays using a p53 responsive p21 promoter element, LANA2 inhibits p53-mediated transcriptional activity. Transient transfection of LANA2 into SAOS-2 cells show inhibition of doxyrubicin induced, p53 mediated apoptosis. Although we detect LANA2 interaction with the amino terminus of in vitro translated p53, we are unable to demonstrate in vivo association to p53 in KSHV-infected cell lines.
These findings show that KSHV has tissue-specific latent gene expression programs and identify a new latent protein which may contribute to KSHV tumorigenesis in hematopoietic tissues via p53 inibition.

N°. dell'Accordo di Collaborazione. 30C.58

CONCENTRATION OF STAVUDINE IN BLOOD AND SEMEN FROM HIV-1 INFECTED PATIENTS

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Purpose of the study: The efficacy of antiretroviral drug therapy depends on the ability of the drugs to penetrate in the relevant parts of the body. Very limited data regarding the pharmacology of antiretrovirals in semen are available. It has recently been reported that ritonavir and saquinavir have limited ability to concentrate in semen. Zidovudine and lamivudine achieve high concentrations in semen and significantly reduce HIV-1 RNA . Data regarding the penetration of stavudine in semen are still scarce. Objective of this study is to measure the concentrations of stavudine in semen and blood samples from HIV-1 patients treated with stavudine.
***Methods*:** We examinated stavudine concentrations in blood and semen in 14 men who received stavudine and other drugs. Blood and semen were collected a median of 2 h after the morning dose taken at home. Stavudine concentration has been determined by high pressure liquid chromatography (HPLC). Samples were grouped according to time of production and allocated to the time periods 0-2 h, 2-4 h, 4-6 h post stavudine ingestion. To date 14 semen and 14 blood samples have been analysed.
***Summary of results*:** Median stavudine blood levels at 0-2 h, 2-4 h, 4-6 h were 0.30 μg/ml (range 0-0.73), 0.31μg/ml (0-0.68) and 0.05 μg/ml (0.01-0.09) respectively. The corresponding stavudine semen levels were 0.36 μg/ml (range 0.25-0.47), 0.33 (0.05-0.57), and 0.22 in only one patient at 5 h.
Stavudine demonstrates good penetration into semen with semen/blood ratios ranging from 0.27 to 2.44 depending on the time post drug ingestion. Four subjects showed stavudine concentration not determinable in blood, where stavudine concentration in semen was ranging from 0.27 to 0.57 μg/ml.
Conclusions: The results should encourage further study of the penetration of antiviral drugs in semen and the development of strategies to determine whether antiviral drugs can be used to reduce the sexual transmission of HIV-1.

Accordo di collaborazione N. 30C.59

PREDICTIVE VALUE OF "AgNOR" TECHNIQUE ON THE THERAPY EFFICACY DURING ACQUIRED IMMUNODEFICIENCY SINDROME

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Our research group has shown that the rapid T cell turnover observed in the phase of acute viral replication in HIV-infected patients induces important perturbations of cell cycle control in peripheral blood lymphocytes. Among these perturbations, the presence of increased concentrations of less degradable cyclin B appears to be prominent. This condition in turn results in a persistent activation of the p34cdc2 kinase which phosphorylates various substrates at inappropriate stages of cell cycle and ultimately results in an increase of apoptotic cell death of T cells. In our study, intracellular concentration of cyclin B was detected by both western blot and densitometric quantitation as well as flow cytometry.

The aim of the present project is to assess the clinical relevance of our scientific observations by performing an integrated analysis of cell cycle using cyclin B measurement as well as the study of the AgNOR number and area of ditribution.

AgNOR staining has been used for several years as a marker of cell turnover and cell proliferation in clinical oncology. AgNOR staining is based on a colloidal silver impregnation of nucleolar proteins, mostly nucleolin, which constitute the so-called Nucleolar Organizer Regions. AgNORs correspond to the DNA regions coding for ribosomal RNA, and therefore represent a reliable marker to quantitate the level of cell activation when one evaluates parameters such as dot number and area of distribution. Resting cells (G0) typically show one small AgNOR dot of area between 0,6 and 0,8 μ^2. As activation progresses, AgNOR number and area of distribution increase. AgNOR number is therefore an index of activation, while the AgNOR area of distribution is an index of the rate of cell proliferation. When these parameters are evaluated on a cell population, i.e. peripheral blood lymphocytes, a statistical analysis is usually required to properly describe their features.

Results obtained using AgNOR staining were confirmed by confocal microscopy analysis which allowed us to assess the subcellular localization of nucleolin in peripheral blood lymphocytes from HIV-infected patients and healthy controls. The combined analysis by western blot and confocal microscopy has revealed important details of the intracellular turnover of nucleolin.

Our results indicate that peripheral blood lymphocytes from HIV-infected patients show some degree of T cell activation that is not present in lymphocytes from healthy individuals. Interestingly, AgNOR parameters in HIV-infected individuals that are successfully treated with anti-HIV drugs, or in those defined as long term non-progressors, are similar to those of controls.

Normalization of AgNOR parameters might therefore be an index of response to therapy in AIDS patients even when the results of therapy are discordant in terms of suppression of viral replication and immunological reconstitution. These "discordant" patients show either viral suppression without increase of CD4+ T cell count or lack of viral suppression with significant CD4 rebound. Analysis of AgNOR staining might represent an effective index of therapy responsiveness in these patients and perhaps suggest alternative therapeutic strategies, i.e.e immunosuppression, in selected HIV-infected patients.

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CYTOKINES AND T CELL ACTIVATION IN HIV+ INDIVIDUALS RECEIVING INTERMITTENT CYCLES OF INTERLEUKIN-2 (IL-2) AND ANTIVIRALS.

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Sixty-one antiviral-experienced HIV-infected individuals were randomly assigned to one of the following arms: A. Antiretroviral therapy (ART) plus IL-2 (12 MIU continuous intravenous infusion, followed by 7.5 MIU/BID, sc, every 8 weeks); B. ART plus IL-2 (7.5 MIU/BID, sc, every 8 weeks); C. ART plus IL-2 (3 MIU/BID, sc, every 4 weeks); D. ART alone. A significant increase of circulating $CD4^+$ cells was observed in IL-2-treated individuals in comparison to those receiving ART alone. In spite of the incomplete suppression of virus replication, IL-2 administration together with ART did not increase either plasma viremia or cell-associated HIV DNA levels. Low doses of intermittent sc IL-2 administration induce a stable increase of peripheral $CD4^+$ cells that was indistinguishable from those associated with higher, less tolerated doses of the cytokine. A decrease overtime of the circulating levels of IL-2 was observed in individuals receiving the highest doses of the cytokine, but not in those belonging to the low-dose (LD) sc arm. In a post-hoc analysis, IL-2-related toxicity significantly correlated to IL-2 and tumor necrosis factor-α (TNF-α) serum levels, and was significantly lower in individuals receiving the lowest dose of the cytokine. The percent of CD4+ T cells bearing surface CD25 molecules sharply increased shortly after IL-2 administration. Conversely, the percent of CD4+/HLA-DR+ T cells decreased significantly, whereas the percent of CD8+/HLA-DR+ T cells remained stable with the exception of the LD sc IL-2 arm in which it also decreased. These results reinforce the hypothesis that intermittent cycles of «low dose « IL-2 (6 MIU/die) are equally potent and less toxic than higher dosages for HIV infected individuals.

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PEPTIDES MIMICKING THE DOCKING SITE OF MET RECEPTORS BLOCK IN VITRO HGF-DEPENDENT BIOCHEMICAL AND BIOLOGICAL RESPONSES AND IN VIVO GROWTH OF KAPOSI'S SARCOMA.

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The Met receptor tyrosine kinase and its ligand, Hepatocyte Growth Factor (HGF) have been implicated in the tumorigenesis and metastasis of different cell types, with different mechanisms. In the case of Kaposis's Sarcoma (KS), the most common neoplam found in patients with acquired immune deficiency syndrome, paracrine and/or autocrine loops seem to be involved, although this receptor/ligand pair is only one among the different factors implicated. KS cells express Met (Maier et al., 1996a), and we have previously shown that HGF stimulation of an established KS cell line (KS-IMM cells) induces a series of biological effects, including cell migration, proliferation and invasiveness (Prat et al., 1998; Montaldo et al., 2000). Moreover, an autocrine loop has been shown to sustain the proliferation of human KS cells and of spindle-shaped cells derived from KS-like lesions developed in BKV/tat transgenic mice (Maier et al., 1996b). Molecules interfering with the biological behavior of the neoplastic cells could represent valuable therapeutic agents. All Met-mediated biological responses depend on trans/autophosphorylation of the receptor on two pairs of tyrosines located in the kinase activation loop (Y_{1234}-Y_{1235}) and in the C-terminal tail docking site (Y_{1349}-Y_{1356}) respectively. To inhibit this activation process, we have developed 19 aminoacid peptides that mimick these receptor motifs linked to the membrane translocation signal of the Drosophila Antennapedia homeo-domain 3 (Bardelli et al., 1999) and tested their effect on KS-IMM cells. At micromolar concentrations, peptides mimicking the docking site, but not peptides mimicking the activation loop, blocked HGF-dependent Met phosphorylation and downstream signaling, and inhibited ligand-dependent in vitro "wound" healing, proliferation and invasiveness. In vivo, the same peptide significantly retarded KS-IMM tumor growth, when injected every other day both from the beginning of the experiment or after 10 days of tumor growth. These data suggest a possible pharmacological mechanism to interfere with key biological properties of Kaposi's sarcoma cells, to decrease their malignant phenotype and tumor expansion (Morini et al., 2001).

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ITALIAN REGISTRY OF ANTIRETROVIRAL PROPHYLAXIS AFTER OCCUPATIONAL AND NON-OCCUPATIONAL EXPOSURE TO HIV

Registro Italiano delle profilassi con antiretrovirali dopo esposizione occupazionale, sessuale o parenterale ad HIV
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Objective: To monitor prophylaxis with combined antiretroviral agents after occupational and non-occupational exposures to HIV (PEP), in Italy.
Methods: Prospective, open study. Data collected by the Italian PEP Registry have been analysed.
Results: On a total of 773 reports: 639 (82%) were health care workers (HCW); 37 (5%) public-safety workers, and 96 (13%) other non-occupational. No treated subjects seroconverted; one possible abortive infection was observed (Inf Contr Hosp Epidemiol 2000; 21:529-31); 26 subjects were lost to follow up. Overall, routes of exposure were: 430 needlesticks, 2 needlesharing, 82 cuts, 121 mucous and 89 skin contamination, 5 bites, 35 sexual exposure, and 9 other/not available. Among non-occupational exposures, PEP were started after 24 injuries with abandoned needles, 2 needlesharings, 12 fights, 4 first aids for street accidents. Among the 35 sexual exposures (10 men, 25 women), 14 were in serodiscordant couples, and 17 due to condom breakage. The acceptance/uptake rate among HCW increased from around 20-29% in 1994-97 to 42-45% in 1998-2000. Mean time (hours) from exposure was 4 for HCW, 17 for sex exposures, 6 for other non-occupational exposures. The initial treatment regimens included 2 nucleoside reverse transcriptase inhibitors (NRTI) in 347 cases (group A), plus 1 protease inhibitor (PI) in 409 (group B). A significant increasing use of group B drugs as initial regimen was observed (52% in 1997, 60% in 1998, and 63% in 1999-2000; $p = 0.02$ by chi square for trend), not depending from exposure characteristics. Overall, mean length (days) of PEP was 15 for group A; it was 19 for the 2 NRTIs component and 17 for the PI component of group B, respectively. Overall, mean length (days) of PEP was 27 among sex exposures, and 22 in others non-occupational PEP. In 223 cases PEP was interrupted after a mean of 3 d because the source tested negative; in this group 8% of subjects developed side effects. No significant differences were found in the proportion of HCW experiencing side-effects and discontinuing PEP among the two groups (Lancet 2000; 355:1556-7). All constitutional and lab adverse effects were mild and reversible at the treatment interruption. A clinically irrelevant trend toward higher triglyceride levels was observed in both groups, which was statistically significant in gr. B compared to gr. A (AIDS 2000; 14:2407-8).
Conclusion: An increasing use of PEP, has been observed, including treatments after non occupational exposure. Highly active PI containing PEP seems increasingly used as initial treatment. Efforts should be made to assure a rapid assessment of source serostatus in order to limit unnecessary PEP, and related adverse effects. Short term toxicity was frequent, mild, reversible and not unusual. The observed rates of PEP discontinuation, similar in the two groups, do not suggest per se the use of a less potent PI sparing regimen.

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DRUG SURVEILLANCE STUDY OF ADVERSE REACTIONS OF PROTEASE INHIBITORS TREATMENT.

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Background: in the age of HAART, the occurrence of adverse events is one of the main factors limiting therapy compliance in HIV infected patients. As these are life-long therapies, it is extremely important to know the real impact of this problem.
Objective : to assess the probability that the protease inhibitor (PI) therapy might be discontinued because of adverse events; to evaluate the incidence rate of adverse reactions during PI treatment.

Design: since september 1997 to September 2000 we have been carrying out a prospective cohort multicenter study on HIV positive patients starting treatment with at least one PI. A total of 1477 patients have been enrolled having an average age of 37.1 years (SD ± 8.1), 1066 are male. Where risk factors for HIV infection are concerned, the distribution was as follow: 48.1% intravenous drug users, 31.6 % heterosexual contacts, 16.2 % homosexual males and 0.7% blood transfusion. Average CD4 + lymphocyte count at enrolment was 265 cell/mm³ (SD ± 201). Average follow-up time is equal to 17.8 months (range 1 – 32). 865 study subjects have been trated with indinavir, 380 with saquinavir-HCG , 279 with ritonavir, 348 with nelfinavir and 146 patients have taken a combination of ritonavir-saquinavir.

Statistical analysis: The incidence rate (IR) of reactions is calculated as the number of reactions in relation to the person/time of observation forming the risk period. The rates are calculated in person-months. Confidence intervals (CI) at 95% for incidence rates are calculated based on Poisson's binomial distribution.

Results: During the study period under consideration, 52.06% of the patients presented at least one adverse reaction of any grade. The average number of reactions per person was 0.9 (range 0 – 10). Respectively, 12.7%, 16.5% and 18.1% of patients discontinued the first treatment within 6, 12 and 18 months. In particular, at 18 months 48.1% of ritonavir treated patients discontinued therapy This happened also in 37.4% of patients treated with Ritonavir-Saquinavir, in 21.6% with Indinavir,in 12.7 % with Saquinavir and in 10% with Nelfinavir.
The second cause for therapy change was therapeutic failure which was observed in 13.5%.
The incidence rate (IR) for any grade of adverse events for each single drugs, calculated per 100 person-years, is the following: Ritonavir 118.9 (95% CI 117.5 – 120.3), Ritonavir + Saquinavir HCG 104.6 (102.1 – 106.4), Nelfinavir 62.2 (61.3 – 63.2), Indinavir 60.3 (59.8 – 60.7), Saquinavir HCG 35.4 (34.8 – 35.9). The IR of serious adverse events (grade 3 or 4) is the following : Ritonavir 31.2 (30.5 – 31.9), Ritonavir-Saquinavir HCG 21.8 (21.06 – 22.6), Indinavir 12.9 (12.7 – 13.1), Nelfinavir 8.5 (8.1 – 8.8), Saquinavir HCG 9.9 (9.6 – 10.2).

Conclusions : 1 – We confirm the high frequency of adverse reactions in HAART still being primary cause of treatment discontinuation. 2 – Either Ritonavir alone or associated with Saquinavir HCG is the PI that most frequently generates adverse events in HAART treated patients; Nelfinavir presents the lower IR for severe adverse events and seems to be the most tolerate drug.

Accordo di collaborazione n. 30C.65

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MODULATION OF NMDA RECEPTOR FUNCTIONS BY THE ENVELOPE GLYCOPROTEIN GP120 IS MEDIATED BY PROTEIN KINASE C ACTIVITY

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HIV-1 infection is characterized by multiple neurological syndromes occurring at all stages of infection: among these, the AIDS dementia complex is typified by cognitive decline, motor dysfunction and behavioural abnormalities. As HIV-1 does not infect neurons, the viral protein gp120 has been proposed to indirectly affect neuronal functions, in concert with neurotoxic compounds released by infected macrophages, by potentiating glutamate effect on NMDA receptors. This potentiation leads to increase of cytosolic calcium concentration followed by activation of Ca^{2+}-dependent enzymes, including protein kinase C (PKC) . Accordingly, PKC activity in the CNS of gp120 transgenic mice was founded to be up-modulated and brain tissue from patients with HIV-1 encephalitis showed increased PKC activity.

We recently provided evidences that gp120 can also act directly on neurons, by potentiating the NMDA mediated functions. gp120 , following recognition by its V3 sequence of the glycine site of NMDA receptors, concentration-dependently (10pM-1nM) potentiates the 100μM NMDA-induced [^{3}H]NA release from superfused isolated nerve terminals (synaptosomes) of rat cortex and hippocampus and of human neo-cortex. As PKC-dependent phosphorylation of NMDA receptors is an important mode of regulation of the receptor activity , the possible involvement of PKC in the potentiating effect mediated by NMDA in presence of gp120 was investigated.

Exposure of superfused hippocampal synaptosomes to the PKC inhibitors GF 109203X (0.1μM) or H7 (10μM) , selective antagonists at the ATP binding site, halved the effect of 100μM NMDA plus 1nM gp120 on the [^{3}H]NA release. On the contrary, sphingosine, which blocks PKC at the diacylglycerol-binding site, failed to affect the evoked release of [^{3}H]NA. Similar results were obtained when studying the [^{3}H]NA release induced by 100μM NMDA plus 1μM glycine. Finally, the PKC antagonists under study failed to affect the [^{3}H]NA release induced by 100μM NMDA alone.

Our results suggest that the potentiation of the NMDA-evoked [^{3}H]NA release induced by gp120 is a PKC-dependent process, involving Ca^{2+}-dependent diacylglycerol-independent PKC isoform, the activation of which might lead to a reinforcement of the NMDA receptor functions .

This work was supported by ISS-“Programma nazionale di ricerca sull’AIDS - Progetto Patologia, clinica e terapia dell’AIDS (30C.66).[#] Recipient of a study bourse aims at AIDS researches (n 818-17/7/97) of the Italian Ministry of Health ISS.

HIV-1 TAT PROTEIN, THROUGH ITS 61-80 AMINOACIDIC SEQUENCE MODULATES THE RELEASE OF [^{3}H]ACETYLCHOLINE FROM CORTICAL NERVE ENDINGS.

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Increasingly attention has recently focused on the non-structural viral protein TAT , a 82-102 aa regulatory protein produced by HIV-1 infected lymphocytes.. The protein is extracellularly released and, once in the biophase, it could i) be internalized by cells and localized in the nucleus, ii) bind to and depolarize neuronal membranes, iii) increase intracellular calcium concentration allowing either calcium entry through receptor-operated calcium channels or calcium mobilization by IP3-sensitive internal stores.

As Tat can excite both human and rodent cortical neurons , we studied the effect of the protein on the neurotransmitter release from rat superfused isolated nerve endings (synaptosomes). TAT_{HxB2} concentration-dependently increases the release of [^{3}H]acetylcholine ([^{3}H]ACh) from preloaded rat cortical synaptosomes. The protein exerted the releasing effect when applied in the nanomolar range (100 pM – 1 nM), concentrations compatible with the one expected to be present in the CNS during HIV-1 infection . The potentiation could be prevented by either protein heat denaturation or protein immunoprecipitation and it appears to be insensitive to the presence of extracellular Ca^{2+} .

The releasing effect was maintained by the sequence TAT_{LAI} 49-86 (100 pM – 1 nM) , while TAT_{LAI} 32-62 (1 nM) was unable to affect the basal release of [^{3}H]ACh.

To determine the sequence responsible of the potentiating effect induced by Tat on the [^{3}H]ACh release, synaptosomes were exposed to shorter 20-mer peptides which overlap by 10aa each and which represent the entire sequence of TAT_{LAI} 1-86 .All the sequences studied were applied at the final concentration of 1 nM. The tritium release was potently increased by Tat_{61-80} and, to a lower extent, by the flanking fragments Tat_{51-70} and Tat_{71-85}.On the contrary the peptide Tat_{1-20}, Tat_{11-30}, Tat_{21-40}, Tat_{31-50} and Tat_{41-60} failed to affect the basal [^{3}H]ACh release.

These results suggest that Tat can release [^{3}H]ACh following recognition by the sequence 61-82 of a high affinity binding site located on the cortical terminals of cholinergic neurons .

Impairments of the central cholinergic functions might be therefore provoke by the protein and play a role in the cognitive deficits that typifies the HIV-1-associated dementia.

This work was supported by ISS-“Programma nazionale di ricerca sull’AIDS - Progetto Patologia, clinica e terapia dell’AIDS (30C.66).TAT fragments were kindly gifted by “AIDS Reagent Project” (ARP7004.2 and 7005) and by European Programme EVA (779.1/779.8).

IMMUNOLOGICAL RECONSTITUTION DURING HAART IN HIV-1 VERTICALLY INFECTED INFANTS.

Cancrini C., Romiti M.L., Castelli-Gattinara G., Bernardi S., Di Cesare S., Rossi P.,

The aim of the study was to evaluate early triple combination therapy (HAART) effects on immune system development in HIV infected new-borns. Immunological, virological findings and CDR3 fragment lengths analysis were performed before initiation of therapy and during 2 years follow-up in two patients, one classified as N1 and the second classified as C2.
Baseline viral load values were 624.609 viral copies/mm^3 (log 5.79) and 490.000 copies/mm^3 (log 4.71) respectively. In new-born N1 a marked reduction of viral load value was observed within 4 weeks of therapy and HIV-1 RNA copy number were undetectable (<50 copies/ml) in the following two years with an almost constant number of CD4+T cells. In newborn C2 a reduction of HIV-RNA level was detected at 4 weeks (80.000 viral copies/ml) to achieve an undetectable level at 5 months of therapy maintained during. Furthermore a marked increase of CD4+T cells was observed during the follow-up. A third HIV-1 vertically infected and symptomatic newborn was recently enrolled since the first month of age.
At baseline CD4 TCR repertoire was normal (gaussian-like distribution) in child N1 and resulted highly conserved during the therapy while it was widely altered in child C2, but normalizing since the 6th month of therapy. However the CD8 TCR distribution was deeply altered in both children with a significant number of oligoclonal and monoclonal patterns. During the follow-up, the CD8 TCRBV patterns were completely normalized in child N1 and partially normalized in child C2. Preliminary analysis on the third patient at baseline showed CD4 TCR repertoire more perturbed than previously analyzed patients and CD8 TCR repertoire highly perturbed as already reported. Conclusion: Early HAART given to an HIV-1 infected newborn after 24 months, completely
restore immunological parameters.

Accordo di Collaborazione n. 30C.67

IMPLICATION OF HIV-1 TAT IN AIDS-ASSOCIATED NEURODEGENERATION

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Infection of HIV-1 is frequently associated with neuronal death in cortical brain areas, that could be responsible for the development of neurological deficits associated with the AIDS dementia complex (ADC). However, neurons do not appear to be the main target of the virus, and the main neuropathological finding is the localization of HIV-1 to blood-derived macrophages, resident microglia and multinucleated giant cells. Thus, it has been hypothesized that an indirect mechanism may exist to explain the neuronal cell death that occurs in patients infected by HIV-1. Several reports, indicate the viral proteins gp120 and Tat as the main candidates as mediators of HIV-1 neurotoxicity. However, the site of interaction of gp120 and Tat on neurons and astrocytes, to mediate the cellular death is still unknown. The aim of this study is to determine a possible role for the regulatory protein Tat in neuropathological phenomena associated with AIDS. Our results show that the treatment with synthetic Tat_{1-86} peptide induces apoptotic death in cultured rat hippocampal neurons. This effect is dose- and time-dependent and occurs with the same intensity in pure neuronal cultures and in the presence of a glial cocolture, suggesting that the viral protien could act directly on neurons. Microfluorimetric studies demonstrated the ability of Tat to induce in hippocampal neurons calcium currents that require extracellular calcium and are partially blocked by ω-conotoxin and nicardipine. Moreover, we also observed an indirect mechanism that might mediate Tat toxicity: both cortical and hippocampal neurons, following a 24 hours stimulation with Tat, show an increased sensitivity to the excitotoxic insult caused by glutamate.

N° dell'Accordo di Collaborazione 30C.68

CHEMOKINES AND AIDS DEMENTIA COMPLEX: A POSSIBLE ROLE FOR SDF-1 IN THE AIDS-REACTIVE GLIOSIS

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Infection of HIV-1 is frequently associated with neuronal death responsible for AIDS dementia complex (ADC). The current opinion supports the hypothesis that an indirect mechanism exists to explain the neuronal cell death and that the viral protein gp120 is one of the main candidates as mediator of HIV-1 induced neurotoxicity. In previous studies we have demonstrated that gp120 is toxic for cortical neurons cultured in presence of type I astrocytes. Considering the chemokine roles in the HIV infection, we analysed in particular the production and functions of SDF-1 and its receptor CXCR4 in CNS cells.
We have demonstrated that CXCR4 and SDF1 are expressed in cultured type I rat astocytes, cortical neurones and cerebellar granule cells, and that this receptor responds to SDF1 stimulation in astrocytes and cortical neurons. Moreover we demonstrated that SDF1 is secreted by astrocytes after induction with LPS and, on the contrary, cerebellar granule cells secrete this chemokine in basal conditions. It has been proposed that the CXCR4 receptor is overexpressed and required for proliferation in human glioblastoma tumor cells. We have investigated whether SDF1α is involved in the control of cell proliferation in cultured type I rat astrocytes. Our results indicate that SDF1 induces a dose-dependent cell proliferation. Moreover we analysed the intracellular pathway activated by SDF1α upon CXCR4 binding and observed, ERK1/2 activation in astrocytes, after SDF1α treatment. Furthermore the astrocytes proliferation can be reduced by PD98059, a MEK inibitor, indicating that ERK1/2 plays a role in the SDF1α induced proliferation. We demonstrated also that PTX treatment reduced MAPK activation suggesting a role for the $G\alpha_i$ protein to link CXCR4 receptor to this intracellular pathway and cell proliferation. In conclusion activation of CXCR4 receptor by SDF1α results in astroglial proliferation via stimulation of the MAPK pathway, suggesting the possible involvement of the CXCR4 in the gliosis induced in the ADC progression.

N° dell'Accordo di Collaborazione 30C.68

SENSITIVITY OF THE HUMAN HERPESVIRUS 8 (HHV8) INFECTED CELL LINES TO THE CYTOLYTIC ACTIVITY MEDIATED BY NATURAL KILLER CELLS IS INVERSELY RELATED TO THE SURFACE EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX-CLASS I ANTIGEN.

Sirianni M.C. [(a)], Libi F. [(a)],Scaramuzzi D. [(a)], Topino S. [(a)], Pierdominici M. [(a)], Mezzaroma I. [(a)], Tedesco T. [(a)], Carbonari M. [(a)], Monini P. [(b)], Ensoli B. [(b)]
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Infection by Human Herpesvirus 8 (HHV8) is associated with Kaposi's Sarcoma (KS), primary effusion lymphomas (PEL) and some variants of the multicentric Castleman's disease. Several cell lines have been derived and established from patients affected by PEL. These cell lines as well as the 'spindle'-like cells of endothelial or monocyte origin, present in KS lesions, are latently infected by HHV8. Several herpesviruses have the potential to interfere with the surface expression, on infected cells, of Major Histocompatibility Complex (MHC) class I antigens, thus inhibiting the recognition of these cells by cytotoxic T lymphocytes (CTL). The aim of our study was to assess the MHC-class I expression on the surface of some HHV8 latently-infected cell lines and to correlate the sensitivity of these cell lines to the cytolytic activity mediated by peripheral blood mononuclear cells (PBMC) from normal donors, both unpurified or highly enriched for natural killer (NK)- or T-CD8$^+$-cell populations. BCBL-1, BC-1, BC-2 and BC-3 PEL cell lines, established from HHV8 infected patients and obtained through the National Institute of Health (Bethesda, MD), were grown in the presence of β-mercaptoethanol or sodium pyruvate. Other HHV8 uninfected human cell lines examined were K562 (erythroleukemia derived) and Raji (B lymphoblastoid and infected by the Epstein Barr virus). MHC (human leukocyte antigen, HLA) class-I expression on the cell lines was assessed by indirect immunofluorescence (IFL). W6/32 (Dako, Glostrup, DK) and an isotype control monoclonal antibodies (mAbs) were detected with a goat anti-mouse serum, phycoerythrin-conjugated (GAM PE, Southern Biothechnology, Birmingham, AL). PBMC were isolated on a Ficoll-Hypaque gradient and then enriched for, respectively, NK cells or T-CD8$^+$ cells by the use of the StemSep^{TM} tetramers technology (StemCell Technologies Inc., Vancouver, Canada). This procedure resulted in viable populations of cells, with a 98% purity for each desired population. The percentage of purification was assessed by the surface expression of CD3, CD16, CD56, CD4 and CD8 on unpurified and purified cells by flow cytometry. The cytolytic activity of PBMC and purified populations was assessed in a 4 hour ^{51}Chromium release assay by the various cell lines. The relative fluorescence intensity of the MHC-class I molecules expression, by the cell lines examined, was the following: K562 1.57, BCBL-1 24.23, BC-1 47.8, BC-2 180.2, BC-3 125.1, Raji 36.27. K562 and BCBL-1 were sensitive to the lysis mediated by PBMC and there was a twofold increase of their sensitivity when highly-enriched populations of cells bearing markers of NK lineage (CD3$^-$, CD16$^+$, CD56$^+$) were used as effectors in the assay. This was seen at all the effector : target cell ratios tested (10:1, 5:1, 2.5:1, 1.25:1). The remaining cell lines were poorly or not sensitive to the lysis. On the contrary, purified T-CD8$^+$ cells were scarcely able to lyse the BCBL-1 cell line. Our results confirm the finding that HHV8 is able to reduce MHC class I expression on infected cells, like BCBL-1 (Brander C. et al., J. Immunol. 165, 2077-2083, 2000). This interferes with the recognition by CTL, allowing a mechanism of viral escape from the immune response. On the contrary NK cells, which mediate a non-MHC restricted cytotoxicity, may have a role in the control of some HHV8 related diseases, as suggested by our present results, which confirm our previous observations in Human Immunodeficiency Virus infected patients, who developed KS. In some of these cases we showed that a good clinical response to the highly active antiretroviral therapy (HAART) was associated to a recovered cytolytic activity to K562 and BCBL-1 and to a reduced antibody titer to HHV8 lytic antigens. Moreover, Interleukin (IL)-2 was able to improve the cytolytic activity of PBMC from normal donors to BCBL-1, although KS can develop even in patients treated with HAART+IL-2 or in long-term-non-progressors. Our findings have implications both to explain the relationship between HHV8 and the immune response and for the development of future, novel therapeutic approaches.

30C.69

SEROLOGIC MARKERS OF IMMUNE-ACTIVATION IN KAPOSI'S SARCOMA (KS), WITH SPECIAL FOCUS FOR HUMAN IMMUNODEFICIENCY VIRUS-ASSOCIATED KS.

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Several lines of evidence suggest that inflammatory cytokines (IC), which are increased both at the systemic level and in tissues from Kaposi's Sarcoma (KS) patients, are able to trigger a cascade of events, which ultimately leads to the recruitment of inflammatory cells in tissues and to neoangiogenesis, that are prominent hstologic hallmarks of KS lesions. These processes are mediated by chemokines and angiogenic factors that, in turn, are induced by IC. Our previous studies have shown, in fact, that these molecules are produced in KS lesions or by KS 'spindle' cells, cultured from KS lesions. Thus, the link between the chemokine and the cytokine system and KS development seems to be strict. The aim of our study was to assess the rate of immune-activation in KS patients and in individuals at risk of KS, by measuring the serum levels of factors, such as soluble CD8 (sCD8) and neopterin, Th1- or Th2-type cytokines and β chemokines. The groups of patients studied were: 51 patients affected by Human Immunodeficiency Virus (HIV)-associated KS, 53 patients affected by classical, not-HIV related KS (C-KS), 6 patients affected by post-transplant KS and 42 HIV positive patients, not affected by KS. A group of 14 normal blood donors (NBD), mean aged 30 years, gender- and age matched with the HIV-KS patients and a group of 68 elderly NBD (mean age: 82$\pm$18.7 years, range 54-104), age matched with the C-KS patients, were also studied. SCD8 and neopterin serum levels were measured by ELISA, through commercial kits (Endogen, Woburn, MA for sCD8 and ELItest Neopterin, Brahms, Berlin). In elderly NBD and in HIV-KS the serum levels of γIFN, IL-4 and, in HIV-KS, the levels of MIP1α and β were also measured (ELISA Quantikine, R&D Systems, Minneapolis, MN). Mean levels of neopterin (nmol/L) were the following: young NBD 5.4$\pm$1.9, elderly NBD 16.8$\pm$20.9 (with a trend to the increase with age), C-KS 11.2$\pm$12.3, HIV-seropositives 14.8$\pm$20.8, HIV-KS 14.1$\pm$15, with a reduction of the values (7$\pm$4) in patients undergoing highly active antiretroviral therapy (HAART) from at least 6 months. Mean levels of sCD8 (IU/ml) were: young NBD 286$\pm$105.1, elderly NBD 427.5$\pm$174.1, C-KS 333.6$\pm$152, post-transplant KS 275.2$\pm$86.9, HIV-seropositives 731.6$\pm$385.5, HIV-KS pre-HAART 756.4$\pm$576.9, post-HAART 364.2$\pm$162.7. Seven individuals with HIV-KS undergoing HAART were longitudinally followed for at least 8 months and sCD8 values were 903.3$\pm$379 (pre-therapy) and 525.4$\pm$205, post-HAART. In the latter cases, we also found no IL-4 in the serum and decreasing levels of γIFN and β chemokines, during HAART. Notably, the type 1 and type 2 cytokine profile, assessed in elderly NBD, showed the presence of detectable serum levels of γIFN (mean values: 24.3$\pm$10.8 pg/ml) and no detection of IL-4. In HIV-KS patients undergoing HAART the decrease of immune-activation, as assessed by a reduction of serum levels of neopterin, sCD8 and chemokines was accompanied by a reduction of the antibody titers to HHV8 lytic antigens and by a decrease of the HIV load. Our previous studies have shown that IC, particularly γIFN, play a key role in KS pathogenesis. Here we show that immune-activation, leading to increased levels of Th1-type cytokines, but not IL-4, is a common trait of patients with KS or at risk of KS. Notably, neopterin and sCD8 are also elevated in elderly NBD, who were age-matched to C-KS patients. The use of these serologic markers, which overcome the need for cell cultures, may be of help for monitoring KS course and therapy, as indicated by the reduction of sCD8, γIFN and β chemokines in patients undergoing HAART.

30C.69

POTENTIATION OF INHIBITION OF WILD TYPE AND MUTANT HIV-1 REVERSE TRANSCRIPTASES BY COMBINATIONS OF NON-NUCLEOSIDE INHIBITORS AND D- AND L-(β)-DIDEOXYNUCLEOSIDE TRIPHOSPHATE ANALOGS.

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Combinations of reverse transcriptase (RT) inhibitors are currently used in anti-HIV therapy in order to prevent or delay the emergence of resistant virus and to improve the efficacy against viral enzymes carrying resistance mutations. Drug-drug interactions can result in either positive (additive or synergistic inhibition) or adverse (antagonistic interaction, synergistic toxicity) effects. Elucidation of the nature of drug interaction would help to rationalise the choice of antiretroviral agents to be used in combination. In this study, different combinations of nucleoside and non-nucleoside inhibitors, including D- and L-(β)- deoxy and -dideoxynucleoside triphosphate analogues, have been tested in in vitro RT assays against recombinant RT either wt or bearing clinically relevant non-nucleoside inhibitors resistance mutations (L100I, K103N, Y181I) and the nature of the interaction (either synergistic or antagonistic) of these associations were evaluated. The results showed that: i) synergy of a combination was not always equally influenced by the individual agents utilised; ii) a synergistic combination could improve the sensitivity profile of a drug resistant mutant enzyme towards the single agents utilised; iii) L-(β)-enantiomers of nucleoside reverse transcriptase inhibitors were synergistic when combined with non-nucleoside reverse transcriptase inhibitors and iv) inter- and intra-combination comparisons of the relative potencies of each drug could be used to highlight the different contributions of each drug to the observed synergy.

References: Maga et al, Antimicrob. Agents and Chemother. (2001), in press

Accordo di Collaborazione N.:30C.70

ROLE OF THE TESTIS AS HIV-RESERVOIR

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In previous papers (Muciaccia et al Faseb J, 12: 151-163, 1998; Muciaccia et al. J Reprod Immunol. . 41: 81-93, 1998), we demonstrated the presence of HIV-1 infected germ cells in the testes of HIV-seropositive non-Aids subjects, and we formulated the hypothesis that the testis may represents a cellular and anatomic reservoir. In fact, by in situ PCR and RT-PCR it was observed the presence of HIV virus in spermatogonial cell populations, which are known to cointain the germ stem cells, as well as in primary spermatocytes which are located in the adluminal compartment of the seminiferous epithelium, i.e. on the other side of the blood-testis barrier. The possibility that the testis may represent a HIV-reservoir, suggested us to monitorize testicular HIV-1 infection during pharmacological treatment of HIV-seropositive subjects. To this purpose, due to the difficulties encountered to obtain testicular bioptic material, we decided to evaluate the testicular reservoir, by studying the presence of HIV in the ejaculated spermatozoa from HIV-seropositive subjects treated with specific pharmacologic therapies.
Presence of HIV-1 in ejacualated spermatozoa from infected men has been object of a long and not yet solved controversy, due to the possibility that viral DNA revealed by PCR in sperm samples may actually derive from contaminating somatic cells.We decided, therefore, to face the pitfalls connected with heterogenity of the cell population present in the seminal fluid, where lymphomonocytes are frequently observable, particularly in the presence of a uro-genital tract infection. Several approaches were used to separate the spermatozoon cell population from “contaminating” somatic cells and to evaluate the purity of the cell population obtained by gene expression screening. The best results were obtained by treament with osmotic shock after Percoll gradient and swim up isolation of spermatozoa. Purity of the cellular fraction obtained, has been assessed by RT-PCR amplification of mRNAs spcifically expressed in lymphomonocytic cells and never in spermatozoa, such as those for CD4, and of mRNAs expressed only by spermatozoa, among which those for PMR2. This approach allowed us to obtain spematozoal populations PMR2 positive and CD4 negative after RT-PCR , therefore completely deprived of contaminating lymphomonocytic cells. However, we are experimenting methods to increase the efficency of mRNA recovery from spermatozoa, since low amount of mRNA are known to be present in these cells and, in addition, HIV-seropositive subjects have frequently low amount of spermatozoa in the semen. We have then utilize ejaculated spermatozoa from healthy men in experiments of DNA genomic amplification by PCR, obtaining adequate results for b-globin and PMR2 genes, both in the soluble fraction and in situ. To eventually evaluate HIV-1 proviral DNA integration into the sperm genome, we tested the *Alu*-LTR PCR method on 8E5/LAS cell line, obtaining positive results. In conclusion, during the last year of activity, we have solved the methodologic problems inherent in our experimental model. We have now adequate tools to verify of the presence of HIV-1 in ejaculated spermatozoa from seropositive subjects by PCR and in situ PCR.

N°. dell'Accordo di Collaborazione. 30C.71

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) SIGNIFICANTLY IMPROVES DISEASE FREE SURVIVAL (DFS) IN PATIENTS (PTS) WITH HIV-RELATED NON-HODGKIN'S LYMPHOMA (HIV-NHL) TREATED WITH CHEMOTHERAPY (CT).

Vaccher E., Spina M., Bernardi D., Talamini R., Tavio M., Nasti G., di Gennaro G., Simonelli C., Schioppa O., Juzbasic S., Vultaggio G., and Tirelli U., for the GICAT.
Division of Medical Oncology A, National Cancer Institute - Aviano, Italy.

To assess the impact of HAART on the outcome of HIV-NHL, we analyzed our monoistitutional study of 235 pts with systemic HIV-NHL, diagnosed and treated between September 1987 and December 1999. Two hundred and six pts were included in prospective studies with combination CT and they are object of this study. Seventy-nine (34%) pts received at any time (i.e. prior, during and after CT) HAART while 156 pts did not receive HAART because not yet available. HAART, clinical findings and laboratory data were evaluated by univariate and multivariate analyses to investigate prognostic factors potentially influencing DFS and overall survival (OS). CR occurred in 49% of pts (no difference in those treated with or without HAART, while the 3-year DFS and OS were 73% and 19%, respectively. In the univariate analysis, female sex, age < 35 yrs, no intravenous drug use, normal LDH value and HAART use were favorable prognostic factors for DFS, while no prior AIDS, ECOG PS < 2, no immunoblastic histology, no B symptoms, CD4 $\geq$ 100/μL, normal LDH value, no extranodal disease and HAART use were favorable prognostic factors for OS. As far as the multivariate analysis, HAART use significantly increased DFS and OS as well, as shown in the table.

	Hazard ratio* (95% CI)	P value
DFS		
LDH normal value	0,3 (0,1 – 0,8)	0,001
HAART	0,3 (0,1 – 0,8)	0,02
OS		
CD4 $\geq$ 100/μL	0,6 (0,4 – 0,8)	0.004
LDH normal value	0,5 (0,3 – 0,7)	$\leq$ 0.001
HAART	0,3 (0,2 – 0,4)	$\leq$ 0.001

* Cox proportional hazard model adjusted

In conclusion, our study shows for the first time that the use of HAART may significantly prolong DFS in systemic HIV-NHL pts, suggesting a direct impact of HAART on the natural history of HIV-related NHL.

N° dell'accordo di collaborazione: 30C.73

FEASIBILITY OF THE INTEGRATION OF STANFORD V CHEMOTHERAPY (CT) REGIMEN WITH HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) AND G-CSF IN PATIENTS (PTS) WITH HODGKIN'S DISEASE AND .HIV INFECTION (HD-HIV).

Spina M., Gabarre J., Vaccher E., Nasti G., di Gennaro G., Schioppa O., Juzbasic S., Scalone S., Di Lauro V., Vultaggio G., Tirelli U., for the GICAT.
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The outcome of pts with HD-HIV is still poor, mainly because the duration of complete remission (CR) is quite short. In order to try to improve the prognosis of HD-HIV, a feasibility study with the intensive 12 week CT regimen with adjuvant radiotherapy, Stanford V {S.J. Horning et al, Ann Oncol 7: Sl05-8, 1996) and concomitant HAART was started in previously untreated HD-HIV pts with bulky limited stage or stage III-IV. HIV+ pts with pathologjcal diagnosis of HD were treated with CT (mg/m2) including doxorubicin 25 and vinblastine 6 on wks l, 3, 5,7, 9, 11; nitrogen mustard 6 wks l, 5, 9; etoposide 60 x 2 wks 3, 7, 11; vincristine 1.4 (max 2) and bleomycin 5wks 2, 4, 6, 8, 10, 12 and prednisone 40 qod. G-CSF was given at the dose of 5 mcg/kg/day sc, days 3-13 (omitted on day 8) and days 17-26 (omitted on day 22) in each cycle. HAART (triple-drug combination including 1 protease inhibitor) was given concomitantly from the beginning of CT, irrespectively of CD4+ cell count and HIV viral load, and was selected based on the pts prior antiretroviral exposure. Pts received daily oral thrimetoprim-sulfamethoxazole to prevent PCP. Since April 1997, out of 43 pts entered, and all are now evaluable for toxicity and response. The median age was 36 yrs (28-63). All pts but one were males, 20 were IVDUs, 13 homosexuals and 10 heterosexuals. The median PS was 1 (0-3). The median CD4+ cell count at entry was 227/mm3 (32-1008) and 24 pts had a detectable HIV viral load (median 2450 copies/μL (range 60- 455000). Systemic “B” symptoms were found in 72% of pts. Stage III and IV disease was present in 34 pts. Histologic subtypes were: MC 17, NS 11, LD 3, not determined 12. As far as toxicity, no toxic death was observed, while an absolute neutrophil count < 500 was observed in 24 out of 43 pts. Eleven pts (26%) had febrile neutropenia with 2 documented bacterial sepsis. Grade 3-4 anaemia was documented in 15 pts. A grade 2-3 peripheral neuropathy was observed in 11 pts. Ileus occurred in 4 pts. As far as response, CR was obtained in 35 pts (81%) and PR in 3 (7%). Six CR pts relapsed (17%). The actuarial overall survival and disease free survival at 24 months are 57% and 66%, respectively. Our preliminary data demonstrated that the abbreviated CT regimen, Stanford V, in combination with HAART is feasible and active in pts with HD-HIV. The amount of neutropenia was as expected while neurologic toxicity could be related to concomitant use of vinca alkaloids and stavudine (D4T).

N° dell’accordo di collaborazione: 30C.73

PILOT TRIAL OF RITUXIMAB AND CHEMOTHERAPY WITH INFUSIONAL CYCLOPHOSPHAMIDE, DOXORUBICIN, AND ETOPOSIDE (CDE) IN HIV-ASSOCIATED NON-HODGKIN'S LYMPHOMA (NHL).

Spina M., Vaccher E., Tavio M., Nasti G., Di Gennaro G., Bernardi D., Simonelli S., Schioppa O., Vultaggio G., Milan I., Zanetti M., Martellotta F., Tirelli U., for the GICAT.
Division of medical Oncology A, National Cancer Institute – Aviano, Italy.

Background: The objective of this trial was to determine the feasibility of combining rituximab with infusional CDE (Proc ASCO 18; 12a, 19991 abstr 41). The rationa1e for adding rituximab include its activity in refractory lymphoma, its non-overlapping toxicity and differing mechanism of action.
Methods: Thirty patients (pts) with HIV-associated B-cell NHL received infusional CDE [cyclophosphamide (200 mg/M2/day), doxorubicin (12.5 mg/M2/day), and etoposide (60 mg/M2/day) given by continuous intravenous infusion for 4 days (96 hours)] every 4 weeks for up to 8 cycles plus rituximab (375 mg/M2) by one of two schedules: (l) prior to each cycle of CDE (N = 25), (2) on day -8 and day -1 prior to cycle 1, just prior to cycles 3 and 5, then on days 28 and 35 after the last cycle (N = 5).
Results: Pt characteristics: median CD4 -132/μL (range 3-470); stage IV (N = 13 [45%]); intermediate (N = 14 [48%]) or high-grade (N = 16 [53%]) histology. The median number of rituximab doses given was 5 (range 2-6) and of CDE was 5 (range 3-6). There were 10 grade 3-4 infections (34%). The incidence of grade 3-4 toxicity for CDE rituximab compared with historical data for CDE alone (Proc ASCO 18; 12a, 1999, abstr 41) was comparable for infections (34% vs. 27%), neutropenia (79% vs. 85%), thrombocytopenia (34% vs. 75%), and mucositis (17% vs. 12%). Twenty-four of 28 patients (86%) have had a complete response, and only 1 pt has relapsed after a median of 4 months (range 1-13 months).
Conclusions: These findings suggest that the addition of rituximab to infusional CDE does not substantially increase the risk of infection in patients with HIV-associated NHL, and that the combination is effective and merits further study.

N° dell'accordo di collaborazione: 30C.73

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) IS AN EFFECTIVE ANTI-KAPOSI'S SARCOMA (KS) THERAPY AFTER DEBULKING CHEMOTHERAPY (CT).

Tirelli U., Juzbasic S., Vultaggio G., Nasti G., di Gennaro G., Fasan M., Ridolfo A., Nigro L., Bernardi D., Tavio M., Spina M., Vaccher E., for the GICAT.
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HAART may inhibit the progression of HIV-related KS. However, the clinical benefit of HAART after systemic CT or maintenance (M)-HAART in rapidly progressive KS patients (pts) has not been evaluated. To investigate the anti-KS activity of M-HAART, the Italian Cooperative Group on AIDS and tumors (GICAT) performed a prospective study in advanced KS pts, who had obtained an objective response after systemic CT. Before CT, all pts had received HAART as anti-KS therapy, but it was not effective. Antiretroviral activity of M-HAART was evaluated by HIV-RNA viral load (b-DNA assay), but not by CD4 cell count because of the immunosuppressive effect of prior CT. From September 1998 to July 2000, 18 pts entered this study: 14 males, 4 females with median age of 36 yrs (range 22-52). Prior CT ACTG stages were T0-1 I1 S0-1 in 10 pts (56%), T1 I0 S0 in 4 (22%) and T0 I0 S0 in 4 pts (22%). Sixteen pts (89%) had previously received one line of CT (TAX-VNB 9 pts, ABV 7 pts) and 2 pts > 2 lines of miscellaneous CT regimens. After CT, median HIV-RNA viremia was < 500 cp/μL (range < 50-189400) and median CD4 cell count 211/μL (range 1-565). The table shows response rates after CT and after M-HAART (median duration 21 months [mos], range 2-39). All new CR pts had achieved PR > 75% after CT and subsequently obtained CR with M-HAART that was not effective as anti-KS therapy prior to debulking CT. Median duration of CR was 24+ mos (range 9,5+ - 32+) and median progression free survival was 21+ mos (range 2+ - 39+). Only 1 pt (6%) developed severe opportunistic infections during M-HAART. No correlation was found between anti-KS response and virological response to M-HAART (p = 0.2). In conclusion, our data show that M-HAART is an effective anti-KS therapy after debulking CT, while not effective in pts with rapidly progressive KS as primary anti-KS therapy.

Table

Response to CT	#	Response to M-HAART n° New	Response to M-HAART n° SD	Response to CT and M-HAART n° (%) TOT
CR	2	7	2	9 (50)
PR	15	-	7	7 (39)
SD	1	-	1	1
PRO	-	1	-	1

CR = complete remission; PR = partial remission; SD = stable disease; PRO = progressive disease.

N° dell'accordo di collaborazione: 30C.74

TAXOL (TAX) AND VINORELBINE (VNB) IN COMBINA ION WITH HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) IN KAPOSI'S SARCOMA (KS): AN EFFECTIVE AND SAFE REGIMEN.

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TAX (Gill P.S. et al, J CO, 1999) and VNB (Errante D. et al, AIDS, 1996) are active single agents in KS. Moreover, preclinical data showed synergistic interactions when VNB preceded TAX exposure. We performed a phase II study to evaluate response and toxicity of a regimen of VNB (25 mg/m2 15 min. i. v. infusion) followed by TAX (100 mg/m2, 3 hours i.v. infusion) every 3 weeks with concomitant HAART in patients (pts) with advanced KS. Triple antiretroviral regimens including protease inhibitors were selected based on prior patient therapy. G-CSF (5 μg/kg) was given on day 2-10. Since October 1996, 39 pts were enrolled (M 36, F 3, median age 42 yrs, PS 0-1 82%, ACTG stage T0 I0 S0 2%, T1 I0 S0 26%, T0-T1 I1 S1 69%). Thirty-six per cent of pts had received prior chemotherapy. At baseline, median CD4 cell count was 162/μL (range 3-915) and HIV- RNA viremia (b-DNA) 25.000 cp/mL (range < 50-534.000). A median of 4 (range 1-6) cycles has been delivered to date. Of the 30 (77%) evaluable pts, 2 pts (7%) achieved CR (duration 26+ months), 18 (60%) PR (overall response rate 67%), 4 (13%) SD and 6 (20%) progressive disease. The median time to progression was 12+ months (range 3+-34+). The main toxic side effects (WHO criteria) were: leukopenia (GI-G2 25%, G3-G4 50%), anemia (Gl-G2 42%, G3-G4 31%), thrombocytopenia (GI-G2 28%; G3-G4 22%), nausea-vomiting (GI-G2 22%, G3-G4 8%), mucosites (G3-G4 17%), peripheral neuropathy (GI-G2 11%), autonomic neuropathy (Gl-G2 6%, G3 6%), and liver toxicity (G1-G2 11 %, G3-G4 4%). One toxic death occurred due to the bone marrow and liver toxicity .These results suggest that TAX -VNB plus HAART is an effective treatment in KS with relatively durable response and acceptable toxicity.

N° dell’accordo di collaborazione: 30C.74

NON AIDS-DEFINING OTHER THAN HODGKIN'S DISEASE (HD) IN HIV-INFECTED INDIVIDUALS: GICAT SERIES.

Tirelli U., Spina M., Tavio M., Nasti G., Di Gennaro G., Bernardi D., Simonelli S., Schioppa O., Vultaggio G., Milan I., Zanetti M., Martellotta F., Vaccher E., for the GICAT.
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The full spectrum of HIV-induced malignancies have now been fully elucidated but a large variety of cancers other than AIDS-defining tumors have been diagnosed in HIV-infected patients (pts). The widespread use of HAART in the industrialized countries as resulted in substantial improvement in the survival of HIV-infected pts. It is likely that in the future, cancers associated with long-term mild immunosuppression will occur at an increased rates in long-term survivors of HIV infection. Among 218 cases of HIV-related tumors collected by the GICAT at september 2000, 491 were non-AIDS defining tumors, including 206 cases of HD and 285 cases of solid tumors (ST). The table reports data on the distribution of ST in pre-HAART and post-HAART years (yrs).

Tumor site	Total N.	pre-HAART yrs (86-96) N. (%)	post-HAART yrs (97-2000) N. (%)
Cervix	80	64 (80)	16 (20)
Lung	56	27 (48)	29 (52)
GI tract	29	18 (62)	11 (38)
Head-neck	19	9 (47)	10 (53)
Skin	18	11 (61)	7 (39)
Other	83	40 (48)	43 (52)

In this communication we summarize the pathological, epidemiological, clinical features and the therapy of the most frequently reported non-AIDS ST in Italy.

N° dell'accordo di collaborazione: 30C.75

SIMIAN VIRUS 40 FOOTPRINTS IN HUMAN LYMPHOPROLIFERATIVE DISORDERS OF HIV$^-$ AND HIV$^+$ PATIENTS AND IN DERIVED LYMPHOBLASTOID B-CELL LINES.

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SV40 sequences were investigated by PCR amplification, followed by filter hybridization, in a series of human lymphoproliferative disorders, obtained from human immunodeficiency virus (HIV)-seronegative and HIV-infected patients, and derived lymphoblastoid B-cell lines. Our PCR and filter hybridization conditions allowed to detect SV40 sequences in the range of $10^{-4}\div10^{-2}$ genome equivalents per cell. In non-Hodgkin's lymphomas (NHL) from HIV$^-$ patients, SV40 footprint was found in 11/79 (13.9%) samples, while in NHL from HIV$^+$ patients, SV40 DNA sequences were detected in 2/16 (12.5%). In Hodgkin's disease (HD), 7/43 (16.3%) from HIV$^-$ and 1/12 (8.3%) from HIV$^+$ patients, respectively, were SV40$^+$. A slightly higher prevalence of SV40 footprint was observed in reactive lymphoadenopathies in both HIV$^-$(3/9, 33.3%) and in HIV$^+$ (6/17, 35.3%) patients. SV40 sequences were also detected in 22/42 (52.3%) of human lymphoblastoid B-cell lines, with a similar prevalence in lymphoblastoid B-cell lines obtained by spontaneous in vitro outgrowth (7/15, 46.7%) and in lymphoblastoid B-cell lines induced with the prototypic B95.8 EBV strain (15/27, 55.5%). No evidence of SV40 sequences was found in the marmoset B95.8 cell line from which infectious EBV virions were produced. Moreover, SV40 Tag sequences were detected in 2/8 (25%) (Namalwa, HBL-2) Burkitt's lymphoma cell lines investigated. SV40 Tag sequences correlated with the presence of the viral DNA sequences in the B-lymphocytes or tissues from which the lymphoblastoid B-cell lines originated. Expression of SV40 Tag sequences was found in 5/5 SV40$^+$ lymphoblastoid B-cell lines by RT-PCR with oligonucleotides specific for SV40 early region mRNA. Moreover, the Tag expression was detected by immunohistochemistry in 5/18 SV40 DNA-positive lymphomas analyzed. However, few tumor cells (<1%) in 3/5 samples displayed the SV40 Tag, while this viral oncoprotein was revealed in several reactive histiocytes present in all five SV40 positive tissues. In situ hybridization experiments showed that, in SV40$^+$ lymphoblastoid B-cell lines, SV40 DNA was present in the nucleus, but in a small fraction of cells (1-2 out of 500). SV40 Tag coding sequences were retained for more than 6 months of continuous culture, without gross variations in the viral DNA load. The tumorigenicity of both SV40$^+$ and SV40$^-$ lymphoblastoid B-cell lines were assayed in SCID mice. Although a higher percentage of SV40$^+$ B-cell lines was tumorigenic (4/5, 80% vs. 2/4, 50%), the total number of tumor masses induced by SV40$^+$ and SV40$^-$ B-cells was comparable (12/25, 48% vs. 7/20, 35%), ruling out that a low SV40 viral load might enhance the lymphoblastoid B-cell tumorigenicity in SCID mice. SV40 DNA was retained in tumors induced by SV40$^+$ lymphoblastoid B-cell lines, with no evident increase of viral DNA load. Sequence analysis established that the amplified PCR products belong to the SV40 sequences. SV40 prevalence and load were similar in samples from HIV-seronegative and HIV-infected individuals, suggesting that SV40 probably does not undergo strong reactivation phenomena in the context of HIV-related immunosuppression. These results suggest that the lymphoid tissue and B-lymphocytes could represent a reservoir for SV40, support the hypothesis that this oncogenic virus may be transmitted through blood cells and may constitute the first step in understanding whether this DNA tumor polyomavirus has a role in the pathogenesis of human lymphoproliferative disorders.

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SOME CLINICAL AND IMMUNOLOGIC RESULTS OF ANTIRETROVIRAL THERAPY MAY BE DUE TO ITS INHIBITORY EFFECTS ON PROTEASOME ACTIVITY.

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Background. Proteasome is the major nonlysosomal endoprotease of eukaryotic cells. Cell-cycle, antigen presentation, cell surface receptors, cytokine production, and apoptosis are all regulated by proteasome activity. We previously showed that prophylaxis with highly active antiretroviral therapy (HAART) to seronegative, HIV-1-exposed subjects inhibited the production of TNF-alfa, IL-2 and IFN-gamma. To further investigate possible direct immunomodulatory effects of antiretroviral agents we assessed whether they inhibit human proteasome activity.
Methods. Chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolysing activities of human 26S and 20S proteasomes, the latter alone or complexed with 11S regulator, were assessed after incubation with indinavir, lamivudine, and zidovudine at 10-80 μM in various combinations. Furthermore, accumulation of ubiquitinated proteins, a hallmark of proteasome inhibition in vivo, and of Ik-B and NF-kB p105, elective intracellular proteasome substrates, were assessed in Jurkat cells incubated for 6 hours with the same concentrations of the drugs, alone and in combination.
Results. Chymotryspsin-like and, partially, trypsin-like activities of 26S proteasome were inhibited by each drug, particularly at 40 μM. These inhibitory effects increased when the drugs were used in double and mostly in triple association. Peptidyl-glutamyl-peptide hydrolysing activity was refractory to the drugs as well as the multipeptidase activity of 20S proteasome, both alone and complexed with the 11S regulator. Preliminary data indicate that accumulation of ubiquitinated proteins as well as of Ik-B and NF-kB p105 occurs in Jurkat cells incubated with 20 μM of the drugs in triple association.
Conclusions. The 26S proteasome activity is impaired by certain antiretroviral agents, particularly when used in association. This inhibitory effect may result in a reduced possibility of viral replication, decreased production of pro-inflammatory cytokines, and lower apoptosis in patients treated with HAART. The clinical and immunologic response to therapy in patients with virologic failure may be due the direct effects of HAART on host cell.

N°. dell'Accordo di Collaborazione. 30C.77

USE OF AN INTEGRATED HIV RESISTANCE MUTATION DATABASE FOR UNCOVERING ASSOCIATIONS BETWEEN MUTANTS AND TREATMENTS: RT MUTATIONS AT CODONS 44 AND 118 ARE PREFERENTIALLY SELECTED BY STAVUDINE AND DIDANOSINE.

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Since genotypic antiretroviral resistance testing is now considered an integral part of the clinical management of HIV-infected patients it is crucial to understand the specific association between mutations and decreased susceptibility to anti-HIV drugs. Recent evidences have revealed a greater level of cross-resistance than previously thought for nucleoside reverse transcriptase inhibitors (NRTIs). Examples include resistance to abacavir in the presence of zidovudine and lamivudine resistance mutations, selection for 'zidovudine-specific' RT mutations by stavudine and decreased sensitivity to lamivudine when novel mutations at codons 44 and 118 are detected in the context of zidovudine resistance. Using a resistance mutation database generated by sequencing 3,195 RT genotypes in clinical practice, we have confirmed that V118I and E44D are virtually absent in drug-naïve subjects and are associated to zidovudine-resistance mutations (especially L210W). V118I and E44D were present in 408 (12.8%) and 166 (5.2%) sequences, respectively. The variant 44A was far less prevalent (n=26, 0.8%) and strongly linked to a 43E low-frequency mutation (also drug.-selected). Both V118I and E44D/A were also linked to H208Y, a mutation previously reported to confer resistance to foscarnet. Analysis of a subfile containing 2378 sequences obtained from subjects for whom the treatment history was available showed that the presence of 118I and 44D was far more associated with stavudine and didanosine, rather than lamivudine, therapy. Examination of treatment history of the patients with multiple sequence data identified 38 cases with a shift from absence to presence of 118I or 44D associated with a NRTI switch. The NRTIs switched to were stavudine and/or didanosine in 33 (86.8%) patients while only one (2.6%) patient switched to lamivudine. Thus, the novel RT mutations at codons 44 and 118 shown to confer moderate resistance to lamivudine are preferentially selected by stavudine and didanosine, suggesting that susceptibility to these compounds is also decreased. The use of a growing number of antiretroviral compounds in the absence of effective long-lasting suppression of HIV replication has made a basis for drug resistance to evolve with increasing complexity. Databases linking genotype with treatment history may be valuable tools in this context.

N°. dell'Accordo di Collaborazione: 30C.79

FRACTALKINE AS AN AMPLIFICATION CIRCUIT OF POLARIZED TH1 RESPONSES

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Fractalkine (FKN) is a membrane-bound CX3C chemokine induced by primary proinflammatory signals in vascular endothelial cells. Here we examined whether FKN is part of induction or amplification circuits in polarised Th1 or Th2 responses. Proinflammatory signals, including LPS, IL-1, TNF and CD40 ligand induced FKN in human endothelial cells. FKN was also induced by interferon-γ (IFNγ) which had synergistic activity with TNF. IL-4 and IL-13 did not stimulate the expression of FKN and markedly reduced induction by TNF and IFNγ, alone or in combination. TNF alone and in combination with IFNγ also induced release of soluble FKN, which was inhibited by IL-4 and IL-13. The finding of a differential regulation of FKN by master cytokines of polarised responses prompted an analysis of the interaction of FKN with polarised T cell populations. The FKN receptor CX3CR1 was preferentially expressed in Th1 compared to Th2 cells. Th1, but not Th2 cells, responded to FKN. NK cells expressed high levels of CX3CR1 and responded to FKN. By immunohistochemistry, FKN was expressed on endothelial cells in psoriasis, a Th1-dominated skin disorder, but not in Th2-driven atopic dermatitis. Similarly, endothelial cells in Mycobacterium tuberculosis granulomatous lymphadenitis, but not those in reactive lymph node hyperplasia or in Castelman's disease, showed immunoreactive FKN. These results indicate that regulated expression of FKN in endothelial cells is part of an amplification circuit in polarised type I responses in vitro and in vivo.

It has recently been observed that HIV infected patients homozygous for the CX3CR1 I-249 M-80 allele show rapid progression to AIDS (Faure et al.Science 287: 2274, 2000). The CX3CR1 I-249 M-80 haplotype is associated with reduced affinity and surface expression of the receptor. Given the protective role of polarised Th1 responses against many opportunistic pathogens and HIV itself, it is tempting to speculate that the rapid progression to AIDS of CX3CR1 I-249 M-80 homozygous individuals might depend on a defective capacity to mount fully effective type I responses.

Accordo di collaborazione n.30C.80

ANTIAPOPTOTIC EFFECT OF HIV-1-PROTEASE INHIBITORS: INTERACTION WITH CELLULAR PROTEASE-SYSTEMS

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Background:
In previous studies we demonstrated immunoreconstitution of phagocyte functions in HIV-infected patients treated with HAART. For neutrophils this phenomenon was correlated with rapid decrease of spontaneous apoptosis and was observed even in patients with virological failure.

Objective:
To study the direct effect of HIV-1-protease inhibitors (PI) on apoptosis of PBMC and PMN isolated from healthy subjects and HIV+ patients. In order to clarify the cellular target on which PI could act, we are studying different cellular protease-systems apoptosis-related in cell line U937.

Methods:
Appropriate cellular cultures were carried out to test the effect of indinavir and ritonavir on spontaneous and puromycin (PMC)-induced apoptosis. Specific caspases and calpains inhibitors were used in parallel with PI. Apoptosis was evaluated by Hoechst staining and tunel assay. After treatment cells were lysed to test caspase activity by using specific fluorimetric substrates. The direct effect of PI on caspase 3, 6 and 8 was studied in cell extracts by enzymatic inhibition assay. Moreover inhibitory effect was evaluated on recombinant caspase 3.

Results:
PI showed a dose-dependent anti-apoptotic effect on PMN and PBMC from healthy controls. At concentration of 100nM a reduction of apoptosis rate of 30-45% for indinavir and 22-40% for ritonavir was found. Similar results were obtained on cells from HIV infected patients. In controls treated with z-VAD-fmk, a broad-spectrum caspase inhibitor, apoptosis decreased with a reduction rate of 65%. On the other hand a specific calpain-inhibitor showed a reduction similar to PI (28-40%).
PMC-induced apoptosis was decreased also in PI-treated U937. The cell lysates analysis showed a reduction of caspase 3, 6 and 8 activity (30, 32 and 10% respectively in PI-treated cells). On the contrary the addiction of indinavir and ritonavir (1-500 nM) after lysis of untreated PMC-induced U937 did not decrease caspase activity. Recombinant caspase 3 preserved its activity even if preincubated with different concentration of PI.

Conclusion:
These data confirm the anti-apoptotic effect of HIV-1 protease inhibitors and exclude a direct interaction with the main caspases studied. A similar apoptosis reduction rate observed in cells treated both with calpain inhibitor and PI suggests a possible implication of calpain system. Further studies are in progress to identify the cellular target of PI.

N. dell'accordo di collaborazione: 30C81

The National research program on AIDS

(Extramural research projects)

Project

PATHOGENESIS AND IMMUNITY FOR INDIVIDUATION OF NEW CHEMOTHERAPY, IMMUNOTHERAPY AND VACCINE PREVENTION TARGETS

Scientific Coordinator: **Paola VERANI**

Projects financed N° 94

REVERSE CORRELATION BETWEEN HTLV-II REPLICATION AND EXPRESSION OF CIITA, THE MAJOR REGULATOR OF HLA CLASS II TRANSCRIPTION

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HTLV-II retrovirus infects and propagates mainly in T cells with a CD8-positive phenotype. However HTLV-II can also be adapted to grow in B cell hosts. We recently succeded in adapting HTLV-II Mo strain to replicate in isogenic B cell hosts, Raji and RJ 2.2.5, whose only difference is the expression of HLA class II molecules present in Raji and absent in RJ 2.2.5. The RJ2.2.5 phenotype is due to a mutation in the AIR-1 locus which encodes CIITA, the major transactivator of HLA class II gene transcription. Unexpectedly, it was found that RJ 2.2.5 cells were massively infected and died after 3 weeks in culture whereas Raji cells were only marginally infected by the retrovirus. Restoration of an HLA class II positive phenotype in RJ2.2.5 by CIITA transfection completely abolished the HTLV-II sensitivity of the isogenic B cell mutant. Similar results were found in the T cell line MOLT-4 which supported HTLV-II replication but failed to do so when transfected with CIITA. Inhibition of HTLV-II replication correlated with a CIITA-positive and not with an HLA class II-positive phenotype, as the class II negative B cell line BLS-1, whose phenotype is caused by a defect in the transcription factor RFX-ANK, and which expresses normal level of CIITA failed to support viral replication.
The results presented in this report establish for the first time a direct correlation between the expression of the major regulator of MHC class II gene expression, the AIR-1 locus encoded transcriptional activator CIITA, and the susceptibility of B and T cells to actively replicate the HTLV-II retrovirus. The molecular basis of the CIITA-mediated inhibition of HTLV-II replication is presently under investigation. Modulation of AIR-1 gene expression can therefore be envisaged as a tool to counteract and possibly inhibit HTLV-II replication and spreading in susceptible hosts.

N°. dell'Accordo di Collaborazione : 40C.1

THE AIR-1 LOCUS-ENCODED HLA CLASS II TRANSCRIPTIONAL ACTIVATOR (CIITA) INHIBITS HIV-1 PRODUCTIVE INFECTION BY BLOCKING THE FUNCTION OF HIV-1 TAT.

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Constitutive and IFN-γ induced expression of HLA class II genes is under the control of the AIR-1 locus product CIITA, a transcriptional activator whose function is highly conserved across species. Here we show that expression of CIITA inhibits HIV-1 viral replication in human T cells, such as Jurkat and Molt-4. It must be stressed that inhibition of viral replication in human T cells stably expressing the human AIR-1 gene product was achieved in presence of CIITA levels comparable to those physiologically expressed in human B cells and in human macrophages treated with IFN-γ.The molecular mechanism by which CIITA inhibits viral replication consists in an inhibitory effect on the viral transactivator Tat, achieved by competing for cellular transcriptional cofactors used by both the class II transactivator and Tat. CIITA-mediated inhibition of Tat function was assessed and shown by several experimental approaches in several cellular targets:

1) by transient cotransfection of CIITA and Tat together with HIV-LTR-CAT construct in human T cells, B cells and Hela;
2) by addition of biosynthetic Tat protein in cultures of Hela cells stably transfected with HIV-1 LTR and CIITA.

At the conditions used, inhibition of Tat function by CIITA could not be obtained in Cos cells, suggesting a different sensitivity of distinct cell types and/or distinct species.
These results indicate that expression of the human HLA class II gene transactivator could be at the basis of the observed inhibitory effect on viral replication in APC activated by IFN-γ, and may open the way to new alternative approaches for controlling viral infection and spreading by modulating the expression of the AIR-1 gene.

N°. dell'Accordo di Collaborazione. 40C.1

HIV-1 NEF PROTEIN INDUCES THE ACTIVATION OF THE SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION STAT1 IN HUMAN MONOCYTE/MACROPHAGE PRIMARY CELLS.

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Nef is a 27-34 kDa HIV/SIV protein expressed in abundance during the early phases of viral replication. It is largely accepted that Nef plays a pivotal role in AIDS pathogenesis possibly by altering cell functions. For example, Nef expression down regulates both CD4 and MHC Class I molecules. In addition, Nef is able to up regulate the expression of Fas ligand and to interact with specific cellular signalling proteins such as Lck, Hck and Vav. Considering that some authors claimed that the expression of Nef could be also at the basis of an altered pattern of secreted cyto/chemokines, we investigated the possibility that Nef influences the JAK/STAT cytokine signal transduction pathway. We demonstrated that the expression of HIV-1 Nef in *ex vivo* cultures of human monocyte/macrophages (m/m) activates STAT1 but not STAT3. These results were obtained by infecting m/m with a VSV-G pseudotyped *nef* deleted HIV and confirmed by treating m/m with soluble recombinant Nef (rNef). In fact, we already showed that m/m efficiently internalize rNef, that disposes in an intracytoplasmic pattern highly reminiscent of that reported for cells endogenously expressing Nef. Western blot analysis of STAT1 activation and expression in cell extracts of rNef treated m/m showed a strong activation signal after 2 hrs followed by the increase of STAT1 expression. STAT1 activation was further confirmed via band shift experiment using both the GAS element of the IRF-1 promoter and the SIE element of the c-fos promoter. Immunodepletion of rNef from the rNef containing medium used to treat m/m failed to induce STAT1 activation, demonstrating the specificity of the observed effects. STAT1 serine phosphorylation was also increased suggesting that rNef treatment induces both the formation of the GAF DNA binding factor and its transactivating function on gene transcription. Accordingly, the expression of the transcription factor IRF-1, that is transcriptionally regulated by STAT1, is also increased. Nef induced STAT1 activation appears mediated by the secretion of a still unidentified soluble factor(s) in a cycloeximide dependent manner. Taken together, our data are consistent with the hypothesis that Nef expression leads to an alteration in the secreted cytokine pattern, at least in monocyte/macrophages. Our model represents a tool of discovery of still unrevealed soluble factor(s) important for AIDS pathogenesis.

Accordo di collaborazione n° 40C.2

T CELL RESPONSES TO HIGHLY ACTIVE ANTIRETROVIRAL THERAPY DEFINED BY CHEMOKINE RECEPTORS EXPRESSION, CYTOKINE PRODUCTION, T CELL RECEPTOR REPERTOIRE AND ANTI-HIV T-LYMPHOCYTE ACTIVITY

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The immunological correlates of highly active antiretroviral therapy (HAART)-induced suppression of human immunodeficiency virus type 1 (HIV-1) replication have been investigated. Twenty HIV-1-infected patients with mean CD4+ T cell count of 298/μL, plasma viral load of 4.7 $\log_{10}$ copies/mL and naive for protease inhibitors (PI) were studied during 12 months of HAART.
After 12 months of HAART a significant increase of both percentage and absolute count of CD4 and CD8 naive cells was observed (for CD4: from 52±15% to 58±15% and from 148±68 cells/μl to 287±104 cells/μl; for CD8 from 38±13% to 49±20% and from 234±75 cells/μl to 399±170 cells/μl). The frequency of CCR5-expressing cells increased after HAART in both CD4 and CD8 subsets, while the absolute numbers increased only up to t6. Similarly, the percentages, but not the absolute counts, of CXCR4-expressing cells decreased at all time points studied within both CD4 and CD8 subsets.
The peripheral distribution of CD95-expressing T cells remained unchanged. Single cell analysis of cytokine production after 12 months of HAART showed an increased number of interleukin (IL)-2-, but not IL-4- and IFN-γ-producing T cells and a decreased percentage of CD8+IFN-γ+ + cells. A correlation between the frequency of IFN-γ-producing T cells and that of memory, CCR5+ and CD95+ T cells was demonstrated in both CD4+ and CD8+ subsets.
Anti-HIV-Gag and Pol specific CTLp were detected in all patients under investigation before HAART. A marked interpatient variability was seen ranging the number of CTLp/10^6 PBMC from 79 to 1142. After HAART the number of anti-HIV Gag and Pol cytotoxic T lymphocytes precursors (CTLp) decreased and highly correlated with the CD8 IFN-γ response. A perturbed repertoire was observed, at baseline, in 90% of patients, both in CD4 and, to a lesser extent, in CD8 cells.
The diversity of CD4+ repertoire was significantly improved by 12 months of HAART: 46 expansions at t0 vs 30 at t3 (p=0.71), 32 at t6 (p=0.64) and 7 at t12 (p=0.002). A transient increase of BV expansions was seen, within the CD8 subset, at t3 (39 expansions at t0 vs 44 at t3, p=0.018) followed by a decrease, at both t6 and t12, that did not reach statistical significance (39 expansions at t6 p=0.11 and 16 expansions at t12, p=0.99). Perturbed CDR-3 profiles were observed in CD4 cells of patients with CD4 count <200 cells/μl and in CD8 cells from all studied patients. However, the level of CDR-3 perturbation was only partially modified by 12 months of HAART.
In addition, a sustained decrease of spontaneous T cell apoptosis was observed, reaching levels consistent with those determined in normals. This was associated to progressive increments of both spontaneous and activation-induced production of IL2 and IL4 by PBMC, though only the latter was found significantly defective at enrollment. In contrast, both spontaneous and inducible production of IFNγ appear further decreased by HAART. Blocking of either spontaneous IL2 or IL4 production by PBMC using neutralizing antibodies, restored high levels of T cell apoptosis. These data suggest that the increased levels of both IL2 and IL4 produced by PBMC during HAART can provide anti-apoptotic signals that directly contribute to an increased survival of T cells and may play a part in long-term immune reconstitution. These results confirm previous observations on the involvment of cytokine unbalance in T cell loss and AIDS pathogenesis and provide evidence of a supporting role of endogenous cytokine production in peripheral T cell repopulation during an effective and prolongued viral suppression. Taken together our observations indicate that a better restoration of immunity may be obtained in patients starting HAART at less advanced stages of the disease.

N° 40C.3

PROGENITOR CELLS ACTIVITY AND ROLE OF ACCESSORY CELLS IN BONE MARROW OF HIV-1 INFECTED PATIENTS TREATED WITH HAART.

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Background: In patients with HIV-1 infection, hematological abnormalities are frequently observed. Suppression of HIV-1 replication by HAART can result in improved clinical conditions and restoration of immunological functions. Little attention has been dedicated to the effects of HAART on the bone marrow (BM) progenitor cell compartment and on BM stromal cells.
Methods: We studied the phenotype of BM progenitor cells, their differentiation capacity in a colony-forming cells (CFC) assay and the growth of the most immature progenitor cells (LTC-IC) in 10 HIV-1 patients (pts), before and after HAART. To determine if progenitor cells harbor proviral DNA, we analyzed CFU-C and LTC-IC derived colonies by polymerase chain reaction (PCR) analysis. In 7/10 patients, BM mononuclear cells (BMMCs) were cultured in long-term BM culture (LTBMC) until stromal confluence and stained by immunohistochemistry for CD34, CD68, S100 and p24.
Results: Pre-therapy assays showed reduced numbers of CFC and LTC-IC with respect to normal controls. After 6 months of HAART, the CFC number enhanced in all pts. On average, 10^5 plated BMMCs yielded a mean value of 7 CFU-E pre-therapy vs. 17 post-therapy, 34 vs. 85 BFU-E, 21 vs. 76 CFU-GM and 2 vs. 6 CFU-GEMM, respectively. A significant increase in LTC-IC was observed in 6/10 patients. In addition, there was a recovery of BMMCs after BM aspirates, while percent of CD34+/CD45+ progenitor cells did not significantly change. These findings were associated with undetectable plasma HIV-1 RNA levels and a rise in CD4+ T cell counts post-HAART. HIV-1 DNA was not detected by PCR in analyzed colonies. However, p24 was expressed in LTBMC-derived stromal cells and macrophages (CD68+ p24+). Interestingly, a high number of p24+ macrophages correlated with elevated plasma HIV RNA levels.
Conclusions: Reduced number of BM committed and primitive progenitors was observed in HIV-1 pts, which was restored after HAART. Our data suggest a role for HIV-1 infection of BM accessory cells in the alterations of the hematopoietic compartment.

N° 40C.3

HIV-TAT AFFECTS PMN MIGRATION AND ACTIVATION: POSSIBLE ROLE IN AIDS-ASSOCIATED PATHOGENESIS

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Polymorphonuclear cells (PMN) show an abnormal behavior in HIV infection; most studies indicate that neutrophils are in a primed state during the asymptomatic phase with normal CD4+ counts, however these phagocytes become "indolent" with progression to clinically manifested AIDS (Elbim C. et al. J Cardiovasc Pharmacol 1995; 25:S66. Pitrak DL. et al. J Clin Invest 1996; 98:2714. Wenisch C. et al. Aids 1996; 10:983). The hyperactivity of PMN in HIV infected individuals appears to lead to increased production of inflammatory cytokines and amplification of reactive oxygen species production. The consequence may be an enhancement of the systemic immune cell activation which favors HIV infection, a general oxidative stress, and eventual loss of PMN effectiveness contributing to the frequent opportunistic infections often observed in AIDS patients.
The HIV-Tat protein shows several extracellular activities, including induction of angiogenesis and stimulation of monocyte migration. Tat has been shown to induce a rapid neutrophil infiltration in a rat model of AIDS-dementia (Jones M, et al. J. Neuropathol. Exp. Neurol. 1998; 57:563). We observed a strong neutrophil infiltration into matrigel sponges containing Tat in murine models. In these models the infiltrating PMN appear to be the first cells recruited towards the Tat stimulus, while macrophages and endothelial cells appear later. In vitro the Tat protein induced a strong, dose-dependent migration of purified PMN, beginning at doses as low as 0.3 nM. Chemokine receptor stimulation cause a rapid and transient increase of cytoplasmic Ca^{2+}. Tat induced a Ca^{2+} flux in PMN similar to that induced by control chemokines. A series of overlapping peptides covering the entire Tat protein sequence were used in equimolar concentrations to test the involvement of functional regions of Tat in PMN migration. The peptide encompassing the cysteine-rich and core domains ($CysL^{24-51}$) proved to be the only peptide able to induce a strong, dose-dependent and reproducible PMN migration and calcium signaling.
Tat induced a significant release of VEGF and IL-8 by PMN, with the maximal response observed after 5 hours. The $CysL^{24-51}$ peptide was also able to induce VEGF and IL-8 release, while the other peptides were inactive. IL-8 release by Tat-stimulated PMN was 15 fold higher than untreated controls after 5 hours. Since stimulation of chemokine receptors leads to "respiratory burst" activation in human neutrophils, we investigated whether PMN produce superoxide anion (O^{2-}) in response to Tat. Tat induced a significant increase in superoxide anion production, with amounts similar to that induced by the chemokines SDF-1α or IL-8 at two minutes of stimulation. After two minutes the O^{2-} concentration was still elevated in Tat-treated samples, while they rapidly decreased in the presence of SDF-1α or IL-8. The $CysL^{24-51}$ peptide sustained high superoxide production for several minutes, similar to that observed for Tat.
Tat effects on PMN may contribute to the immune-disregulation observed with HIV infection and progression to AIDS, and could be a factor favoring in the frequent opportunistic infections observed in AIDS patients.

N°. dell'Accordo di Collaborazione 40C.4

IDENTIFICATION OF IMMUNO-DOMINANT EPITOPES IN TAT-TOXOID VACCINATED HEALTHY AND HIV INFECTED VOLUNTEERS

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HIV-1 Tat is an 86-101 amino acid protein that plays a key role of Tat in viral replication and HIV pathogenesis. Tat is also relatively well conserved, indicating it may be an effective target for anti-HIV strategies as a target for vaccine development. Simian models indicated that immunization with Tat could produce full or at least partial protection against subsequent SHIV challenge. Since the extensive biological activities of Tat suggested it could have significant collateral effects an inactivated Tat Toxoid has been developed that retains its immunogenic potential. Clinical studies on safety of the Tat toxoid have been initiated in both HIV infected patients and healthy volunteers demonstrating a strong cell mediated and humoral immune response. Tat appears to have immuno-dominant regions, we recently demonstrated 3 immuno-dominant epitopes in mice (amino acids 1-9, 52-55 and 81-86). In humans, normal healthy sera showed no reactivity with Tat, while HIV positive patients showed variable, low levels of reactivity. We found that anti-sera of healthy volunteers vaccinated with the Tat toxoid also showed preferential reactivity with amino acids 1-24 and 46-60, corresponding to the N-terminus and basic domains of Tat. In contrast, while all the sera from HIV positive patients vaccinated with the Tat toxoid showed strong reaction with the 1-24 N-terminus, most sera also recognized peptides encompassing all the different domains of Tat. Since we observed reactivity with a peptide from amino acids 79-101, and the Tat toxoid immunogen was only 86 amino acids, we isolated the reactive antibodies by peptide affinity. These antibodies clearly recognized an antigen localized at amino acids 79-86. Finally, we have shown that Tat forms stable dimers that are recognized by some antibodies. Western blotting clearly demonstrated that the sera of Tat toxoid vaccinated individuals recognized both monomer and multimer forms of Tat. Our data clearly demonstrate that Tat toxoid vaccination induces antibody responses to two immuno-dominant epitopes for HIV-1 Tat in healthy subjects, whereas vaccinated HIV infected patients show a broader response to Tat epitopes.

N°. dell'Accordo di Collaborazione 40C.38 and 40C.4

THE BINDING SUBUNIT OF PERTUSSIS TOXIN (PTX-B) AS A NEW AND POWERFUL ANTI-HIV COMPOUND EFFECTIVE IN PRIMARY $CD4^+$ T LYMPHOCYTES AND MACROPHAGES AND CHRONICALLY INFECTED U1 CELLS.

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We have previously shown that the binding subunit of pertussis toxin (PTX-B) inhibits HIV replication at multiple steps. PTX-B inhibits entry and trascription of R5-HIV, and post-entry events in the case of X4-HIV strains in activated T lymphocytes. HIV entry inhibition was not mediated by down-regulation or decreased binding of CCR5 but by CD4-CCR5 colocalization and consequently viral envelope-cellular membrane fusion inhibition in primary $CD4^+$ lymphocytes (M. Alfano et al, J. Exp. Med., 1999, M. Alfano et al. J. Virol., 2000).

In the present study we demonstrate that PTX-B profoundly impairs entry and replication of the HIV-1_{ADA} (R5), as well as of HIV pseudotyped with either Murine Leukemia Virus or Vescicular Stomatitis Virus Envelopes, in monocyte derived macrophages (MDM).
PTX-B inhibited HIV replication in MDM previously infected and treated with AZT, supporting the evidence of additional post-entry inhibitory effect of this agent. In addition, PTX-B strongly inhibited X4 HIV-1 replication in U937 promonocytic cells and virus expression in the U937-derived chronically infected U1 cell line stimulated with cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Of interest, TNF-α-mediated activation of the cellular trascription factor NF-kB was unaffected by PTX-B. In IL-6 stimulated U1 cells, PTX-B inhibited the accumulation of multiply spliced but increased the levels of unspliced viral RNA concomitantly with inhibition of viral protein synthesis, suggesting a possible effect of PTX-B on the Rev-RRE axis of the virus expression.

These findings strongly support the potential testing of PTX-B as antiretroviral agent. This hyphotesis is greatly facilitated by the existence of a genetically modified form of pertussis toxin, PT-9K/129G, lacking the toxic but retaining all the non-toxic or binding subunit properties of pertussis toxin. This molecule is currently safely administered in vivo against the Bordetella Pertussis infection, and we have shown that retains all the PTX-B antiviral properties in vitro (M. Alfano et al, J. Immunol., 2001 in press).

N° dell'accordo di collaborazione: 40C.5

STUDIES ON LYMPHOTACTIN IN HIV INFECTION

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(Grant no. 40C.6)

Lymphotactin (LPTN) is a γ-chemokine, exerting its activity mainly on NK cells/$CD8^+$ T cells. This molecule may be particularly important in cancer immunotherapy, where a potentiating effect on T cell priming against tumor antigens was demonstrated. In view of data also suggesting its possible role in HIV infection, we addressed the study of this molecule; given the paucity of available reagents, the last few months were devoted to setting up appropriate tools for its biochemical and functional characterization.

LPTN sequence isolation and transfection: In order to have a suitable LPTN source, its sequence was derived from $CD8^+$ T cells stimulated with PMA and A23187. After RNA extraction and retrotranscription, the cDNA was cloned in the eukaryotic expression vector pHCMV. The production of LPTN in the supernatant of tranfected 293T cells was demonstrated by Western blotting.

Production of anti-LPTN monoclonal antibodies: Groups of Balb/c mice were injected with LPTN; following boosting, spleen cell fusion with the appropriate partner and screening with ELISA and Western blotting using a LPTN-GST fusion protein, 30 anti-LPTN antibody-producing clones were stabilized. When they were tested for the ability to inhibit LPTN chemotactic activity (in collaboration with P. Allavena, M. Negri Institute, Milan), 7 clones were able to neutralize at variable extent LPTN activity in an in vitro NK cell migration assay. Work is in progress to also characterize their ability to stain LPTN-producing cells in immunohistochemistry approaches.

Real-Time PCR for LPTN mRNA quantification: A Real-Time PCR was set up to quantify LPTN expression in diffent cell populations; in fact, its major cellular source is as yet unclear. Preliminary data indicate that freshly isolated $CD4^+$ T cells produce little if any LPTN, and in vitro stimulation is associated with scanty increase in production of this chemokine. On the other hand, freshly isolated $CD8^+$ T lymphocytes show high LPTN expression, and a 100-fold increase is observed following in vitro activation. Surprisingly, most LPTN expression is confined to $CD8^+$ cells who lack CD5 antigen expression; this is noteworthy, as we showed that HIV^+ patients have a tremendous increase in the $CD8^+CD5^-$ T lymphocyte subset in circulation. Studies are in progress to further characterize this subpopulation, also increased in peripheral blood of immunosuppressed bone marrow transplant recipients.

In addition, this study intends to further characterize the possible role of LPTN in HIV infection by: (1) evaluating LPTN expression in HIV infected patients, and correlating it with surrogate markers of disease progression; (2) investigating its role in HIV spread into $CD4^+$ T cells in vitro; (3) evaluating its role in naïve $CD8^+$ T cell priming by dendritic cells.

VALUE OF PROVIRAL DNA SEQUENCE IN PATIENTS ON HAART

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Prolonged antiretroviral treatments may lead to the emergency of viral variants bearing gene mutations responsible for drug-resistance and therapy failure. Early recognition of these mutations is of critical importance in order to improve the long-term response to antiretrovirals. Most studies focused on genotype and/or phenotype of plasma derived HIV; however, at present this approach is limited to patients with detectable HIV-1 RNA, and only estimates the actually replicating virus without any information on the archived virus.

We studied the pattern of resistance-associated mutations in plasma RNA and proviral DNA extracted from paired peripheral blood mononuclear cells (PBMCs) and rectal mucosa samples of patients while receiving HAART. Rectal biopsies were decided as they represent a tissutal lymphoid compartment which can be repeatedly sampled by a painless, minimally invasive procedure.

A total of 20 HIV-1 seropositive individuals were studied who were classified as “responders” (undetectable plasma HIV-1 RNA, 7 patients) and “non responders” (detectable plasma HIV-1 RNA, 13 patients). Paired blood and rectal mucosa bioptic samples were obtained from each patient; by direct sequencing of the pol gene (reverse transcriptase and protease domains), HIV-RNA and proviral DNA were analyzed .

As expected, in non responders patients, sequences from plasma RNA showed a higher degree of mutations, consistent with the current drug regimen; although a general concordance was seen among RNA and DNA sequences, mutations were less frequently detected in proviral DNA. Nevertheless, in proviral DNA samples we detected mutations that were absent in plasma RNA. In patients responders to therapy (for whom only DNA from PBMCs and rectal mucosa was analysed) mutations compatible with their current antiretroviral regimen were observed even in the absence of detectable virus in plasma. Interestingly, certain drug-resistance associated mutations were found in rectal mucosa, but not in the corresponding PBMC sample.

Data presented here indicate that the analysis of proviral DNA sequences may constitute an useful clinical tool for the detection of drug-resistance associated mutations in patients on HAART. The use of rectal biopsies, moreover, might provide additional information, since mutations can be observed at the mucosal site that are not detectable in peripheral blood.

Accordo di Collaborazione 40C.7

N,N-DISUBSTITUTED DIHYDRO-ALKYLAMINO-BENZYL-OXOPYRIMIDINES , A NEW CLASS OF POTENT AND SELECTIVE ANTI-HIV-1 AGENTS BELONGING TO DABOS

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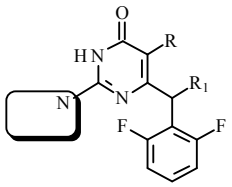
Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) were reported by our group in 1992 as a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) class. Since then, a great number of oxopyrimidines were synthesized and tested as anti-HIV-1 agents with the aim to obtain more potent and selective derivatives.[1-8] Structure-activity relationships (SARs) of DABOs, supported by molecular modeling investigation on their putative binding mode, have shown that the presence of a C2-alkoxy (DABO) or C2-alkylthio (S-DABO) side chain is a structural determinant for the antiviral activity of these derivatives, with the length and size of the C2 side chain having only modulatory effects on potency. Moreover, the introduction of a 2,6-difluoro substituent at the C6 phenylmethyl moiety of S-DABOs, generating a favorable π-stacking interaction with the Tyr188 side chain, afforded compounds active in the nanomolar range (difluoro-S-DABOs, F_2-S-DABOs).

On the basis of these findings, we planned the synthesis of a novel series of DABO derivatives bearing, beside the "classical" C5-H and C5-methyl substitutions, the highly favorable 2,6-difluorophenylmethyl moiety at C6 position, and characterized by the replacement of the C2-alkoxy/alkylthio side chain with the isoster C2-dialkylamino side chain (amino-DABOs).

The new DABOs were tested in cell culture using MT-4 cells and both wild-type virus and virus containing mutations in residues 181, or 181 plus 103, known to be crucials to confer NNRTI resistence in treated patients, in comparison with nevirapine and efavirenz as controls.



N,N-DABOs

$R = R_1$ = H, Me, Et
= pyrrolidine, (substituted)piperidine, (substituted)piperazine, morpholine, thiomorpholine, tetrahydroazepine, dialkyl

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Accordo di collaborazione N° 40C.8

3,4,5-TRIHYDROXYCINNAMOYL MOIETY: A POTENT PHARMACOPHORE FOR ANTI-INTEGRASE AGENTS ACTIVE EITHER IN ENZYMATIC OR IN CELL-BASED ASSAYS

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The highly active antiretroviral therapy (HAART),[1] based on a combination among a nucleoside reverse transcriptase inhibitor, a non-nucleoside one and two protease inhibitors is nowadays the most effective approach in treatment of AIDS. HAART does not totally defeat HIV-1 infection, nevertheless it is more effective than monotherapy due to the multidrug approach targeted at two different HIV-1 enzymes: reverse transcriptase (RT) and protease (PR). No drug active against HIV-1 integrase (IN) is today under clinical trials, so IN is still an attractive target for the development of new drugs useful in AIDS multitherapy. Recently, several families of hydroxylated aromatic compounds have been shown to potently inhibit HIV-1 IN including caffeic acid phenethyl ester (CAPE)[2], curcumin[3] and L-chicoric acid[4].

However, the great majority of them are unable to prevent HIV-1 multiplication when tested in cell-based assays. Recently we reported on polyhydroxylated aromatic compounds containing the 3,4-dihydroxycinnamoyl moiety which inhibit the rIN activities at submicromolar concentrations, but failed to selectively inhibit the HIV-1 multiplication in acutely infected cells probably due to the high cytotoxicity[5].

With the aim to knock down both the unresolved problems of cytotoxicity and inactivity in cell-based assays and to increase SAR investigations, we prepared novel cynnamoyl derivatives in which the catechol moiety is replaced either by other 3,4-disubstituting phenyl groups or by the parent 3,4,5-trioxyphenyl moiety. Moreover we synthesized novel derivatives having two 3,4,5-trihydroxycinnamoyl pharmacophores and carboxyl groups, that would be essential for enhancing inhibitory activity.

As expected from the rationale that have guided own research, the 3,4-dichlorobenzylidene derivatives were not active both in the enzyme and cell-based assays and showed in general high cytotoxicity. The same results were found for nitrohydroxy derivatives.

Among the test series good activity was assured by trioxyderivatives, which inhibited HIV-1 integrase in vitro in the range 0.3-6.0 μM and were found to prevent HIV-1 replication in acutely infected cell cultures. The best results were found with trioxyderivatives with a carboxy or carbethoxy group that, differently from their catechol counterparts, were able to block the replication of HIV-1 in MT-4 cell-based assays. The good activity of these compounds points out that the introduction of the carboxylic group in is a determinant to increase the anti-HIV-1 activity in tissue culture.

A comparison between curcumin and chicoric acid used as reference drugs, and our compounds, showed novel derivatives to be much more potent against IN than curcumin and somewhat more potent or as potent as L-(-)-chicoric acid. Moreover some derivatives resulted good inhibitors of viral replication in cell culture assays, showing SI ranging from 1.4 to 17.5. Based on these results we can conclude that title compounds are members of a novel class of potent anti-IN capable to inhibit the HIV-1 replication in acutely infected MT-4 cells. Among them compounds carboxy derivatives are promising lead compounds for further synthetic and biological studies.

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Accordo di collaborazione N° 40C.8

DIARYLMETHOXYETHYLMETHYLNITROIMIDAZOLE (DAMNI) DERIVATIVES, NOVEL POTENT INHIBITORS OF THE ANTI-HIV-1 REVERSE TRANSCRIPTASE

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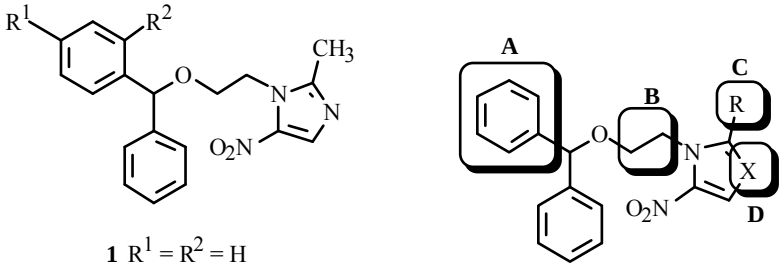
DAMNIs are a potent class of inhibitors of the Reverse Trancriptase (RT) enzyme, involved in the replicative life-cycle of the Human Immunodeficiency Virus (HIV) [1]. In the in vitro assays on MT-4 cells (MTT method), the lead compound 1-[2-(diphenylmethoxy)ethyl]-2-methyl-5-nitroimidazole (1) showed CC_{50} > 200 μM, EC_{50} = 0.2 μM, S.I. > 1000 and IC_{50} = 0.05 μM values. SAR results pointed out that the substitution on the imidazole ring was essential for the anti-HIV activity. Appropriate substituents on the phenyl ring , e.g.: R^1 = F, Cl, CH_3 or C_6H_5, exerted a favourable effect on the antiviral acitivity, exhibiting EC_{50} values near to those of 1. Introduction of a second atom, e.g.: R^1 = R^2 = Cl, reinforced the anti-HIV potency. Substitution R^1 = phenyl ring was less cytotoxic than R^1 = tert-C_4H_9, thus suggesting a better fitting for an aromatic ring with respect to ramified alkyl susbtituents.

Figure 1



1 $R^1 = R^2 = H$

Novel DAMNI derivatives were planned with the aim to evaluate: (A) the indroduction of halogen atoms, small size alkyl groups and heteroaromatic rings (e.g. pyrrole) at the position ortho, meta and para of the phenyl ring; (B) the optimization of the methoxyethyl linker lenght; (C) the evaluation of the effect of the methyl group at position 2 of the imidazole ring; (D) the comparison between the imidazole (X = N) derivatives and the pyrrole counterparts (X = CH, Figure 1). The synthesis of DAMNI derivatives was performed by reaction of the appropriate diaryl carbinol or diarylbromomethane with suitable 1-(2-hydroxyethyl)-1H-azole in the presence of PTSA acid or potassium carbonate, respectively. The anti-HIV activity of the novel DAMNI derivatives is at present under investigation.

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Accordo di collaborazione N° 40C.8

SPREADING OF HIV-SPECIFIC $CD8^+$ T CELL REPERTOIRE IN LONG-TERM NON-PROGRESSORS AND ITS ROLE IN THE CONTROL OF VIRAL LOAD AND DISEASE ACTIVITY.

APOPTOTIC CELLS OVEREXPRESS VINCULIN AND INDUCE VINCULIN-SPECIFIC CYTOTOXIC T CELL CROSS-PRIMING, DEPENDING ON THEIR CAPACITY TO ACTIVATE DENDRITIC CELLS.

Propato A, Schiaffella E, Francavilla V, Baloni L, Paroli M, Finocchi L, and Barnaba V.
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Long-term non-progressors (LTNP) represent a minority of human immunodeficiency virus (HIV)-infected individuals characterized by stable or even increasing $CD4^+$ T cell count and by stronger immune responses against HIV than progressors. Here, we found that HIV-specific effector $CD8^+$ T cells, as detected by both a sensitive ex vivo enzyme-linked immunospot (ELISPOT) assay and specific major histocompatibility complex (MHC)-peptide tetramers, were at a low frequency in the peripheral blood of LTNP, and recognized a lower number of HIV peptides than their memory resting cell counterparts. Both factors may account for the lack of complete HIV clearance by LTNP, who could nonetheless control the viral spread, and displayed a higher magnitude of cytotoxic T lymphocyte (CTL) responses than progressors. By combining cell purification and ELISPOT assays we showed that both effector and memory resting cells were confined to a $CD8^+$ population with memory $CD45RO^+$ phenotype, with the former being $CD28^-$ and the latter $CD28^+$. Longitudinal studies highlighted that a relative stable HIV-specific effector repertoire, viremia and $CD4^+$ T cell counts were all correlated to maintain non-progressor status. In conclusion, the analysis of HIV-specific cellular responses in these individuals may help to define clear correlates of protective immunity in HIV infection.

The second study demonstrates that cells induced into apoptosis overexpress vinculin and are ingested by dendritic cells (DC), which subsequently cross-prime vinculin-specific cytotoxic T lymphocytes (CTL). Successful cross-priming requires that the apoptotic cells provide maturation signals to DC via CD40-CD40 ligand (CD40L) interactions. If apoptotic cells are CD40L-negative, the help of a third party T cell is needed for priming, suggesting a regulatory role of the apoptotic cell in determining priming or tolerance. Vinculin-specific CTL priming is also apoptosis-related in vivo, since the frequency of specific CTL depends on both the viral load and the proportion of peripheral apoptotic cells in human immunodeficiency virus (HIV)-seropositive individuals.

N°. dell'Accordo di Collaborazione: 40C.9.

HEARTENING RESULTS FROM THE FIRST FIELD TRIAL OF AN ANTI-FIV VACCINE

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Attempts to develop effective anti-FIV (feline immunodeficiency virus) vaccines have been numerous because this virus represents a useful model for HIV as well as an important pathogen for its natural host. To date, the experimental vaccines tested in controlled laboratory settings have afforded inconsistent levels of protection. However, a vaccine we have developed employing cell-associated FIV-M2 strain (clade B) and fixed with paraformaldehyde has provided so satisfactory results when tested in the laboratory (1-3) as to suggest the opportunity of performing a first limited trial under natural conditions of infection. Subject cats were in a private shelter that was endemic for clade B FIV; prevalence 29-58% and incidence 9-17% over an 8 year observation period. Cats roamed freely from the shelter through the surrounding countryside but returned for food and shelter. After ensuring that cats were FIV negative, they were immunized using 6 doses of vaccine over a 16 month period, returned to their normal life style, and observed for 28 months after the initiation of immunization. Twenty six cats (12 immunized and 14 non-immunized controls) were followed for a minimum of 22 months. Immunized cats did not experience appreciable adverse effects of vaccination and developed both antibodies and cellular immunity to FIV, although individual responses varied greatly. At the conclusion of the study, 0/12 immunized cats had evidence of FIV infection, while 5/14 control cats were infected. Thus, the vaccine was safe, immunogenic and did not transmit infection. Furthermore, vaccinated cats did not develop FIV infection in a limited clinical trial over an extended time period. Thus, the data suggest that a fixed, FIV-infected cell vaccine has potential for preventing natural FIV infection and encourage toward more extensive testing in field cats.

1. Matteucci et al., J.Virol. 70:617-622, 1996.
2. Matteucci et al., J.Virol. 71:8368-8376, 1997.
3. Mazzetti et al., J.Virol. 73:1-10, 1999.

Accordo di Collaborazione N° 40C.10 - 40C.11

IMMUNOGENICITY OF A FIXED VIRUS-AUTOLOGOUS CELL BINDING COMPLEX AS A POTENTIAL FUSION-COMPETENT ANTI-FIV VACCINE

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Most anti-lentiviral vaccines tested to date have failed to elicit the production of antibodies capable of neutralizing a wide spectrum of primary virus isolates. This might be related to the cryptic nature of the relevant protective epitopes, which might remain hidden or be lost in conventional inactivated and subunit vaccines but might become exposed following virus interaction with susceptible cells (fusion-competent epitopes; 1). In an attempt to develop a potential fusion-competent vaccine, we inactivated a primary FIV-M2 isolate with 2,2'-dithiodipyridine to maintain the conformational and functional integrity of the Env glycoproteins, reacted it with autologous lymphoblasts obtained from each individual SPF cat to be vaccinated, and finally fixed the virus-cell complex with 0.2% paraformaldehyde. In some such preparations, synthetic peptides believed to inhibit different steps of FIV binding to cells were also used, in the attempt to freeze the fusion process in a vaccine-useful conformation. Another vaccine preparation consisted of autologous lymphoblasts infected with active FIV-M2 and fixed with 1.25% paraformaldehyde. Autologous lymphoblasts processed exactly as for the fusion competent vaccines except for virus omission served as mock vaccine. Parameters of virus-cell interactions such as saturability of binding, amount of absorbed virus, and percentage of FIV-immunoreactive cells were evaluated by real-time quantitative PCR, Western blotting and immunofluorescence assays. Virus inactivation was tested in vitro and in vivo.

Groups of 5 SPF cats were inoculated subcutaneously with the above immunogens five times and then challenged with fully virulent ex vivo homologous virus. The anti-FIV immune responses generated by each immunogen will be described. These include binding antibodies to whole FIV, FIV glycoproteins and immunodominant linear epitopes measured by ELISA, neutralizing antibodies tested by a lymphoid cell-based assay, and T helper activity assayed by lymphoproliferation to whole FIV. The results of challenge are currently under evaluation.

1. LaCasse et al., Science 283:357-361, 1999
2. Matteucci et al., J.Virol. 70:617-622, 1996.
3. Matteucci et al., J.Virol. 71:8368-8376, 1997.
4. Mazzetti et al., J.Virol. 73:1-10, 1999.
5. Matteucci et al., J.Virol. 74:10911-10919, 2000.

Accordo di Collaborazione N° 40C.10. - 40C.11

PLANT CHIMERIC VIRUS PARTICLES AS IMMUNOGENS FOR INDUCING MOUSE AND HUMAN IMMUNE RESPONSES AGAINST HIV-1.

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The ideal requisites of any vaccine in inducing protective systemic and mucosal immunity include safety, efficacy and low-costs. Plants and plant viruses have recently been considered as attractive systems for expressing and delivering foreign proteins or peptides as immunogens to be used for the development of new vaccination strategies. The employment of plants for the production of therapeutic proteins offers several advantages such as absence of mammalian pathogens, cost-effectiveness, large-scale production and relative ease in expression and purification.
Plant virus coat proteins (CP) are particularly suitable carriers to present immunogenic peptides to the immune system. When properly fused at different positions on the capsid proteins, exogenous sequences are expressed in plant, originating recombinant viral coat proteins able to self-assemble and generate chimeric virus particles (CVPs) displaying the foreign sequence on their outer surface. The possibility to carry out a mucosal delivery of vaccine-expressing plants, potentially resulting also in the activation of the mucosal-associated immune system, is particularly important for viruses transmitted mainly via mucosal surfaces such as human immunodeficiency virus type 1 (HIV-1). Protective humoral immune response against HIV-1 requires antibodies able to properly bind to the virus envelope under physiological conditions. Up to now, the only epitopes clearly identified as being well exposed are those recognized by the neutralizing monoclonal antibodies (mAbs) 2F5, 2G12 and b12. Among these, mAb 2F5 recognizes the highly conserved linear epitope ELDKWA (2F5e), located in the membrane proximal part of the gp41 ectodomain. Therefore, we assayed the immunogenicity of PVX-derived CVPs displaying this epitope as an interesting candidate for the preparation of a vaccine against HIV-1. We modified the PVX CP encoding gene by linking the sequence encoding the HIV-1 gp41-derived 2F5e and recombinant virus was used to infect Nicotiana benthamiana plants. Leaves from plants showing infection were collected, CVPs (PVX-2F5E) purified, and assessed by ELISA for the correct display of the HIV-1 epitope on their outer surface using the human mAb 2F5. Then, we immunized mice i.n. or i.p. with purified CVPs, with wild type PVX (WT-PVX) or PBS as controls. Sera from both intranasally or intraperitoneally PVX-2F5E-immunized mice showed high levels of IgG specific for a synthetic peptide containing 2F5e, while no reactivity was found in the sera of control animals. Anti-H66 IgG titres, calculated as endpoint dilution, ranged from 2,000 up to more than 30,000 for the intranasally immunized group and from 2,000 to 15,000 for the intraperitoneally immunized animals. Mice immunized via mucosal route showed anti-2F5e IgA presence in the serum and in faecal samples. Isotyping of the IgG response showed that the IgG2a subclass was dominant suggesting a bias toward a Th-1 type immune response. Newly developed human vaccines are generally tested in normal mice before clinical experimentation and concerns have been recently raised regarding the predictive value of studies in mouse models, due to the marked differences in the regulation of the immune response between mice and humans. Therefore, it would be desirable, where possible, to extend the evaluation of candidate human vaccines from normal mice to animal models in which human primary immune responses can be studied. For this reason, we investigated the human immune response to PVX-derived CPVs displaying the 2F5e in severe

combined immunodeficient mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID). We provide evidence hu-PBL-SCID mice immunized with CVPs-pulsed autologous dendritic cells (DCs) are able to mount a specific human primary antibody response against the HIV-1-derived epitope. Remarkably, sera obtained from both normal and hu-PBL-SCID mice were endowed with anti-HIV-1 neutralizing activity.
Here, we report also the use of PVX as vector to express the regulatory Tat protein in plant. To this end, the sequence encoding Tat was fused at the 3'end with c-myc and His tags, and finally cloned in the viral vector as a single ORF (PVX-tat). Upon systemic infection of N.benthamiana plants, the expression of the foreign sequences has been verified by Western blot. Our results have shown that the accessory ORF tat did not interfere with the correct assembly of virions. Western blot analysis of fractionated protein extracts revealed that polyclonal anti-Tat antibodies recognize a 14 KDa (corresponding to monomeric Tat -faint signal-) and a 48 KDa (strong signal) bands. This result was also confirmed using monoclonal anti-c-myc antibodies. We have estimated that the expression level in infected plant tissues is around 0,2% of the total proteins. Ongoing studies will evaluate if the immune response elicited by Tat protein expressed in plant tissues is comparable or better to that induced by E. coli-derived Tat protein.

N°. dell'Accordo di Collaborazione. N. 40C.12.

CHANGES IN GP120 CONFORMATION INDUCED BY THE VIRAL INCORPORATION OF HLA CLASS I MOLECULES: IMPLICATIONS FOR VACCINE DESIGN.

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Assembly of HIV occurs at the level of the plasma membrane of the host cell. During this process HIV incorporates significant quantities of cell-surface-derived molecules into its lipid bilayer, including HLA class I and II antigens. Several studies indicate that virion-associated host molecules are functional and affect the biological properties of HIV. While HLA class II antigens have been shown to increase the infectivity of HIV through binding to CD4, HLA class I act in a different way, i.e. they directly associate to the viral envelope glycorprotein gp120 and change its conformation. Using HLA class I negative cell lines (LCL.721.221) co-transfected with the HLA Cw4 and the human CD4 gene we demonstrated that the infectivity of primary HIV-1 X4 isolates is greatly increased by the incorporation of HLA C molecules. The same effect was also seen on T cell line adapted strains (TCLA). However, of the four TCLA strains tested, two (NDK and NL-4-3) were not influenced by HLA C incorporation. The increase in infectivity was due to an accelerated entry kinetic and was clearly associated to changes in the viral gp120 conformation which included an increased exposure of the V3 loop and the 1.7b epitope, which correspond to the co-receptor binding site. These changes were observed only in the TCLA strains whose infectivity is increased by HLA C incorporation but not in those that were not susceptible to HLA C incororation. The changes in gp120 conformation are due to a direct physical association between HLA C and gp120, which was demonstrated by the co-precipitation of the two molecules by either antigp120 or anti-HLA C antibodies. The TCLA strains which are susceptibility to HLA C incorporation display an extended V-1 V2 loop.
The data show that virionic HLA class I molecules directly contribute to the assembly of a functional viral envelope most likely as part of a multimolecular complex including gp120/41 and possibly other host molecules.
In order to establish the relevance of these findings for HIV vaccine design we plan to perform immunization experiments using cells expressing endogenous gp120 in the presence or absence of HLA class I. Monkeys will be immunized with these cells and the neutralization titers as well as the fine specificity of the resulting antibodies will be measured. Due to their effect on gp120 conformation (enhanced expression of the co-receptor binding site) HLA class I may molecules may result to be an essential component of immunogens targeted at the induction of antibodies with broad neutralization activity.
We demonstrated that using HLA class I negative cell lines (LCL.721.221) co-transfected with the HLA Cw4 and the human CD4 gene. These cell lines express constitutively the CXCR4 coreceptor and can be used to assess the infectivity of TCLA as well as primary isolates which use CXCR4.
The infectivity of all primary X4 isolates tested was greatly enhanced by HLA Cw4 incorporation. This was shown by the kinetic of p24 antigen production in culture and by an entry PCR assay.

Accordo di collaborazione scientifica 40C.13

THE LD78β ISOFORM OF MIP-1α, THE MOST POTENT HIV-1-INHIBITORY CHEMOKINE, IS SPONTANEOUSLY SECRETED FROM CD8+ T LYMPHOCYTES OF HTLV-II INFECTED INDIVIDUALS

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Direct evidence that HTLV-II has the potential of inhibiting HIV-1 replication via a chemokine-mediated pathway has been recently provided by our team (Casoli et al., Blood 91:2296,2000). Through optimization of a co-cultivation system based on IL-2 stimulated-PBMCs isolated from individuals both mono- and co-infected with HIV-1 and HTLV-II, we have demonstrated a negative interference between these two retroviruses via up-regulation of HIV-inhibitory CC-chemokines and particularly MIP-1α, which alone seemed to account for most the anti-HIV activity. Human MIP-1α is encoded by 2 highly related non-allelic genes producing 2 different isoforms designed LD78α and LD78β. Because of variation in the site of signal peptidase cleavage between the two isoforms during secretion, LD78α is produced without the 4 anticipated amino-terminal aa. (MW ~7.5 kDa, 66 aa.), whereas the natural LD78β isoform is characterized by a full-length NH_2-terminal region (~7.8 kDa, 70 aa.). Interestingly, MIP-1α has been shown to be produced naturally by $CD8^+$ T cells, the main target of HTLV-II, and in greater quantity as a –4 aa. variant. It has been independently reported that the LD78β isoform shows 50-fold higher antiviral activity against M-tropic HIV-strains compared to RANTES and LD78α on PBMC infection. In the present study we have investigated whether different isoforms of MIP-1α could account for its biologic preponderance.

The spontaneous proliferation in short-term cultures of PBMCs isolated from HTLV-II mono-infected individuals is associated with significant secretion levels of LD78β isoform in the medium at concentrations comparable to those of the LD78α isoform. The MIP-1α isoforms from patients' PBMC were fractionated and purified to homogeneity by RP-HPLC. Electrospray ionization mass spectrometry allowed us to identify the α and β forms. Similar isolation procedures of defined concentrations of human recombinant MIP-1α isoforms (purchased from R&D) and their mixture were also performed for standardization purposes. From both standard and PBMC-derived supernatants, mass spectra of the chromatographic peaks with a retention time of 12.88 and 13.41 min. provided evidence of coexistence of 66 and 70 aa. forms respectively. These spectra, of similar amplitude, provided an ion envelope of multiply-charged ions typical of proteins. The measurement of the two mass spectra, obtained by deconvolution whit MaxEnt1 (Micromass) software, provided a degree of confirmation for the identity of α and β forms on the basis of their theoretical molecular weight (α form:7456 and 7446 Da, β form: 7846 and 7848 Da for standard and patients, respectively). Quantitative analysis of purified LD78 forms from patients, as well as testing of their antiviral activity assayed as ability to induce-down-regulation of the CCR5 receptor in primary T lymphocytes, are in progress.

Putative regulatory sequences of the LD78 genes have been detected in the promoter regions of human GM-CSF and human γ-interferon (IFN-γ), two cytokines that can modulate the susceptibility of macrophages to HIV-1 infection. Spontaneous proliferation of PBMC from HTLV-II-infected individuals was associated with the similar kinetics of GM-CSF and IFN-γ secretion.

These findings altogether highlight novel and important interaction between HTLV-II and HIV-1, and indicate potential interference modalities through which HTLV-II may delay HIV disease progression in vivo.

N.° Accordo di Collaborazione: 40C.14 e 40C.73

HTLV-II-MEDIATED CORRELATES OF HIV-1 PROGRESSION IN CO-INFECTED INDIVIDUALS

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To evaluate the reciprocal influence of HTLV-II and HIV-1, we have recently developed PBMC cultures from both mono- and HIV-1/HTLV-II co-infected patients (Casoli et al., Blood 91:2296, 2000). In order to further investigate the mechanism by which HTLV-II might alter the natural course of HIV-1 infection in vivo, we have investigated both retroviral replication, cytokine, and chemokine expression in PBMC from mono- and co-infected patients. All the co-infected individuals belong to the previously described cohort of IVDUs (total number = 40; follow-up = 15 ± 2.5 years; 8 asymptomatic in the absence of HIV therapy; 13 slow and 19 progressors; all of them in therapy). Three HTLV-II- and 10 HIV-1 -mono-infected patients, as well as 10 healthy seronegative blood donors, were included in the study as controls.

HIV-1 infection is characterized by a general decrease in the expression of type 1 T-helper cytokines, an increase in the expression of pro-inflammatory cytokines, a possible increase in type 2 helper cytokines, and increased expression of antiviral interferons (IFNs). The levels of several cytokine transcripts in PBMCs from mono- and co-infected patients belonging to our cohort were determined by Taqman-based real-time PCR and quantified in terms of fold induction vs. the corresponding mRNA levels of the corresponding gene in unstimulated PBMCs of healthy donors. A substantial variability in terms of cytokine expression was observed in PBMCs from co-infected individuals. Expression of IL-1α (range 0.2-6472), IL-1β (1-116) and IL-8 (1-223) were found significantly enhanced in PBMCs of patients characterized by a high HIV-1 viremia/HTLV-II proviral DNA ratio and advanced HIV-1 disease progression. In mono-HTLV-II infected individuals, IL-1α (range 3.7-17), IL-12 (4-7.1) and IFN-γ (1.8-4) were found to up-regulated in comparison to the controls, whereas IL-2, IL-4, IL-5, IL-8 and TNF-α were found in the normal range, and IL-1β, IL-10 and IL-15 were down-regulated (up to 10-fold).

In addition, we have evaluated the expression of the CCR5 and CXCR4 in the PBMCs of our study population. By using FACS analysis of freshly isolated PBMCs we have observed that the expression of CCR5 on CD4+ T cells was significantly lower in HTLV-II mono- and co-infected individuals than in HIV-1 mono-infected or in uninfected individuals (mean 2.47 % ± 0.6 vs. 14.6% ± 2.8 or 6.5% ± 1.2, respectively; $p < 0.05$). The level of CCR5 on CD8+ T-cells and CD14+ monocytes was down-regulated in comparison to healthy controls but the difference was not significant. CXCR4 expression on both CD4+ and CD8+ T-cell surface was significantly reduced with respect to that of uninfected individuals (64% vs. 9.6% and 62% vs. 17.8%, respectively), but not with respect to HIV-1 mono-infected patients. In contrast, CXCR4 expression on CD14+ monocytes in co-infected subjects was significantly increased (60.2%), corresponding to the level observed in uninfected individuals (69.2%), when compared to HIV-1 mono-infected group (10.2%).

Some of us (CB, EV, and GP) have reported that a truncated isoform of signal transducer and activator of transcription (STAT5) is selectively observed activated in most HIV-1 infected individuals (C. Bovolenta et al., Blood, 1999) Preliminary observations on the constitutive state of STAT proteins in HTLV-II mono- and co-infected individuals suggest that this activity is not expressed in vivo. These results provide novel elements of investigation of the interrelationship between HTLV-II and HIV-1 in vivo and may provide new tools for monitoring the natural evolution of both infection or of the co-infection as well as their response to anti-retroviral therapy.

Accordo di collaborazione scientifica n. 40C.14 e 30C.61

BOOST OF X4 HIV-1 REPLICATION AND sCD30 SECRETION VIA CD30 LIGATION

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We have previously shown that CD30 triggering induced HIV expression in a model of chronic HIV-1 infection by acting at the transcriptional level. We here investigated whether signalling via CD30 affected acute in vitro infection with HIV-1, encompassing also early steps of the viral replication cycle. Several cell lines, including Molt-3, U937 plus, MT-2, known targets of X4 HIV-1 infection, resulted CD30 positive. Ligation of CD30 induced an upregulation of viral replication which varied substantially among the cell lines, from strong in Molt-3 to undetectable in MT-2 cells, paralleling the effect of TNF-α. Interestingly, we observed that the enhancement of X4 replication inversely correlated with both the density of CD30 on the cell surface and with constitutive NF-kB activation. We are currently investigating the levels of the proteins TRAF-1 and TRAF-2, members of the TNF receptor-associated factor family, involved in the signalling pathway of TNF R1 and CD30. Concomitantly, the soluble form of CD30, sCD30, was specifically boosted upon CD30 triggering in all our non-Hodgkin $CD4^+$ cell lines as well as on primary γδ T cell clones. Our data suggest that the interaction of CD30L, present on activated macrophages, B cells and constitutively on granulocytes, with CD30, expressed by activated T cells, may result in the amplification of HIV-1 X4 replication and contribute to the generation of plasma levels of sCD30.

Accordo di Collaborazione Scientifica n. 40C.15

THE UROKINASE RECEPTOR (CD87), CHEMOTAXIS AND AIDS.

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The urokinase receptor (uPAR) has multiple functions in cell migration and adhesion, pericellular proteolysis and tissue remodeling. Urokinase (uPA) induces chemotaxis in a large number of cell types, including monocytes, neutrophils and epithelial cells. We have previously determined that this activity requires the presence and a conformational change of the uPA receptor, uPAR/CD87. We have later determined that the conformational change consists in the cleavage of uPAR by uPA between domains D1 and D2. In the last year, we have also identified several steps of the signal transduction pathway elicited by the binding of uPA to uPAR (1).

We have now observed that a soluble form of uPAR (suPAR) present in plasma/serum is increased in HIV-1 infected individuals. Plasma suPAR levels were measured by ELISA in untreated HIV-positive patients collected before the introduction of the HAART treatment, and compared with healthy blood donors. suPAR plasma level of HIV-patients is significantly ($P<0.0001$, Mann-Whitney U-test) elevated compared to healthy donors (median values respectively 3.83 ng/ml (n=191) and 1.61 ng/ml (n=30)). High plasma suPAR-levels are strongly indicative of HIV infection with 91.6% sensitivity and 93.3% specificity. To evaluate the prognostic value of serum suPAR in HIV-patients we performed Kaplan-Meier survival analysis. The overall survival is progressively shorter with higher levels of plasma suPAR suggesting a correlation with disease progression. Cox multivariate analyses indicates that suPAR is a strong prognostic factor for survival, independent of CD4 counts, HIV viral load, β2-microglobulin and all other tested parameters . This paper has been now published (2).

We have also determined that monophagocytic and lymphocytic cells transfected with HIV-1 but expressing the virus at low levels show a very low level of expression of uPAR. However, under conditions in which virus replication is induced (i.e. with PMA), uPAR level increases and part is also secreted in the medium. Interestingly, the released suPAR is also cleaved between domain D1 and D2. This is important, since the cleavage between domain D1 and D2 converts suPAR into a strong chemoattractant (see above). In addition, suPAR is found cleaved in the blood of acute leukemic patients that are refractory to therapy (3). It will be important, therefore, to investigate whether the presence of cleaved suPAR in the blood of AIDS patients undergoing HAART may predict a relapse of the disease. We are also carrying out experiments to directly assess the role of the uPA/uPAR system on virus replication/infection.

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N°. dell'Accordo di Collaborazione. 40C.16

USE OF RIBOZYMES AND RNA DECOYS FOR THE GENE THERAPY OF HIV INFECTIONS

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Several approaches have been utilized in order to interfere with HIV-1 replication. The most powerful ones consisted in the delivery of therapeutic RNA molecules inside the nucleolar compartment. Through the utilization of genes coding for small RNAs which have nucleolar localization (snoRNAs) chimeric RNAs containing either specific ribozymes for the HIV pre-mRNA or RNA decoys against the Rev protein have been stably expressed in T-cells. Such chimeric molecules have been shown to specifically localize inside the nucleoli of permanently transformed cells and to produce dramatic suppression of HIV-1 replication. The results obtained in this study not only demonstrate the effectiveness of the strategy utilized but also suggest a trafficking of HIV-1 RNA through the nucleoli of human cells, thus posing a different paradigm for lentiviral RNA processing.
Since we have shown that the nucleolar compartment is very effective for directing therapeutic molecules against the HIV-1 pre-mRNA, we have extended the use of snoRNAs for the delivery in this compartment of other types of anti-HIV RNAs: antisense and methylating RNAs. Antisense have been designed such as to prevent the recognition of specific splice sites, while methylating RNAs have been engineered such as to direct specific 2'O-methylation of the 3' end of the Rev intron. The expected effect of both types of constructs should be the interference with the HIV-1 pre-mRNA splicing and ultimately with viral replication.

N°. dell'Accordo di Collaborazione. 40C.17

IMMUNOLOGICAL CORRELATES OF PROTECTION IN THE NATURAL RESISTANCE TO HIV INFECTION: IDENTIFICATION OF IMMUNOPROTECTIVE CELLULAR EPITOPES FOR THE DESIGN OF INNOVATIVE VACCINES

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Natural resistance to HIV infection has been long described in exposed seronegative individuals (ESN). The biological basis of this experimentum naturae is multifactorial and involves genetic and epigenetic factors. We have been working on some unconventional immune responses to HIV-1 in ESN individuals. We focussed our attention on autoantibodies that can be identified in these individuals, which in vitro inhibit HIV-mediated syncytia formation and HIV replication. The specificity of these autoantibodies is exquisitely targeted to conformational epitopes of the CD4 receptor molecule, being generated upon binding of viral gp120 to cellular CD4 following viral docking to the target cell. This property makes these antibodies not cytophilic and devoid of any self-danger effects (which in contrast has been described for anti-CD4 autoantibodies found in HIV-positive individuals). We demonstrated that the generation of antibodies to conformational epitopes of CD4 follows a peculiar T cell help pathway (intermolecular help). Moreover, these antibodies are subjected to isotype switch and to affinity maturation.

We set up an in vitro method for the quantitative generation of fusion complexes (FC), which represent the cellular correlates of the multistep molecular interactions between HIV and target cells. We used FC as immunogens in Balb/c mice and defined the route of immunisation and the amount of complexes, which best supported the generation of high titers of antibodies to conformational epitopes on the viral/host molecules complex. We screened sera and monoclonal antibodies for interference with the in vitro formation of fusion complexes as well as for inhibition of HIV replication, in standard p24 assays.

We found that antisera with the highest fusion- and proliferation-blocking titers were associated with a prevalent binding to fusion complex (as compared to the single cellular fusion partners) and with a modest capability to interfere with CD4-gp120 binding. Moreover, these blocking capabilities were non-HIV group specific, since cross-clades experiments demonstrated that: i) they were active on fusion partners expressing envelope proteins form different HIV strains (NSI and SI) and ii) they were coreceptor usage independent.

In addition, at the monoclonal antibody level, we found that inhibition of HIV replication does not always correlate with inhibition of cell fusion, indicating that cell fusion (and the formation of syncytia) is a complex phenomenon, with respect to the final outcome of infection.

In conclusion, our results indicate that the generation of FC immunogens can help in the design of vaccines, which could be functionally active on HIV replication. These reagents could overcome the problem of viral escape by targeting the immune response to transiently expressed conformational epitopes that are nor subjected to immunological selection.

MONOCLONAL RECOMBINANT Fab FRAGMENTS AGAINST HIV TAT PROTEIN OBTAINED FROM IgM-RESTRICTED PHAGE DISPLAY REPERTOIRE LIBRARIES CONSTRUCTED FROM SERONEGATIVE PATIENTS.

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A careful study of the role played by antibodies in the history of the disease caused by HIV infection is needed both for development of effective antiviral strategies and for a more complete comprehension of the virus-host interplay The importance of so-called "natural antibodies" (molecules with affinity for HIV antigens present in individuals with no history of contact with the virus) in protecting from the disease and in slowing the progression of the infection from the asymptomatic phase to AIDS is debated and remains still to be determined. Unfortunately the study of this aspect of the humoral immunoresponse is not easy, due to the relative paucity of these antibodies in the serum and to the fact that genuine natural antibodies are present together with other antibodies featuring a polyreactive binding pattern. A great contribution to the knowledge in this field can derive from the application of novel techniques for cloning the antibody repertoire by construction of phage display combinatorial libraries. Production of human monoclonal antibodies representing discrete components of the natural immunoresponse can provide an useful insight of this interesting aspect of the virus-host interaction, defining biological activity and genetic features of these molecules.
A novel strategy for constructing a repertoire library displayed on the surface of a filamentous phage containing genes coding for antibody fragments exclusively of the IgM and IgD isotype was developed during the first part of our project. Furthermore, using as a source a phage display combinatorial library containing the IgM repertoire of a seronegative individual, genes coding for IgM-Fabs correspondent to natural antibodies with affinity for HIV TAT protein were selected and inserted in a Fab labeling expression vector (pComb3/Flag) that allowed to demonstrate and map the binding of these molecules. Sequences of the variable parts of the heavy and light chain were determined and the Fabs were demonstrated to be all identical, featuring an unusual heavy chain derived from a germ line of the Vh6a subfamily with minimal mutations. Binding to the antigen was demonstrated to be specific, being inhibited by antigen excess. Finally, the epitope recognized by these Fab molecules was mapped.
Availability of these molecules can be important for elucidating the role of natural humoral immunity in HIV infection and disease progression.

Accordo di Collaborazione n 40C.19.

INTEGRIN-DEPENDENT ACTIVATION OF VEGFR-2 BY TAT: MOLECULAR AND BIOLOGICAL FEATURES OF A DYNAMIC MODULE IN VASCULAR CELLS.

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Tat exerts its extracellular biological activity through the activation of two family of receptors: the tyrosine kinase VEGF receptors and the G protein-coupled CC chemokine receptors. By structure-activity relationship studies it has been established that the Tat basic domain binds to and activates VEGFR-2 in vascular endothelial cells (EC), and the Cys rich and the core domains represent the molecular determinants responsible for CCR2 and CCR5 engagement (1-3). Besides the activation of an angiogenic program, VEGFR-2 stimulation by Tat or by Basic$^{46-80}$ peptide elicits vascular permeability, production of MCP-1 and PAF and is instrumental in lymphomononuclear cell infiltration in infected tissues. In contrast CysL$^{24-51}$ did not share this activity that may exclude the involvement of chemokines receptors in the response of EC to Tat (4). On the basis of a structure-activity relationship study we demonstrated that the RGD sequence of Tat co-operates with the basic region in the activation of VEGFR-2 and β3 integrin associates to the receptor after Tat challenge (5, 6). In the present study we have transduced porcine EC, which are devoided of VEGFR-2 and β3 integrin, with the wild types and mutated forms in the cytoplasmic tail of both molecules to study the the molecular events leading to the formation of a dynamic module constituted by VEGFR-2, β3 integrin or VE-cadherin, Tat and the tyrosine phosphatase SHP2. We demonstrated that VEGFR-2 may form two different modules (*a complex of different proteins that depicts a critical level of biological organization sometimes exceeding the activity of single components)* depending on the functional state of the cells at the moment of activation. In sparse EC, Tat stimulated VEGFR-2 associates with β3 integrin and migrates into focal adhesions. In cells carrying β3Δ744, this integrin is unable to localize to focal adhesions and the activated VEGFR-2 does not form the complex. In these cells, VEGFR-2 is phosphorylated by Tat but does not transduce biological signals (migration) indication that the complex formation and the localization to the focal adhesion is necessary to the full activation of the receptor. The role of β3 integrin is that of a shuttle molecule and allows SHP2 to reach VEGFR-2 and to modulate its biochemical signals. In confluent EC, VEGFR-2 stimulated by Tat co-immunoprecipitates with VE-cadherin and, through the activation of Akt kinase, activates an anti-apoptotic program. Thus, it is likely that discrete fractions of VEGFR-2 stimulated by Tat, by interacting with VE-cadherin or αvβ3 integrin, participate in two different modules. These would direct growth factor receptor signaling toward distinct pathways depending on the functional state of the cells**.** Furthermore, these data firstly demonstrate that Tat may be a survival factor for EC in some conditions.

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TARGETING HIV-1-INFECTED CELLS WITH PHAGE DISPLAY

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The availability of receptors for HIV-1 binding and entering into host cells is crucial for infection and propagation of the virus. CD4 is the cell membrane receptor for HIV-1, and co-receptors have been identified among chemokine receptors, mainly CCR5 and CXCR4. The initial targets are memory T cells that express both CD4 and CCR5, but both naive and memory CD4 T cells are targeted by viruses capable of using CXCR4 for entry, and macrophages become the primary target cells when most CD4 T cells are depleted. Compelling evidence is emerging that the availability of target cells for infection is a limiting factor for the spread of the virus. HIV-1 Tat is a powerful nuclear trans-activator. It has been demonstrated that Tat is able to induce the expression of some HIV-1 co-receptors in host cells themselves, thus enhancing the efficiency of virus infection. So, infected cells express a peculiar pattern of surface molecules, making them a suitable target for anti-viral therapy. We established an *in vitro* model reproducing the high levels of Tat expression observed during HIV-1 infection. Using a retroviral system, we transduced U937 monocyte cells with Tat full-length cDNA. The expression of Tat was evaluated by Northern Blot analysis and its activity confirmed by a CAT assay. In our system, the overexpression of Tat does not modulate the expression of the known co-receptors for HIV-1, i.e. CCR5 and CXCR4, as assayed by FACS analysis. So, we decided to look for novel binding sites using the phage display technology. Peptide phage libraries can be generated to display up to 10^9 random peptide permutations on the minor coat protein pIII of bacteriophages, and are largely used to obtain defined peptide sequences interacting with a peculiar molecule. Looking for Tat-induced cell membrane markers, we screened two phage display peptide libraries ($CX_{10}C$ and $CX_3CX_3CX_3C$) on U937/Tat cells. Five sequences were selected from the $CX_{10}C$ and four from the $CX_3CX_3CX_3C$ library. The specificity was tested by panning single phage on Tat-expressing cells. Interestingly, four of the selected peptides proved to share homology with different regions of HIV-1 gp120env. In particular, one of the selected peptides maps between loop V1 and loop V2, while other two correspond to the end of loop V3 and V4, respectively. The loop V3 has been implicated in binding to chemokine receptors, but the role of the other loops remains uncertain. Our results suggest that *i)* the selected regions of gp120env could actually be implicated in binding to cellular co-receptors for HIV-1, possibly different from chemokine receptors, and whose expression is modulated by Tat; *ii)* the soluble peptides could inhibit HIV-1 infection by competing with gp120env for binding sites on the host cells. To further investigate this second hypothesis, soluble peptides with the selected sequences have been synthesized, and will be tested for their ability to interfere with HIV-1 entry in peripheral blood mononuclear cells from healthy donors.

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HIV-SPECIFIC T-HELPER AND CTL RESPONSE FOLLOWING ART IN CHRONIC AND PRIMARY HIV INFECTION

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Maintenance or restoration of HIV-specific T cell responses are tought to be necessary immunological correlates to favourable clinical evolution of HIV infection, as shown by studies on subjects with long-term non progressive disease, and are the major target of vaccine-based strategies.
Anti-retroviral therapies (ART), very efficient in suppressing virus replication, have been shown to influence HIV-specific T cell responses. A better understanding of the extent and of the mechanisms of ART-induced immune reconstitution is thus mandatory not only for vaccine development, but also as a guidance for crucial decisions on initiation, maintenance or structured interruption of therapy.
In order to contribute to this goal, we studied the evolution of HIV-specific T cell functions in 57 previously untreated individuals with chronic HIV infection (CDC class A1=17; A2-B2=27; A3-B3=13) undergoing different ART regimens, and in 5 subjects with primary HIV infection (PHI) treated with AZT+3TC+IDV within 34 days (median, range 30-52 days) from the onset of symptoms.
Significant increase in CD4 counts and reduction in HIV viremia have been observed in most of the patients with chronic disease and in the 5 individuals with PHI, two of whom have been followed up for 52 weeks to date.
Analysis of HIV-specific CTL dynamics in response to ART in patients with chronic HIV disease, performed by LDA, showed rapid and persistent down-modulation of effector (in vivo activated) CTL response, whereas increase of gag-specific memory (precursor) CTL was observed in few patients and only after 6-12 months of successful therapy. Spontaneous treatment interruption in one patient resulted in drop of CD4 count, rebound of HIV viremia and eCTL increase within 7 weeks. Restart of the same treatment led again to CD4 increase, undetectable viremia and down-regulation of eCTL.
HIV-specific CD4-mediated lymphoproliferative responses (LPR) were also assessed, together with responses to recall antigens and mitogens. Recovery of LPR to Candida, CMV and Influenza antigens were observed, whereas no HIV-specific T-Helper activity was detected neither before nor after 12 month of therapy in patients with chronic infection.
In contrast, HIV p24-specific T-Helper response was present before therapy in 4 out of 5 patients with PHI. In 3 of them analysis of TCR beta chain repertoire on CD8 and CD4 cells was performed at baseline by cytometry: no significant perturbations in CD4 and CD8 subpopulations were detected in 2 patients, whereas the third showed a CD8 oligoclonal expansion. ART-induced modifications on TCR perturbations will be assessed quarterly, as follow-up proceeds.
These results show that the possibility that ART may preserve CD4-mediated HIV-specific T-Helper function seems to be strictly related to the time elapsed from the initial exposure to the virus and favor early therapy. However, the detection of CD8-mediated HIV-specific memory CTL activity after long-term therapy allows to speculate that recovery of the necessary CD4 T-Helper activity may be achieved at least in some patients with chronic infection. Further insights on the dynamics of CD4 and CD8-mediated HIV-specific response will be obtained from protocols of structured treatment interruptions and immune-based interventions, allowing expand the links among pathogenesis and treatment of HIV-induced immunodeficiency.

Grant n° 40C.21

LONG-TERM DYNAMICS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 EXPRESSION IN HUMAN MACROPHAGES AND ITS MODULATION BY ANTIVIRAL COMPOUNDS.

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The long-term dynamics of HIV-1 replication and cumulative virus production were evaluated in human macrophages under treatment with antiviral compounds. Unspliced (US) and multiply-spliced (MS) HIV-RNA linearly increased after infection, reaching at day 10 levels of 1.5×10^8 and 2.8×10^5 copies/10^5cells respectively, and plateauing thereafter. HIV-RNA production at day 50 was still 1.7×10^8 and 3.9×10^5 copies/10^5cells for US and MS-RNA. Virus release in supernatants paralleled the production of viral transcripts, with a cumulative production of p24 Ag and genomic HIV-RNA at day 50 of 10^7 pg/ and 10^{10} copies/10^6cells respectively, corresponding to about 100 particles produced daily by each infected macrophage. Number of macrophages remained stable in cultures during infection. AZT inhibited HIV-1 replication in macrophages up to day 50, with a cumulative virus production of both genomic HIV-RNA and HIV-p24 of 2.5×10^9 copies/ and 1.1×10^6 pg/10^6cells (73.8% and 88.9% inhibition respectively). Ritonavir was inactive upon HIV-RNA production, but was effective upon release of p24, with an inhibition of cumulative virus production at day 50 of 40.1% compared to control (p=0.04). In conclusion, macrophages are able to sustain long-term and high-level of HIV replication. This supports their unique role as a persistently-infected HIV-1 reservoir in the body.

N°. dell'Accordo di Collaborazione: 40C.22

THE LD78β ISOFORM OF MIP-1α IS THE MOST POTENT CC-CHEMOKINE IN INHIBITING CCR5-DEPENDENT HIV-1 REPLICATION IN HUMAN MACROPHAGES.

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The CC-chemokines, RANTES, macrophage inflammating protein-1α (MIP-1α) and MIP-1β, are natural ligands for the CC-chemokine receptor CCR5. MIP-1α, also known as ld78α, has an isoform ld78β, which was identified as the product of a nonallelic gene. The two isoforms only differ in 3 amino acids. Ld78β was recently reported to be a much more potent CCR5 agonist than ld78α and RANTES at inducing intracellular Ca^{2+} signaling and chemotaxis. CCR5 is expressed by human monocytes/macrophages (M/M) and represents an important coreceptor for M-tropic, CCR5-using (R5) HIV-1 strains to infect the cells. We compared the antiviral activity of ld78β and the other CC-chemokines in M/M. Ld78β at 100 ng/ml almost completely blocked HIV-1 replication, while at the same concentration ld78α had only weak antiviral activity. Moreover, ld78β proved to be the most antivirally active of the chemokines when HIV-1 infection in M/M was monitored by a flow cytometric analysis using p24 ag intracellular staining. RANTES, once described as the most potent chemokine in inhibiting R5 HIV-1 infection, was found to be considerably less active than ld78β. Ld78β strongly downregulated CCR5 expression in M/M, thereby explaining its potent antiviral activity. This superior anti-HIV-1 activity of LD78β has to be reconsidered in the light of the previously reported overproduction of the CC-chemokines MIP-1α, MIP-1β and RANTES in HIV-1 repeatedly exposed subjects, that is correlated with the protection against HIV-1 infection, where MIP-1α appeared faster and reached higher concentrations than MIP-1β and RANTES. These clinical data are in agreement with the higher antiviral potency of the LD78β isoform of MIP-1α in macrophages, and confirm the relevance of this study.

N°. dell'Accordo di Collaborazione: 40C.22

INTRASTRIATAL RAT INJECTION OF HIV-1 TAT PROTEIN INDUCES A PARKINSON'S-LIKE DISEASE: EVIDENCE FOR TAT-MEDIATED INHIBITION OF THE TYROSINE HYDROXYLASE GENE EXPRESSION

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Isolated parkinsonian features, such as bradykinesia, postural instability, hypomimetic facies, and tremor are common manifestations of acquired immunodeficiency syndrome (AIDS). HIV-1-infected patients may also present striking sensitivity to dopamine (DA) receptor blockade and show significant lower levels of DA in the cerebrospinal fluid. As Parkinson's deaseas (PD) is characterized by a slow and progressive loss of dopaminergic neurons in the substantia nigra (SN) that project to the striatum, we addressed the issue of whether HIV-1 Tat was able to modulate the expression of tyrosine hydroxylase (TH), the rate limiting enzyme for DA synthesis. Since Tat protein is rapidly taken up by intact cells, we explored whether local administration of Tat into the rat striatum could affect the nigro-striatal pathway. In vivo injection of synthetic Tat protein into the striatum of healthy rats induced a subclinical Parkinson's-like disease, that became manifested when the animals were treated with amphetamine. As early as one week post-injection, the histochemical analysis of the rat substantia nigra showed a reduced staining of neurons expressing TH, which were lost at later time points.

The molecular basis of TH impairment was then investigated on the dopaminergic PC12 cell line. Treatment of PC12 cells with Tat protein or tat cDNA inhibited the expression of TH and the release of DA into the culture medium. The adverse effect of Tat on DA synthesis was shown to be mediated by a c-AMP dependent pathway involving downstream upregulation of ICER, which in turn represses TH transcription and consequently inhibits the hydroxylation of tyrosine to DOPA.

Accordo di Collaborazione: 40C.23

CELL-DERIVED MEMBRANE PROTEINS PRESENT ON CIRCULATING HIV-1 BEFORE INITIATION OF HAART+IL-2 AND AFTER CONTROLLED THERAPY SUSPENSION.

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OBJECTIVES: To determine the profile of cell-derived membrane proteins (CMP) on HIV-1 circulating in the plasma of asymptomatic patients and to analyze possible changes after a cycle of highly active anti retroviral therapy (HAART) plus IL-2.
METHODS: Plasma samples from eight drug-naïve asymptomatic subjects were tested to detect CMP by an immobilized antibody capture assay (IAC), followed by quantitative RT-PCR. Patients were sampled before the initiation of HAART plus IL-2, and after controlled therapy suspension, at the time of viral rebound. Lymphocyte subset markers (CD45RO and CD45RA), activation markers (HLA-DR), adhesion molecules (LFA-3), costimulatory molecules (B7-2), lymph node homing receptors (CD62L) and pro-apoptosis molecules (Fas-L) were considered in the study.
RESULTS: LFA-3 and CD45RO and HLA-DR are the most represented CMP on the surface of HIV-1 present in the plasma of drug-naïve asymptomatic patients, whereas CD45RA, CD62L, B7-2 and FasL are detected in a minority of cases, and to a low extent. After therapy suspension, at the time of viral rebound, a significant reduction in both HLA-DR and CD45RO embedded in virion envelope is observed, whereas LFA-3 content is virtually not affected. At the same time, CD45RA, CD62L, B7-2 and FasL remain not detectable on circulating HIV-1.
CONCLUSIONS: Based on the assumption that CMP present on HIV-1 envelope represent a footprint of the cell actually replicating the virus, activated memory T-cell appear to be the main source of plasma HIV-1 in asymptomatic patients. After therapy suspension, when naïve T cells population is significantly expanded, CD45RA is still virtually absent on circulating virions, indicating that these cells do not became a major source of virus replication. The decreased presence of HLA-DR on HIV-1 after therapy is in agreement with a reduced activation status of the virus-producing cells. These findings can help to identify viral reservoirs responsible for virus rebound after therapy interruption.

N°. dell'Accordo di Collaborazione: 40C.24

MOLECULAR, PHARMACOLOGICAL AND IMMUNOPROPHYLACTIC APPROACHES AGAINST THE TAT PROTEIN OF HIV-1

The objective is to study therapeutic and immunoprophylactic strategies to block tat activity in in vitro and in vivo models. The project is developed in two parts.

(a) ANTI-TAT THERAPEUTIC APPROACH (PHARMACOLOGIC AND GENE THERAPY)

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A1. We have studied the effect of two dystamicin derivatives (PNU 151484, PNU 953429) on the virologic and extra-virologic activities of extracellular Tat. The results indicate that the drugs bind extracellular Tat through its basic region and inhibit extravirologic effects of Tat: they i) block the autocrine and paracrine loop of extracellular Tat on cell survival and proliferation inducing apoptosis of Tat-expressing cells, ii) inhibit the angiogenic activity of Tat released from T53 cells (derived from VBK/tat trangenic mice) and iii) inhibit T53 induced-tumors and metastases in nude mice. The drugs exhibit an inhibitory effect also on extracellular Tat virological activity. So far the results indicate that they block HIV-1 replication, measured both as p24 Ag or RT released in cell supernatant and as proviral DNA in infected cells. Inhibition is dose dependent, being HIV-1 replication slowed down in the presence of 5 μM of drug and completely inhibited at the concentration of 20 μM of drug. Replication studies in the presence of drugs and peptides corresponding to the basic region of Tat are ongoing. Similar studies will be performed on HIV-1 infected cells derived from seropositive patients.
A2. We have proposed to construct and characterize new pseudoviral vectors based on BKV, for transduction of hematopoietic cells, that eventually will be used for delivery of anti-Tat molecules. So far we have constructed eukaryotic vectors expressing the BKV late region, and vectors containing BKV origin of replication and early promoter driving the expression of reporter genes (EGFP, tat or neo). Production and titration of pseudovirions in human cells expressing BKV-T antigen, and their characterization is ongoing.

(b) ANTI-TAT IMMUNOPROPHYLACTIC APPROACH

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We have characterized in vitro and in vivo the biological properties of a new class of synthetic block copolymers for the delivery of DNA for vaccine application. Five block copolymers (K1-K5) were chosen. They self-assemble with DNA in aqueous solution to form micellar-type particles and protect DNA from DNAseI attack. Block copolymers deliver DNA intracellularly and allow release and expression of Tat in cell cultures. In addition, block copolymers, alone or associated to DNA, are not toxic in mice injected s.c. or i.m. The analysis of the immune response to the HIV-1 tat gene in Balb/c mice vaccinated i.m. with tat alone or associated to K1, K2 or K5 indicate that vaccination with tat and the block copolymers induce a strong CTL response against Tat. Other vaccination routes will be studied. Microspheres for the delivery of Tat protein are under investigation. Preliminary results in HL3T1 cells indicate that they i) protect Tat from oxidation and loss of activity; and ii) deliver Tat intracellularly. Vaccination experiments will be carried out.

Accordo di collaborazione N. 40C.25

DEVELOPMENT OF INHIBITORS OF THE NEF PROTEIN.

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We are aiming at developing an innovative strategy to inhibit the activity of any given protein and we are applying it to the HIV protein Nef. To this end we want to control the protein degradation machinery in order to instruct it to phenotypically knock out any given gene product.
The strategy is based on the recent observation that E3 proteins (ubiquitin ligases), which are responsible for the selective degradation of proteins in the cell, are bimodular adapters with one of the module serving to link to the ubiquitination machinery and the other module targeting the protein to be degraded.
According to this model, it should be sufficient to covalently link any protein binding domain X to an E2 recognition module (e.g., Fbox, SOCS boxes etc.) to induce ubiquitination and degradation of the protein ligand of domain X. This approach, if successful, should allow one to degrade in vivo any protein of interest in order to investigate the consequence on the cell physiology.
As a model system we have used a protein recognition module, SH3 domain, that in nature is not exploited by ubiquitin ligases as a target recognition module. The aim of our project is to demonstrate that a viral protein that is not naturally ubiquitinated, the HIV Nef protein, can be degraded by the proteasome by coexpression of a modified E3 ligase.
We have constructed a hybrid β-TcrP were the WD recognition module is replaced by the SH3 domain of the kinase Hck that efficiently binds to Nef.
We have expressed a collection of recombinant proteins and we want assess whether this chimeric E3 ligases can induce the ubiquitination of purified Nef in reticulocyte extracts. Furthermore, we want to show that transfection ,with this constructs, of macrophage cell lines cronically infected with HIV (expressing large amounts of Nef), induce the degradation of Nef via the proteasome pathway. If this strategy will prove successful we will have a tool to specifically knock out any protein from the cell.

N°. dell'Accordo di Collaborazione 40C.26

IDENTIFICATION OF A DOMAIN IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) REV THAT IS REQUIRED FOR FUNCTIONAL ACTIVITY AND MODULATES ASSOCIATION WITH SUBNUCLEAR COMPARTMENTS CONTAINING SPLICING FACTOR SC35.

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The activity of HIV Rev as a regulator of viral mRNA expression is tightly linked to its ability to shuttle between the nucleus and cytoplasm. This property is conferred by a leucine-rich nuclear export signal (NES) and an arginine-rich nuclear localization signal/RNA binding domain (NLS/RBD) required for binding to the Rev-responsive element (RRE) located on viral unspliced and singly spliced mRNAs. Structure predictions and biophysical measurements indicate that Rev consists of an unstructured region followed by a helix-loop-helix domain (HLH) containing the NLS/RBD and a carboxy-terminal tail containing the NES. We present evidence that the loop portion of the HLH is an essential functional determinant that is required for binding to RRE and correct intracellular routing. Data obtained using a CK2 phosphorylation assay indicated that the loop region is essential for juxtaposition of helixes 1 and 2 and phosphorylation by protein kinase CK2. Deletion of the loop resulted in partial accumulation of Rev in SC35-positive nuclear bodies that resembled nuclear structures that form in response to inhibition of transcription. Accumulation of the ΔLoop mutant in nuclear bodies depended on the presence of an intact NES, suggesting that both the loop and the NES play a role in controlling intranuclear compartmentalization of Rev and its association with splicing factors.

Proposal n. 40C.27

HTLV-I TAX BLOCKS CELL DEATH INDUCED BY SERUM STARVATION BY INTERFERING WITH A MITOCHONDRIAL-MEDIATED APOPTOTIC PATHWAY.

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Although the viral transactivator Tax has been established as an essential effector of HTLV-I-mediated pathogenesis, the molecular mechanisms underlying Tax-induced diseases remain to be clarified. Dysregulation of the apoptotic process can lead to pathophysiological changes which result in either degenerative diseases or cancer. As the apoptotic potential of Tax is still debated, we addressed this question by testing the susceptibility of Tax(+) and Tax(-) murine fibroblasts to apoptosis under conditions of growth factor withdrawal or treatment with TNFα, two stimuli that trigger apoptosis via distinct pathways. Results showed that Tax protects cells from apoptotic death induced by serum deprivation but does not affect TNFα-mediated apoptosis. This indicates that Tax has different effects on cell death depending on the apoptotic stimulus used. Analysis of the mechanism(s) involved in Tax-mediated resistance to serum depletion-induced apoptosis indicated that Tax prevents or delays apoptosis by interfering with the release of cytochrome c from the mitochondrial intermembrane space and by blocking the redistribution of Bax from the cytosol to mitochondria.

Proposal n. 40C.27

RECOMBINANT PHENOTYPE OF V3 CLONAL SEQUENCES AND ROLE OF INTERACTIONS AMONG SPECIFIC AMINO ACID SUBSTITUTIONS

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The envelope glycoproteins of HIV-1 interacts with receptors of the target cells and mediates the process of virus entry. For this principal reason, the evolutionary changes characterizing the HIV-1 env gene during the natural history of infection strongly influence the early steps of the viral infection cycle, including coreceptor usage and CD4 independence. In this context, careful analysis of the intra-host evolution of the env gene can be strategic for addressing the relevant features of the virus-host relationships and disease pathogenesis. In addition, HIV-1 entry is an attractive target for new classes of putative anti-retroviral compounds, including inhibitors of HIV-1 binding to CCR5 and CXCR4 coreceptors and fusion inhibitors. In this study, we addressed the genotypic and phenotypic features of the HIV-1 evolutionary potential in vivo and analyzed the biological role of interactions among specific amino acid substitutions in the V3 sequence. Firstly, we developed a new strategy to analyze directly the recombinant phenotype to the HIV-1 coreceptor usage of chimeric viruses bearing V3 clonal sequences selected in vivo, secondly, we used site-directed mutagenesis to evaluate the relevant features of specific V3 motifs. Two viral backbones (designated NLmodΔV3 and NLmodAD8gp120ΔV3) were planned and developed in order to obtain recombinant infectious virions after cloning exogenous V3 sequences. Optimization of the method was achieved using V3 sequences from reference viral strains: AD8 (R5 phenotype), SF2 (R5/X4 phenotype), and NL4-3 (X4 phenotype) documenting that the inserted V3 sequences determined the original tropism in the recombinant viruses generated. The growth characteristics and coreceptor usage of the recombinant viruses were studied following infection of cell lines (PM1 and MT2), PHA-stimulated PBMCs, and cell lines expressing the chemokine receptors (U87-CD4). Clonal V3 sequences from sequential samples of HIV-1-infected subjects were selected and an R5 phenotype was observed in 14/20 recombinant viruses. These viral chimeras showed two distinct patterns of replication (fast and slow). A dualtropic R5/X4 phenotype was also observed, and this virus maintained the original dual tropism after infection of either R5, or X4/U87-CD4. Five recombinant viruses were unable to replicate in any of the cell lines employed. Site-directed mutagenesis of specific amino acid residues was performed and documented the crucial role of specific V3 mutations in driving the relevant features of the HIV-1 replication dynamics and, in some cases, coreceptor usage. The mutual interactions between host's selective forces and HIV-1 variability are believed to influence considerably not only the intra-host evolution of HIV-1 quasispecies, but also disease progression. In this context, the studies addressing viral evolution through parallel analysis of the viral genotype and phenotype could be of considerable help in clarifying the bio-pathological role of intra-host HIV-1 evolution. In the present study, we have developed a new recombinant assay which proved to be useful to study the V3 evolutionary dynamics. Moreover, the method allows the interactions among specific amino acid substitutions to be addressed. The potential use of the method described above to evaluate the viral phenotype to inhibitors of coreceptor usage (thus allowing the identification of resistant strains) should also be addressed in the near future.

No. dell'Accordo di Collaborazione: 40C.28

EVALUATION OF IMMUNE ACTIVATION IN HIV-INFECTED AND -UNINFECTED AFRICAN INDIVIDUALS BY SINGLE-CELL ANALYSIS OF CYTOKINE PRODUCTION.

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Immune activation (robust production of interleukin-2, interleukin-10 and tumor necrosis factor alpha) is reported to be common in both HIV-infected and -uninfected African individuals. This activation is probably secondary to environmental reasons (infections, critical hygienic conditions, nutritional deficiencies) and is suggested to result in profound modifications of the interaction between the immune system and HIV. To better characterize immune activation in Africa we performed in-depth immunological analyses.
Freshly drawn peripheral blood mononuclear cells of HIV-infected African (Gulu department; North Uganda) and Italian (Milan) antiviral naive patients in a comparable clinical stage, and of healthy individuals from Uganda or Italy were used for the study. All individuals were age-and sex-matched and were screened to exclude viral or parasitic infections. Intracellular levels of IL-2, IL-10, and TNFa were measured by FACS in CD4 and CD8 T lymphocytes either in the absence of stimulation or upon stimulation with mitogens (M) or a gp160 peptides (env). M- and Env-specific, IFNg-producing CD8 T cells were measured in an ELIspot assay
The following statistically significant differences were observed in African compared to Italian individuals: 1) CD4+ T lymphocytes: augmented M-stimulated production of IL-10, and TNFα both in infected and uninfected subjects; 2) CD8+ T lymphocytes: augmented M-stimulated IL-10 and TNFα production both in infected and uninfected subjects; augmented env-stimulated IL-10 and TNFα production in HIV-infected patients. Elispot assays showed M- and env-stimulated IFNγ- producing CD8+ T cells to be greatly reduced in African compared to Italian individuals and not increased by preincubation of PBMC with αIL-10mAb.
This is the first set of data reporting immune activation in rural Africa by single cell analysis of cytokine production. These results confirm that abnormal immune activation is present in African individuals independently of HIV infection. The significant increase in IL-10 production might not be responsible for the reduced cytolitic activity observed in African individuals

N°. dell'Accordo di Collaborazione 40C.29

IGA FROM HIV-EXPOSED UNINFECTED INDIVIDUALS INHIBIT HIV-1 TRANSCYTOSIS THROUGH HUMAN EPITHELIAL CELLS

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A minority of individuals remain persistently HIV-1 seronegative despite repeated viral exposure. IgA purified from the plasma and mucosal surfaces of these highly exposed. persistently negative (HEPS) individuals have been shown to neutralize infection of peripheral blood mononuclear cells by HIV-1. We have now investigated the ability of IgA from HEPS individuals to inhibit the transcytosis of primary HIV-1 isolates across a tight epithelial cell layer, in a system which models natural HIV-1 infection across a simple epithelial cell membrane in human mucosal tissue. In a two-chamber well system, a tight polarized human epithelial cell layer was grown on a filter separating the two compartments. In the absence of IgA, HIV-1 primary isolates were actively transported across the epithelial membrane, and released on the opposite side of the barrier. Co-culture with fresh human mononuclear cells demonstrated that these transcytosed HIV-1 particles were still infectious. IgA purified from HEPS individuals was also transcytosed across the epithelial membrane model. By pre-incubation of the epithelial cell layer with salivary IgA, before addition of the HIV-1-primary isolate, resulted in partial or total inhibition of HIV-1 transcytosis. IgA purified from the plasma and genital tract of HEPS individuals demonstrated the same inhibition. IgA purified from low-risk, healthy control subjects had no inhibitory effect on HIV-1 transcytosis.
We conclude that the ability of mucosal and systemic IgA to inhibit HIV-1 transcytosis across the mucosal epithelium may represent an alternative mechanism for protection against the sexual acquisition of HIV-1 infection in HEPS individuals.

N°. dell'Accordo di Collaborazione 40C.29

FAS-REACTIVE IGG AND SERUM ELEVATIONS OF SOLUBLE FAS CONCUR IN T CELL DEPLETION IN HIV-1 INFECTION

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Deregulated apoptosis during both primary HIV-1 infection and full-blown AIDS is considered a crucial factor in the progressive depletion of $CD4^+$ cells. In particular, the overexpression of Fas, a 45 kDa apoptogen receptor, along with its increased susceptibility to oligomerization in response to either agonist or antagonist stimuli, is a major event leading to the accelerated cell death. Recent studies suggest that serum levels of soluble (s) Fas increase during infection. However, its role is presently unclear although the release of membrane receptors could exert a regulatory effect on several cell functions. On the other hand, a potential role as apoptosis blocker was ascribed to sFas for its ability to disactivate Fas-L on cytotoxic cells in vitro.

By investigating 227 HIV-1^+ subjects with different clinical disease, we found that serum levels of sFas and anti-Fas IgG were linearly correlated (R^2 = 0.304) in 17 severely lymphopenic patients. Cytofluorimetric measurement of the subdiploid DNA-containing cell population by both PI and TUNEL methods revealed an increased occurrence of cell death in vitro, especially in patients with the highest sFas titers. Fresh $CD4^+$ cells from these patients showed a high intracellular content of CPP32, namely caspase 3, and its cleavage products such as PARP and CK18. These cells were also suppressed in their proliferative rate in vitro by the addition of recombinant (r) Fas and showed an enhanced intracytoplasmic content of FLICE, the Fas-related caspase.

We have therefore hypothesized that sFas binds its membrane homologous as proposed by recent studies (Papoff et al., J Biol Chem 274: 38241, 1999; Siegel et al., Science 288: 2354, 2000) and that this linkage triggers the apoptotic pathway of T cells through Fas. To elucidate this hypothesis, membrane lysates of Fas^+ T cell lines, including Jurkat and CEM, were adsorbed on Sepharose columns coupled with rFas. Preliminary results showed that the affinity-purified membrane proteins linking rFas on columns had molecular size(s) similar to both monomeric and oligomeric isoforms of Fas. In addition, these protein fragments were reactive in immunoblotting to the anti-Fas MoAb ZB4 and exerted a clear-cut apoptogenic effect on Fas^+ T cell lines.

These data support the apoptogenic role of sFas during HIV-1 infection and suggest that the receptor is released by functionally exhausted T cells to spread an execution signal and amplify the lethal event. This can elucidate the described occurrence of enhanced cell death in advanced infection in association to serum elevations of the soluble receptor.

Accordo di collaborazione: 40C.30

EVELOPMENT AND COMBINED USE OF RECOMBINANT AVIPOXVIRUSES, EXPRESSION PLASMIDS AND VIRUS-LIKE PARTICLES IN «PRIME-BOOST» PROTOCOLS FOR VACCINATION AGAINST SHIV

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Considering that the spread of AIDS is mainly due to sexual intercourse through the transmission of macrophagotropic or dualtropic genetic variants of HIV utilizing preferentially the CCR5 coreceptor, it appears evident the need to develop new immunogens able to induce high levels of the CCR5 natural ligands RANTES, MIP-1 alfa and MIP-1 beta. The utilization of the first immunogen we constructed, a recombinant fowlpox virus expressing the complete env gene of HIV-1_{SF2} (FP $_{HIV\text{-}1env}$), was able to induce a level of immune response in macaques lowering of 3 log the proviral load after SHIV challenge, although insufficient to obtain a sterilizing immunity. This failure has been attributed mainly to the immunization regimen we utilized and, to a lesser extent, to the utilization of a single immunogen, without any booster with purified proteins. For this reason we engineered a new expression vector utilizing the pcDNA3 plasmid by inserting the gag/pol genes of SIV. This construct has been utilized, in combination with a reoptimized fowlpox env recombinant, to formulate a new immunization protocol. In detail, the new fowlpox-env was made by inserting the env gene derived from the highly pathogenic dual-tropic HIV-$1_{89.6P}$ strain into the hemagglutinin gene of the vector. Moreover, to obtain a booster effect, we produced virus-like particles (VLPs) identical to the challenger (but devoid of complete genomic RNA).
Three different immunization protocols were used to prime rabbits using two fowlpox (FP) recombinant constructs and two expression plasmids, able to separately express $SIVmac_{239}$ gag/pol and HIV-1env $_{89.6P}$ genes. Priming was followed by two boosts with virus-like particles (VLPs). The three groups of animals were immunized intradermally:

A. 4 animals (priming with 1 DNA construct + 1 FP construct , boosting with VLPs)
week 0 pcDNA3gag/pol_{SIV} (250 μg/animal) + $FPenv_{89.6P}$ (10^8 PFU/animal)
week 4 pcDNA3gag/pol_{SIV} (250 μg/animal) + $FPenv_{89.6P}$ (10^8 PFU/animal)
week 8 pcDNA3gag/pol_{SIV} (250 μg/animal) + $FPenv_{89.6P}$ (10^8 PFU/animal)
week 16 1° VLPs boost (1 μg/animal)
week 30 2° VLPs boost (1 μg/animal)
B. 4 animals (priming with 2 FP constructs, boosting with VLPs)
week 0 $FPgag/pol_{SIV}$ ($5x10^7$ PFU/animal) + $FPenv_{89.6P}$ ($5x10^7$ PFU/animal)
week 4 $FPgag/pol_{SIV}$ ($5x10^7$ PFU/animal) + $FPenv_{89.6P}$ ($5x10^7$ PFU/animal)
week 8 $FPgag/pol_{SIV}$ ($5x10^7$ PFU/animal) + $FPenv_{89.6P}$ ($5x10^7$ PFU/animal)
week 16 1° VLPs boost (1 μg/animal)
week 30 2° VLPs boost (1 μg/animal)
C. 4 animals (priming with 2 DNA constructs + 2 FP constructs, boosting with VLPs)
week 0 pcDNA3gag/pol_{SIV} (250 μg/animal) + $pNDenv_{89.6P}$ (250 μg/animal)
week 4 pcDNA3gag/pol_{SIV} (250 μg/animal) + $pNDenv_{89.6P}$ (250 μg/animal)
week 8 $FPgag/pol_{SIV}$ ($5x10^7$ PFU/animal) + $FPenv_{89.6P}$ ($5x10^7$ PFU/animal)
week 16 1° VLPs boost (1 μg/animal)
week 30 2° VLPs boost (1 μg/animal)

Preliminary results showed a good immunizing activity in all the three groups (high antibody levels, up to 1:320000, and good lymphocyte proliferation response). Our project is now aimed at determining the homologous and heterologous neutralizing activity, cellular cytotoxicity and cytokine profile by real-time PCR (TaqMan).
Protocols showing the most promising humoral and cellular response will be used to immunize Rhesus macaques, which will be then challenged with 10 MID of $SHIV_{89.6P}$.

Accordo di Collaborazione N° 40C.31

PEPTIDES BASED ON THE N TERMINUS OF CCR5: INHIBITION OR ENHANCEMENT OF HIV-1 ENTRY?

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We demonstrated that a peptide, reproducing the sequence 1-26 of CCR5, is able to increase, in a dose-dependent manner, the infection of HIV-IIIB in an experiment using U87MG $CXCR4^+$ cells (50 times if compared to the control).

RIA tests showed that the peptide (1-26)CCR5 is able to bind to sCD4; this datum is in agreement with recent published results about the association between CCR5 and CD4 in absence of gp120 and the importance of CCR5-CD4 complex in the formation of gp120-CD4-CCR5 complex.

The sCD4 affinity to gp120 is not increased by the presence of (1-26)CCR5. To repeat the experiment using cellular CD4, it was necessary to synthesize an analogue of (1-26)CCR5 selectively marked with biotin. Experiments using HeLa cells either wild-type or expressing CD4 or HIV-env or CD4 and CCR5, showed that biotin-(1-26)CCR5 binds very weakly CD4. Competition experiments with gp120 are in progress.

Because, more recently, different research groups demonstrated that

i) specific amino acids, including acidic residues and tyrosines, located within the CCR5 amino-terminal domain, are essential for CCR5-mediated fusion and HIV-1 entry,

ii) the residues 3, 10 and 14 might be sulfated and several sulfated compounds can inhibit HIV-1 entry,

we synthesized two peptides of 26 amino acids, the first one carrying $Tyr^{15} \rightarrow Ala$ modification and the second one containing sulfotyrosines in positions 3,10 and 14.

The last analogue could help us to explain if the switch between inhibition and enhancement is due to the posttraslational modification of tyrosines.

GRANT N° 40C.32

THE ROLE OF NUCLEOPHOSMIN IN THE REV EXPORT PATHWAY

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Nucleophosmin (NPM) is a nucleolar protein which shuttles between nucleus and cytoplasm. Indirect evidence suggests that it is involved in the transport of preribosomal particles through the nuclear membrane. NPM is also found as part of three different fusion proteins derived from chromosome translocations and associated with: Anaplastic Large Cell Lymphoma (ALCL, t(2;5), NPM/ALK); Acute Promyelocytic Leukaemia (APL, t(5;17), NPM/RAR) and Acute Myeloid Leukaemia (AML, t(3;5), NPM/MLF1), respectively.
Starting from the reported interaction between the HIV-Rev and NPM, we hypothesized a role for NPM in the molecular machinery responsible for nucleocytoplasmic shuttling of Rev. Indeed, we were able to demonstrate that NPM is a cofactor in the Rev-related nuclear import-export machinery. We further demonstrated that the NPM fusion proteins present in leukemia displayed an inhibitory activity on this machinery. In particular, we showed that NPM/ALK expression causes the cytoplasmic delocalization and proteasome-dependent degradation of Rev. Structure-function analysis further revealed the determinant contribution of the NPM moiety for Rev degradation, in the context of NPM fusion proteins. The role of NPM in the coordinated control of nucleocytosolic shuttling, protein degradation and cell proliferation will be discussed.

Grant N. 40C.33

DECAY OF HIV-1 DNA AND DEVELOPMENT OF DRUG-RESISTANT MUTANTS IN PATIENTS WITH PRIMARY HIV-1 INFECTION RECEIVING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY.

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The present study was aimed at describing the effect of HAART in 10 patients with Primary HIV Infection (PHI). Enrollment and treatment of PHI patients were carried out at IRCCS "L. Spallanzani" hospital in Rome.
Clearance rate of HIV-RNA and HIV-DNA in peripheral blood as well as the preexistence and the emergence of drug-resistant strains of HIV were determined over 52 weeks of treatment.

The mean baseline value of HIV RNA copies/mL for PHI patients was 4.9 ± 0.8 Log copies/mL (range: 3.6-5.8 Log copies/mL). After treatment plasma viremia dropped to <200 copies/mL in all patients with a mean time of 60 ± 40.8 days and became <50 copies/mL after 6 months. Plasma HIV-RNA remains undetectable in all subjects, except for one patient (CF) who experienced a rebound of plasma viremia after 12 months of therapy (4.2 Log copies/mL) in spite of proper compliance.
HIV-DNA level was measured in PBMC of PHI patients at baseline (time 0) and during therapy at 3, 6 and 12 months of follow up.

At time 0 mean HIV-DNA was 2.4 ± 0.5 Log copies/10^6 cells (range 1.5–3.4 Log copies/10^6 cells). The mean value decreased at 2.0 ± 0.4 Log copies/10^6 cells (range 1.5-2.8 Log copies/10^6 cells), at 1.9 ± 0.4 Log copies/10^6 cells (range 1.0-3.1 Log copies/10^6 cells), and at 1.7 ± 0.4 Log copies/10^6 cells (range 1.0-2.9 Log copies/10^6 cells) respectively at 3, 6 and 12 months of follow up. The above values were different from baseline values at each point examined, being significantly lower at 6 and 12 months ($p<0.05$).
Nevertheless all patients had detectable level of HIV-DNA copies in PBMC at 12 months of follow up with the exception of patient 1 whose HIV-DNA in PBMC was undetectable (<10 copies/ 10^6 cells).
To establish whether HAART may favor the emergence of drug-resistant strains during PHI, pol gene sequence was analyzed at baseline and during treatment. Analysis of the presence of mutations associated with drug resistance in patients under treatment was performed using DNA from PBMC because of the undetectability of HIV-RNA in plasma; only for patient CF who experienced a rebound of plasma viremia genotypic analysis was carried out both from plasma and PBMC also during treatment (12 months). No mutations in pol gene were found at baseline in patients except in patient CF in which M41L substitutions were found in RT region. Interestingly, in this patient new resistance mutations K70R and M184V in RT appeared after 6 months of therapy in HIV-DNA. Such mutations emerged before the rebound of plasma viremia observed at 12 months.
Mutation M184V was detected also in PBMC of patient DFA at 6 months of follow up although no changing in the level of plasma viremia was observed in this patient even at 12 months. Patient BA, whose plasma HIV-RNA remained <50 copies/mL during the 12 months of follow up, developed a V/I84A mutation in PRO sequence on PBMC-DNA at 6 months.
For the other patients, whose plasma HIV-RNA remained constantly <50 copies/mL during treatment, no mutations were found in pol gene.
In conclusion, our data indicate that HAART is able to induce a suppression of plasma viral load together with a significant decrease, but not a suppression, of PBMC-associated proviral DNA in PHI subjects.
Drug-resistant analysis revealed that 3 PHI patients, showing a complete virologic response, developed mutations in pol gene thus suggesting that a persistent residual replication exists despite a sustained suppression of plasma viremia and-or that strains selected for drug-resistance may establish latency during treatment.

Accordo Collaborazione N° 40C.34

MOST OF HIV-RNA IN PLASMA FROM CHRONICALLY INFECTED PATIENTS IS ASSOCIATED TO ANTIBODY.

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HIV isolation can be readily accomplished, especially under low CD4 + lymphocyte or/and high HIV RNA copy number conditions by culturing PHA activated peripheral blood mononuclear cells (PBMC) from HIV infected patients. In contrast HIV is not easily isolable from plasma even when a relatively high number of HIV RNA copies are detected by quantitative PCR. It is not known whether this failure is due to a low sensitivity of the cell culture system, to the defectivity of most of the circulating virus or, finally, to the presence of plasma associated inhibitors of infectivity. Among these HIV antibody (Ab) should be considered. It is common opinion that the high mutation rate of HIV favours an antigenic drift that is used by the virus to escape neutralization by Ab. If this is true most of the circulating Ab should not be virus-associated. To test this hypothesis we employed an experimental system aimed at: i) absorbing plasma from HIV patients to a solid substrate capable of specifically binding Ab molecules; ii) testing whether HIV RNA is, or is not, associated to the substrate-bound Ab.
Binding experiments performed with plasma of 4 chronic asymptomatic HIV infected subjects gave the following results: the percentage of unabsorbed virus ranges from 16% for patient 1 to < 3% of the input virus for the other 3 patients. Then, except for patient 1, most of virus seem to be captured by the Protein A coated beads, thus indicating that in chronic asymptomatic HIV infection most of the virus is linked to IgG HIV-specific antibody. In contrast, the data obtained from binding experiments performed with plasma virus derived from patients with primary HIV infection show that virus does not bind to Protein A coated beads, thus indicating that at this stage of infection most of the virus is not associated to Ab. Similarly no binding was detected when beads were incubated with a laboratory strain of HIV grown in tissue culture. To show that the binding to Protein A coated beads was specific, we performed competition experiments by adding excess of soluble Protein A to the plasma sample to saturate the Fc fragment of the IgG linked to virus. As control the same experiments were conducted in parallel without Protein A. The data demonstrated that free Protein A is capable of blocking the binding of the virus to the beads. In fact, in the presence of Protein A all the virus was found in the unabsorbed fraction while in control experiments (without Protein A) most of the Ab-virus complexes were found associated to the beads since the first absorption step (71% of the input). Further control experiments showed also that the presence of soluble Protein A did not influence the HIV-RNA quantification. These data confirm that the binding to the Protein A beads is specific for IgG-virus complexes.
In conclusion, our data show that most, if not all, of the HIV RNA circulating in plasma of chronically infected HIV patients "comigrate" with the Ab, strongly suggesting an association between virions and the Ab itself. These observations, other than possessing important pathogenetic implication may explain why virus isolation from plasma is very often negative in chronically infected patients even in the presence of high titer HIV RNA. Studies to evaluate whether the binding Ab have neutralizing activity are in progress.

Accordo Collaborazione N° 40C.34

THE T CELL ACTIVATION MOLECULE H4 AND THE CD28-LIKE MOLECULE ICOS ARE IDENTICAL

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We recently described H4, a surface glycoprotein selectively expressed by activated T cells and mature thymocytes and displaying weak lateral association with CD4. In the mouse, H4 is also expressed at high levels by thymic NKT cells, that are a T cell subpopulation expressing a restricted TCR repertoire and secreting high levels of cytokines. In HIV-1 infection, H4 expression displays a peak in the early phase of primary infection, drops to control levels in the asymptomatic phase, and is newly expressed as AIDS develops. Incubation of T cells from normal donors with HIV gp120 induces transient H4 expression in resting $CD4^+$ T cells and potentiates the H4 lateral association with CD4 in activated T cells. The recently cloned CD28-like molecule ICOS displays striking similarities with H4. Both molecules i) are selectively expressed by activated and germinal center T cells, ii) display similar structure, iii) display costimulatory activities. To evaluate whether H4 and ICOS are the same molecule, we used the C398.4A (binding human and mouse H4) and F44 (binding human ICOS) mAb in parallel experiments on human T cells. ICOS and H4 displayed the same expression pattern in a panel of T cell lines and the same expression kinetics in PHA-activated T cells. C398.4A completely blocked cell staining by F44, whereas F44 partially blocked C398.4A. H4 and ICOS immunoprecipitates displayed identical SDS-PAGE patterns and H4 immunoprecipitation completely removed ICOS from cell lysates. PNGase-F- and endo-H-mediated digestion showed that H4 and ICOS immunoprecipitates display similar glycosylation patterns, which were consistent with the three N-glycosylation sites displayed by the ICOS amino acid sequence. Finally, the C398.4A mAb specifically stained cells tranfected with the human or mouse ICOS. These data prove that H4 and ICOS are the same molecule and that F44 and C398.4A bind partially different epitopes. Interestingly, H4/ICOS stimulation by mAb seems to have peculiar effects on T cells since it selectively increases secretion of IL-10 and expansion of TH2 cells, which may play a role in immune disregulation in HIV infection.

GRANT N°. 40C.35

DECREASED FUNCTION OF FAS IN PATIENTS DISPLAYING DELAYED PROGRESSION OF HIV-INDUCED IMMUNE DEFICIENCY

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Fas/Apo-1 is a transmembrane molecule inducing apoptosis upon interaction with FasL and is involved in shutting-off immune responses. In patients with the autoimmune lymphoproliferative syndrome (ALPS), defective function of Fas, due to inherited deleterious mutations hitting the Fas system, cause non-malignant lymphoproliferation and autoimmune phenomena with similarities to those found in systemic lupus erythematosus. A recent classification named ALPS-Ia and -Ib the disease with mutations of Fas and FasL respectively and ALPS-II the disease without mutations of these genes. Several data suggest that ALPS-II is due to mutations hitting the Fas signaling pathway downstream from Fas, which involves activation of a caspase cascade. We have recently suggested that these Fas inherited defects may be more frequent than the rare ALPS pattern, since we found that a substantial proportion of patients with common autoimmune diseases, such as insulin dependent diabetes mellitus or multiple sclerosis, display decreased function of Fas. In AIDS patients, apoptosis of uninfected lymphocytes may contribute to development of immune deficiency. This process may involve recruitment of Fas by HIV products. In line with this possibility, the viral envelope glycoprotein gp120 does not induce death of T cells from subjects with ALPS. We evaluated the possibility that Fas function defects delay progression of HIV-induced immune deficiency by assessing susceptibility to Fas-induced cell death on T cells from "long-term non progressor", "non-progressor", "progressor" asymptomatic HIV-1-infected individuals, and AIDS patients. We found that Fas-induced cell death was significantly lower in long-term non progressors and non-progressors than in normal controls, progressors, and AIDS. Search for mutations of the Fas gene by SSCP analysis and sequencing of the whole coding region of the Fas gene did not detect any mutation in four representative Fas-resistant LTNP patients. Analysis of the uninfected parents of two long-term non progressors displaying decreased Fas-function showed that the mother of one of them and the father of the other displayed the same Fas function defect as their child. Mutant alleles of genes for CCR5, CCR2, and the CXCR4-ligand SDF1 are associated with delayed progression and CXCR4 seems to be involved in cell death induction in T cells. Therefore, we searched for CCR5-Δ32, SDF1-3', and CCR2-64I mutation in 12 long term non progressors and 1 non progressor to evaluate whether the mutant alleles correlated with defective function of Fas. This analysis did not detect any correlation between mutant alleles and resistance to Fas-induced cells death. Fusion of T cells from Fas-resistant individuals with a Fas-sensitive cell line gave rise to Fas-resistant hybrid lines not carrying HIV, which suggests that the resistant phenotype is due to molecules exerting a dominant negative effect on a normal Fas system. Analysis of the Fas signaling pathway in these lines showed decreased capacity of Fas to activate caspase 8, 9 and 3. These data suggest that Fas-resistance in long-term non progressors is due to inherited alterations of the Fas signaling pathway and may be a novel factor in delayed progression.

GRANT N°. 40C.35

HIV REPLICATION IN NON-LYMPHOID CELLS: CHARACTERIZATION OF HIV PRODUCED BY CELLS OF DIFFERENT HISTOLOGICAL ORIGIN EITHER EX VIVO OR IN VITRO. INTERACTIONS WITH OTHER VIRUSES

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HIV latency and persistence in body tissues is an issue of increasing relevance, due to the "rebound" effect seen in patients at the end of treatments. It is therefore important to identify those cells that, in particular organs and tissues, may be "sanctuaries", where HIV may persist and adapt, leading to selection of variants that may emerge and re-colonise the patient. In this respect, the role of epithelial and connectival cells is poorly studied, if any. We addressed this issue i) by studying HIV replication in a variety of cells from solid tissues (fibroblasts, epithelial, endothelial, amniotic cells, etc.), and establishing also persistent infections; ii) by characterising the HIV emerging from different cells, both ex vivo and in vitro; iii) by studying the interactions of HIV with other co-infecting agents.

In persistently infected Hela-T4 epithelial cells, we selected from the T-tropic HIV-1_{P1} strain an "epithelial" variant (HIV-E). When compared to the original T-cell-derived virus (HIV-T) for efficiency of infection, the HIV-E variant shows a selective advantage for replication in Hela-T4 cells as well as in diploid PEU fibroblasts, as evaluated by HIV RNA accumulation within cells, and detection of virus yields by p24 and infectivity assays. When given at the same multiplicity of infection, both viruses bind preferentially to cells of the same type of the parental one. In epithelial cells, provided that similar amounts of virus are bound, HIV-E yields are more than 3 Log_{10} higher than those of HIV-T. Sequence analysis of HIV-E and HIV-T in the env and LTR regions of the genome revealed that the two variants present identical env V3 loops, containing the nucleotides associated to the syncytium-inducing phenotype. PCR products from the LTR region of HIV-E migrated in gel more slowly than those from HIV-T. Sequencing of the amplified products revealed that HIV-E presents a TAR duplication in the LTR region. Conformational studies indicated that the duplication modifies TAR stem-loop structure of HIV-E, that is present in a two-horned conformation resembling that of HIV-2 TAR. The two tat-binding bulges are presumably in a functional form, since exogenous tat is able to stimulate HIV-E yields and with higher efficiency than for HIV-T, since it occurs with lower tat concentrations and at earlier times.

Since HIV can be transmitted also through breast milk, we analysed HIV infection of adherent cells of human milk (15-25% of milk cells are fibroblasts and epithelial cells). Kept in culture, these cells show epithelial morphology, are >90% cytokeratin-positive, and 42% and 50% positive for HGMF-1 and HGMF-2 (human milk globulin fat antigen 1 and 2), respectively. They are CCR5(+) and CXCR4(+), bind both M-tropic and T-tropic HIV, and produce gag-DNA and gag-RNA (with different efficiency as for HIV-T, HIV-E and HIV-BaL), therefore indicating that these cells may constitute a route of HIV transmission.

LTR is a HIV region sensitive to selective pressures exerted by different nuclear control systems, we analysed its sequence present in autoptical and in bioptical samples from different organs of HIV patients; bioptical samples were also kept in culture, and sub-cultured, when possible, in order to eliminate contaminating blood cells and to try virus isolation. Sequence analysis indicated that the prevalent strains present in different organs of the same individual

differ in LTR sequence; in particular we observed point mutations and/or duplications in the Sp-1 binding site proximal to the NF-κB binding site.

Side studies in the Project were the interactions of HIV with other pre-existing or co-infecting agents. i) In collaboration with the O.U. Orsi, Rome, we analysed HIV interactions with BKV polyomavirus. While setting an epithelial cell system suitable for co-infections, we selected healthy donors presenting in their PMBC BKV DNA genes. Preliminary in vitro studies of BKV expression in presence of HIV, tat or HIV-induced cytokines indicated increased accumulation of T antigen RNA caused by HIV, tat and TNF-α. ii) Experiments of the same type were carried out in cells of individuals releasing the multiple sclerosis-associated retrovirus (MSRV), belonging to the HERV-W family of endogenous retroviruses: while TNF-α and IL-6 increased MRSV yields, as expected, HIV infection or treatment with tat interfered with MSRV release, at variance with the wide-spread activation by HIV of concomitant pathogens.

O.U Dolei: HIV polytropism: study of the factors that regulate infection and persistence in cells from solid tissues and effects of virus phenotype and tropism. Induction of other viruses

N^{o} dell'Accordo di Collaborazione: 40C.36

ROLE OF THE VIRAL NEF PROTEIN, DERIVED FROM PEDIATRIC RAPID PROGRESSOR AND NON PROGRESSOR PATIENTS, IN THE REGULATION OF CELLULAR RECEPTORS OF THE IMMUNE SYSTEM

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Several studies indicate that the Nef protein of HIV/SIV plays an important role in disease progression. However, gross defects or sequence abnormalities in nef alleles are found in some but not all long term non progressor idividuals (LTNP). Besides, the contribution to viral pathogenicity in vivo of each activity of the Nef protein (CD4 and MHC-I down-regulation, enhancement of infectivity, modulation of signalling pathways, induction of lymphocyte chemotaxis and activation) is still unclear. In our laboratory, we want to study the correlation between specific Nef activities on membrane receptor expression/function and disease progression. For this study, we are performing a functional screening of nef alleles isolated at various times from rapid progressor (RP, who became C3 during their first year of life) and non progressor (NP, N1 and A1 despite 12-16 years of HIV-1 infection) pediatric patients. We cloned into a retroviral vector (Pinco, which co-express the GFP-green fluorescence protein) 110 nef allelic variants derived from NP and 95 from RP (based on length size and restriction analysis, six rapresentative Pinco-nef recombinant clones have been selected for each PBMC sample). All nef alleles are being sequenced and their biological activities analyzed in cells, such as CD4+ Hela, A2-RMAS (a mouse T cell lymphoma expressing human A2 class I molecules) and primary CD4+ T lymphocytes, infected by the PINCO-derived recombinant retroviruses. So far, we tested all nef alleles isolated from RP and NP patients for their ability to express a Nef protein and to induce a decrease in CD4 and MHC-I cell surface expression. Our data indicate that: i) nef deletions were found in 1 out of 4 RP patients and in 4 out of 7 NP; ii) the frequency of deleted nef alleles was high (80% instead of 15-20%) only in 1 NP patient; iii) the vast majority of intact nef genes from both RP and NP express a full-length Nef protein; iv) the majority of full-length Nef proteins derived from both RP and NP is active on CD4 and MHC-I, although their relative ability may vary notably; v) sequence analysis of functionally characterized nef alleles suggest a role in Nef protein activity for some aminoacidic residues which were not previously identified.

N°. 40C.37

$CD8^+$ T LYMPHOCYTES FROM HIV-EXPOSED-UNINFECTED INDIVIDUALS SUPPRESS THE GROWTH OF HIV-1 STRAINS EXHIBITING DIFFERENT CORECEPTOR USAGE.

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Some individuals remain uninfected despite repeated exposure to HIV-1 (exposed-uninfected [EU]). Genetic polymorphisms of chemokines and chemokine receptors have been associated with natural resistance to HIV-1 in a proportion of these subjects, but in the majority the mechanism of resistance remains unknown. We studied non-lytic $CD8^+$ T-cell antiviral activity in a cohort of 16 sexually-exposed uninfected partners of HIV-seropositive individuals, in parallel with two groups of controls, including 13 uninfected-unexposed subjects and 24 HIV-seropositive asymptomatic subjects. The HIV-suppressive activity was analyzed against three viral strains displaying different coreceptor usage: one CCR5-tropic (R5), one CXCR4-tropic (X4) and one dual-tropic (R5X4). This inhibitory activity was documented in the absence of exogenous stimuli in vitro, thus reflecting a status of T-cell activation and functional competence in vivo. $CD8^+$ T cells from all the EU subjects analyzed, except one, inhibited the growth of all HIV-1 strains, regardless of their coreceptor usage, with a potency similar to that of asymptomatic HIV-infected patients. By contrast, uninfected unexposed controls showed a significantly lower suppressive activity, particularly against the R5 isolate. Transwell culture experiments showed that the CD8-mediated viral suppression was partially due to soluble factors, although it was more efficient when cell-to-cell contact was allowed. These findings demonstrate that $CD8^+$ T-cells from EU subjects are endowed of strong non-lytic HIV suppressive activity, supporting the notion that $CD8^+$ T cells are implicated in the mechanisms of natural resistance to HIV-1 infection.

N^o. Accordo di Collaborazione 40C.39

CHARACTERIZATION OF A MHC CLASS II X-BOX BINDING PROTEIN ENHANCING THE TAT-INDUCED TRANSCRIPTION DIRECTED BY THE HIV-1 LONG TERMINAL REPEAT

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The X-box element present within the promoter region of genes belonging to the Major Histocompatibility Complex (MHC) plays a pivotal role in gene expression, since it contains the binding sites for several transcription factors.

We have analyzed a randomly selected compilation of viral genomes for the presence of elements homologous to the X-box of the HLA-DRA gene. We found that HIV-1 shows the highest frequency of X-like box elements per 1,000 bases of genome. Within the HIV-1 genome we found an X-like motif in the TAR region of the HIV-1 LTR, a regulative region playing a pivotal role in the Tat-induced HIV-1 transcription (1). Decoy of nuclear proteins binding to this element, namely XMAS for X-like Motif Activator Sequence, by transfection of copies of this sequence into cells carrying an integrated LTR-CAT construct, suggests that this element binds to nuclear proteins that enhance Tat-induced transcription. Furthermore, the XMAS sequence of HIV-1 also inhibited the expression of the human HLA-DRA gene in Colo38 melanoma cells stimulated by γ-IFN, and double stranded DNA mimicking the human HLA X-box sequence inhibited both Tat-induced viral transcription and HLA-DRA gene expression (1). These data suggested the existence of a concerted regulation of viral and human genes, and X-box binding transcription factors may play a pivotal role in this context.

We have characterized two proteins, one binding to the XMAS motif and the other to the flanking regions of XMAS. EMSAs performed on crude nuclear extracts or enriched fractions suggest that similar proteins bind to XMAS of HIV-1 and X-box of HLA-DRA gene. Furthermore, UV cross-linking assay suggests that one protein, showing a molecular weight of 47 kDa and termed FAX (Factor Associated to XMAS) 1, binds to the XMAS of HIV-1 (1). The other protein exhibits a molecular weight of 56 kDa and was termed FAX 2. In a decoy ex vivo experiment, it was found that the decoy molecule requires the simultaneous presence of the two sequences recognizing both proteins in order to inhibit Tat-induced HIV-1 LTR-driven transcription.

Taken together, our data suggest that XMAS and nearest sequences modulate Tat-induced HIV-1 transcription by binding to the X-box binding protein FAX-1 and FAX-2. The sequence-homology between XMAS and X-box leads to (a) binding of a common protein, namely FAX-1, and (b) similar functional role on gene expression. To our knowledge, this is the first report showing that transcription factors binding to the X-box of the MHC class II genes enhance the transcription of HIV-1.

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Programma Nazionale di Ricerca sull'AIDS-1999. Accordo di Collaborazione N. 40C.40

DEVELOPMENT OF DECOY MOLECULES AGAIST NF-KB TRANSCRIPTION FACTORS BASED ON PEPTIDE NUCLEIC ACIDS (PNAS) AND PNA-DNA CHIMERAS

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In vitro transfection of cis elements decoy against nuclear factors leads to alteration of gene expression. For instance, decoy molecules against NF-kB inhibit the expression of NF-kB regulated genes (MHC genes, IL2 receptor α, Igk, IL6, δ opioid receptor, preprogalanin, adhesion molecule-1). More recently, dumbell DNA elements decoy against NF-kB were demonstrated to be active in inhibiting ex vivo transcription driven by the LTR of HIV-1.

We have proposed peptide nucleic acids (PNAs) as alternative reagents in experiments aimed at the control of gene expression involving the decoy approach (1). In PNAs, the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units. PNAs hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices and are resistant to both nucleases and proteases. We designed and tested PNA and PNA-DNA chimeras mimicking the HIV-1 LTR NF-kB binding sites. Molecular modeling was employed for the design of the NF-kB PNA-DNA-PNA (PDP) chimeras (2); prediction of molecular interactions between a double-stranded PNA-DNA-PNA chimera and nuclear proteins belonging to the NF-kB family was performed by energy minimization and molecular dynamics simulations (2); interactions between NF-kB DNA/DNA, DNA/PNA, PNA/PNA, PDP/PDP, DNA/PDP double stranded molecules was studied by electrophoretic mobility shift assay (1), Surface Plasmon Resonance (SPR) based Biospecific Interaction Analysis (BIA) (3), competitive DNase I footprinting (1). Effects of these newly designed decoy molecules were studied by in vitro transcription.

By SPR-based BIA we have found that NF-kB p52 is able to bind to both NF-kB DNA/DNA and DNA/PNA hybrid mimiking the NF-kB target sites present in the HIV-1 LTR. Low binding of NF-kB p52 to PNA/PNA hybrids was on the contrary found. Accordingly, gel shift data demonstrated that the DNA/PNA hybrid molecules are capable to interfere with the binding of NF-kB to the HIV-1 LTR. Furthermore, we analysed the effects of PNA-DNA-PNA chimeras (PDP) mimicking the NF-kB binding sites in interacting with both purified NF-kB p52 and p50, as well as nuclear factors from B-lymphoid cells. We found that both the hybrids between DNA and DNA-PNA chimeras and double stranded PDP/PDP chimeras mimicking the HIV-1 NF-kB binding sites are able to suppress the molecular interactions between HIV-1 LTR and p50, p52 and nuclear factors from B-lymphoid cells. Therefore, the results obtained conclusively demonstrate that the designed NF-kB DNA-PNA chimeras could be proposed as powerful decoy molecules. In addition, we demonstarted that PNA/DNA hybrids (2) and double stranded PNA-DNA chimerase are efficiently vehiculated to taget cells by liposomes, microspheres and nanospheres (manuscript in preparation).

These results are expected to have practical implications. The last findings that DNA-PNA chimeras stably interact with NF-kB transcription factors, allows to propose these molecules for the development of potential agents for a decoy approach in gene therapy of AIDS.

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Programma Nazionale di Ricerca sull'AIDS-1999. Accordo di collaborazione N.40C.40

CELLULAR INTERACTIONS AND INTERCELLULAR TRAFFICKING OF THE HIV-1 TAT PROTEIN

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Our laboratory is interested in elucidating the kinetics of HIV-1 gene expression and understanding some of the molecular properties of the regulatory HIV proteins. After integration, the HIV-1 LTR promoter is present in a nucleosome-bound conformation and it is transcriptionally silent in the absence of stimulation. Activation of HIV-1 gene expression is concomitant with an acetylation-dependent rearrangement of the nucleosome positioned on the viral transcription start site, as determined by quantitative chromatin immunoprecipitation with anti-acetylated histone antibodies. Thus, similar to most cellular genes, histone acetylation is a hallmark of transcriptional activation also for the HIV provirus. This enzymatic activity results from the function of histone-specific nuclear acetyltransferase (HAT) enzymes. We observed that the Tat protein of HIV-1 specifically associate with one of these enzymes, the transcriptional co-activator p300/CBP. Tat and p300 are now found to be part of a multiprotein complex, including other transcriptional co-factors which are required for the expression of other cellular and viral genes.

To obtain further insights into the molecular properties of the HIV-1 Tat protein, we are collaborating with the Physics Laboratory of Scuola Normale Superiore in Pisa, directed by F. Beltram, to exploit high sensitivity optical nanotechnologies for the study of intercellular Tat trafficking and of its intracellular interactions. These studies are facilitated by the development of a novel mutated derivative of the GFP protein displaying the convenient property of escaping permanent photobleaching upon proper laser illumination. We are exploiting high resolution fluorescence resonance energy transfer (FRET) to visualize and quantitatively analyze the interaction between Tat and some of its cellular partners. One of these proteins is human Cyclin T1, a component of the P-TEFb kinase complex. Current understanding suggests that Tat recruits P-TEFb to the viral long terminal repeat to promote transcriptional elongation of viral RNAs. FRET was mapped in different cellular compartments after transfection with proteins tagged by optically matched variants of the green fluorescent protein. Strong energy transfer was observed between the two proteins both in the cytoplasm and in determined nuclear localizations. Cyclin T1 was found to reside in specific subnuclear compartments which are coincident with promyelocytic leukemia (PML) nuclear bodies. The results obtained are consistent with a model by which Tat recruits Cyclin T1 outside of the nuclear compartments where the protein resides to promote transcriptional activation of HIV-1.

Finally, we are also continuing our work on the molecular mechanisms of intercellular Tat trafficking. Recombinant Tat has the unusual capacity of being internalized by cells when present in the extracellular milieu, a property dependent on its interaction with cell surface heparan sulfate proteoglycans. These molecules also mediate physiological internalization of Tat-GFP released from neighboring producing cells. The ubiquitous distribution of HS proteoglycans permits the intracellular delivery of heterologous proteins fused with Tat to different mammalian cell types for pharmacological applications.

Reserch Project N. 40C.41

MOLECULAR AND PHYLOGENETIC CHARACTERIZATION OF *env* (C2-V3 REGION) AND *gag* (p17) OF HIV-1 VARIANTS IDENTIFIED IN ITALIAN PRIMARY HIV-1 INFECTIONS (PHIs).

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The characterization of the genetic evolution and distribution of HIV-1 subtypes is necessary to monitor the introduction/diffusion of different HIV-1 clades within a geographical area and, consequently, has strong implications for the development of potential candidate vaccines.

In this framework, a collaborative study has been set up to evaluate the clade distribution in the PHIs occurring in Lombardia, the Italian region with the highest incidence of HIV-1 infections. The subjects have been enrolled in this study on the basis of two major inclusion parameters: sexual transmission and recent seroconversion (< 1 year).

The hyper-variable C2-V3 region of the *env* gene as well as the more conserved 5' region of the *gag* p17 sequence have been amplified by Polymerase Chain Reaction (PCR) in peripheral blood mononuclear cells (PBMC) from 16 subjects and characterized by direct DNA sequence analysis.

The results show an average divergence of 16,45% in the C2-V3 region and 9,9% in the p17 region. Moreover, in 2/3 of the analyzed samples, there is a parallel genetic evolution with an overall constant equilibrium ratio between the two genes characterized by a significant linear correlation (r=0,96) between the two divergence values. In addition, phylogenetic analyses show that all 16 samples from the PHI-cohort cluster with reference standards of the clade B for both *env* and *gag* sequences. No homogeneous subclusters have been identified, suggesting the absence of a single founder, and the more remarkable inter-dispersed branch distribution of *env* sequences confirms the higher nucleotide divergence within this region.
The overall results obtained in this cohort would confirm that viral regions under pressure of the immune system undergo to a higher nucleotide variability and suggest that, in the Italian region with the highest incidence of HIV-1 infections, the B clade is still prevalently associated with acute PHIs occurring through sexual transmission.

ACCORDO DI COLLABORAZIONE N. 40C.42

INDUCTION OF HUMORAL AND CELLULAR RESPONSE BY AN ANTI-HIV-1 VACCINE BASED ON VIRUS-LIKE PARTICLES (VLPs), EXPRESSING A gp120 MOLECULE FROM AN UGANDAN HIV-1 VARIANT OF CLADE A.

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The vaccine strategy developed in our Laboratory is based on HIV-1 gag-structured Virus-Like Particles (VLPs), expressing a gp120 from an Ugandan HIV-1 isolate of the clade A (HIV-VLP_A), which shows a 90% homology in the C2-V3 region versus *intra*-clade isolates (Buonaguro L. *et al.*, 1995 and 1998).Virus proteins Nef and Pol, mutated in the *protease* and *reverse transcriptase* ORFs, have been included to broaden the spectrum of presented viral antigens (preventive activity) and to test the possibility of modulating the evolution of an established infection (therapeutic activity).

The HIV-VLP_As have been produced in a baculovirus expression system in the Sf9 insect cell line, and the quantitative/qualitative expression of viral proteins has been verified by Western Blot (WB) analysis. The HIV-VLP_As show the appropriate density (1,14-1,18 g/ml) after sedimentation through a continuous sucrose gradient and the morphology has been confirmed by standard transmission EM analysis (Buonaguro L. *et al.*, 2001).

In vivo experiments performed in Balb/c mice have shown the high immunogenicity of VLPs with induction of both humoral and cellular immunity, without adjuvants. Furthermore, immune sera show >50% neutralization activity against both the autologous field isolate and the heterologous T-cell-adapted B-clade HIV-1_{IIIB} viral strain (Buonaguro, L. *et al*., submitted).

The development of a HIV-1 vaccine, specific for the clade A, is driven by epidemiological evidences of its high worldwide distribution. It represents, in fact, the 25% of all identified HIV-1 isolates and is the prevalent clade in Sub-Saharan Countries, where 70% of the global HIV-1 infections occurs. Furthermore, the migration processes lead to develop vaccine strategies based on cocktails of different antigenic variants as well as diverse presentation systems (recombinant proteins, DNA vaccines, canarypox, VLPs), in order to broaden the protective and/or therapeutic vaccine efficacy. As consequence, a vaccine specific for clade A will be necessary, in the next future, also for Western countries where it is already present in well characterized populations (such as Bielorussia).

ACCORDO DI COLLABORAZIONE N. 40C.42

MOLECULAR ANALYSIS AND PROGNOSTIC VALUE OF THYMOPOIETIC POTENTIAL OF HIV$^+$ SUBJECTS IN CHIMERIC FETAL THYMUS ORGAN CULTURES

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Once viral load is brought under control by pharmacological treatment, reconstitution of the immune response is critical for the survival of HIV-infected individuals. The attainment of such a reconstitution could depend on individual thymic function. Indeed, some recent data suggest that the adult thymus may play an important role in the renewal of the peripheral T cell pool. Therefore, identification of molecules able to increase thymic function could be relevant for implementing immune reconstitution.
We used chimeric fetal thymus organ cultures (FTOC) to study the regeneration potential of peripheral hematopoietic precursors of HIV$^+$ patients enrolled in a phase III randomized study aimed at evaluating the effects of IL2 additon to HAART in HIV$^+$ subjects. All the patients enrolled had CD4$^+$ lymphocyte counts comprised beetwen 50-300x10^6/l and plasma HIV-RNA levels of <10.000 copies per milliliter. We analyzed the T cell regeneration potential of 9 healthy individuals, 6 HAART treated subjects and 7 HAART + rIL-2 s.c. treated patients (9 MUI/day for 5 d in 8-week intervals). After 6 months of therapy 60% of the participants showed an increase in T cell development capacity in FTOC both in the HAART and in the HAART + IL-2 treated arms. This increase was accompanied by an increase in the number of CD4$^+$ T cells in the peripheral blood of the individual. Interestingly, while CD4/CD8 ratio of T cells generated in FTOC performed with healthy individuals precursors was 1.7 ± 0.6, in HIV$^+$ subjects this value was less than 1 also in patients negative for HIV-RNA. Experiments are in progress to evaluate if the presence of pro-viral DNA in viral reservoirs (i.e. macrophages, CD34$^+$ cells) could be responsible for the observed phenomenon in FTOC. In addition, we analyse the effect of rIL-7 on the differentiation of T cell precursors in FTOC. We observed an 1.6 ± 0.7 fold increase in the maturation of cells in the healthy group (n=6) and 2.9 ± 2.2 fold increase in the HAART treated group (n=5).

Accordo di collaborazione N° 40C.43

MACROPHAGES PROTECTION AGAINST HUMAN IMMUNODEFICIENCY VIRUS BY RED BLOOD CELL-MEDIATED DELIVERY OF A NEW HETERODINUCLEOTIDE OF AZIDOTHYMIDINE AND 9-(R)-2-(PHOSPHONO-METHOXYPROPYL)ADENINE.

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Purpose. Monocyte-derived macrophages (M/M) are the principal reservoir of virus during HIV-1 infection. Hence, therapeutic strategies must consider the importance of inhibition of HIV replication in M/M. The aim of the present research was the selective delivery in macrophages of the new heterodinucleotide AZTpPMPA, consisting of two anti-HIV drugs (AZT and (R)PMPA) bound together by a phosphate bridge.

Methods. AZTpPMPA was synthesized and encapsulated into autologous erythrocytes (RBCs) modified to increase their recognition and phagocytosis by human macrophages.

Results. When AZTpPMPA was incapsulated into RBCs, it proved to be stable enough for the use as a drug delivery system. Addition of AZTpPMPA-loaded erythrocytes to human macrophages provides an effective in vitro protection against HIV-1 replication.

Conclusions. The experimental data prove that the heterodinucleotide AZTpPMPA, once encapsulated into autologous erythrocytes modified to increase their recognition and phagocytosis, is able to protect macrophages from "de novo" HIV-1 infection and to act as an efficient antiviral prodrug.

Grant No. 40C/44

PHASE I/II OPEN STUDY ON SAFETY OF ANTI-TAT IMMUNIZATION WITH TAT TOXOID, AN HIV-1 REGULATORY PROTEIN, AND COMPARISON OF THE IMMUNOGENICITY OF DIFFERENT DOSES IN ASYMPTOMATIC HIV-INFECTED PATIENTS.

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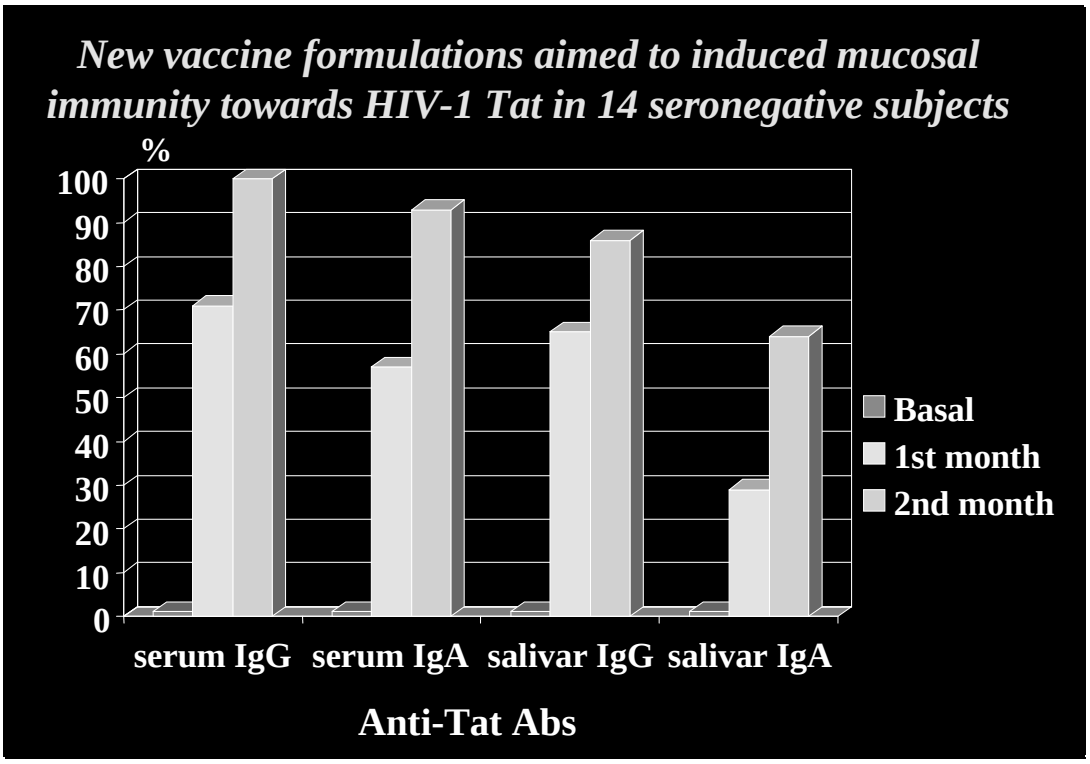
The HIV-1 early regulatory protein called Tat is a true toxin, known to be able to transactivate proviral DNA and to induce immune suppression of non-infected T cells. Since 1997 4 clinical trials in humans have been carried out in order to investigate safety and immunogenicity of an anti-AIDS vaccine using Tat toxoid, a detoxified recombinant Tat protein.

1. a phase I study in 5 HIV-1-negative volunteers showed that Tat toxoid vaccine was safe and immunogenic: it induced high-titre of specific anti-Tat antibodies with neutralizing activity after 1 to 3 weekly or monthly injections. After 3 years, HIV-1-negative vaccinees are still showing high anti-Tat antibody titers and cell mediated immunity.
2. In a phase I/II study all of 14 HIV-1+ patients, whether on HAART or not, responded to immunisation. All subjects showed a significant raise of anti-Tat Ab levels ranging from 2- to 8 fold compared with pre-immunization values. Four of 8 tested patients showed a positive DTH response to Tat and a consistent increase of T cell proliferative response in vitro. CD4+ cell counts increased significantly after immunization ($p<0.001$): the mean difference from pre-immunization values was +60 CD4+ cells/mm^3 (95% C.I.: +34 to +85 cells/mm^3). Moreover, HIV-plasma viremia (HIV-1 RNA PCR) showed a trend towards a decrease ($p=0.07$): the mean difference from pre-immunization values was -6,300 copies/ml (95% C.I.: -13,000 to +600 copies/ml). After 3 years these patients showed a stabilisation of clinical, virological and immunological parameters without any toxicity as compared to open comparison patients.
3. A further phase II study in 30 HIV-1+ patients was designed to evaluate doses and timing in order to optimise the vaccination regimen. Patients were randomised to receive Tat toxoid i.m. 100 mcg monthly, the same dose weekly or 50 mcg monthly. This trial tested the same Tat-toxoid vaccine preparation adjuvanted with Seppic ISA 51 mineral oil (Incomplete Freund's Adjuvant) used in previous trials. The number of priming injections received by each patient ranged from 1 to 7, depending on the vaccine response of the patients and availability of vaccine preparation. Overall 63% of vaccinees showed a specific antibody response, defined as an increase of anti-HIV-1 Tat Ab levels of 2-fold or more from pre-immunization values, after a median of 3 injections, mirroring our previous reports. Vaccine regimens were absolutely comparable as to the response rates. However, if we take into account the time from the first priming injections, the vaccine regimen with Tat toxoid 100 mcg given weekly was indisputably the faster in inducing an antibody response.

4. A new clinical trial have been initiated in order to evaluate safety and immunogenicity of an oral and/or intranasal preparation of Tat toxoid, a detoxified recombinant Tat protein, aimed to induced specific anti-Tat mucosal IgA antibodies. Fourteen healthy HIV-1 seronegative subjects volunteered to participate to this study. All subjects have been given one priming I.M. injection, followed 7 days after by intranasal drops (4 subjects), oral capsules (8

subjects), intranasal drops and oral capsules (2 subjects). The oral and intranasal vaccine preparation consisted of the same detoxified recombinant Tat protein used in previous trials, encapsulated in nanoparticles of PLGA (poly-lactide-coglicolide). Study subjects were assayed for the presence of specific anti-Tat IgG/IgA anti-Tat antibodies in serum and saliva in serum and saliva, together with routine biochemistry for safety evaluation. Intramuscular, oral and intranasal vaccine preparations were proved to be safe and well tolerated. Immunogenicity data after 2 months from priming are available up to date, as shown in the graph.



In conclusion, these vaccine trials in humans showed that Tat toxoid vaccine is long-term safe and immunogenic. To rapidly induce a specific anti-Tat antibody response HIV-1 infected patients require at least 5 consecutive i.m. injections at one-week intervals, whereas in HIV-1 negative volunteers a systemic anti-Tat response can be obtained after 1 to 3 i.m. injections. Preliminary data of oral and intranasal anti-Tat vaccine administration in HIV-1 seronegative humans showed that these preparations seemed safe and immunogenic, being able to induce secretory IgA antibodies.

N°. dell'Accordo di Collaborazione: 40.B.49 (II Programma Nazionale - 1998)
40C.45 (III programma Nazionale - 1999)

DEVELOPMENT OF NEW STRATEGIES FOR THE INDUCTION OF CELL-MEDIATED IMMUNE RESPONSES AGAINST ANTIGENIC DETERMINANTS OF HIV-1 REVERSE TRASCRIPTASE

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In this project, we aimed at further developing our observations concerning the ability of bacteriophage particle to vehiculate, efficiently and safely, a HIV-1-derived antigenic determinant to the MHC class I processing and presentation compartment. Our work concerns the determination of the immunological correlates in vitro, using human PBL and in vivo, in transgenic animals, of the inoculation of phage particles expressing CTL and T helper determinants. This information may be useful to set the
conditions for vaccination trials using these constructs.

N°. dell'Accordo di Collaborazione.: 40C.46

DABOS AS VIRUCIDAL AGENTS TO PREVENT SEXUAL TRANSMISSION OF HIV

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Worldwide, the heterosexual route is the prevalent mode of transmission of AIDS. The absence of an HIV vaccine, the lack of access to effective anti-HIV therapy in Third World countries and the persistency of high viral loads in endocervical fluid and semen of successfully HAART-treated patients, has placed new emphasis on the development of topical agents (microbicides) capable of reducing sexual transmission of HIV. At present, all commercially available products have detergent-type effect. Therefore, they disrupt viral envelops as well as the membranes of epithelial cells and microorganisms of the vaginal flora, causing increased risk of irritation/ulceration and mucosal infections. Hence the need for new compounds with no detergent-type action. Among the various agents proposed are non-nucleoside reverse transcriptase inhibitors (NNRTIs). These antiretroviral agents are thought to be "ideal" topical microbicides for the following reasons: i) act with high potency against a broad spectrum of quasispecies of HIV-1; ii) act directly on their target without first needing to be metabolized. iii) are non-toxic to cells. Following the report of the virucidal activity of the NNRTI UC781, we comparatively evaluated the capability of DABOs and of a variety of other NNRTIs to irreversibly stop the HIV infection in vitro. Unlike potent NNRTIs such as nevirapine and efavirenz, DABOs clear the HIV infection in MT4 cells acutely infected with up to 2500 infectious virions/cell. Pretretment with DABO 1220 renders cells refractory to subsequent HIV infection in the absence of extracellular drug, thus suggesting that it accumulates inside the cell. DABO 1220 is a slow-binding/tight-binding inhibitor of RT, from which it dissociates with a half life of 100 minutes. DABO 1220 lacks inhibitory activity against vaginal commensals, such as Candida spp. and Lactobacillus spp. Thus, DABO 1220 may have considerable promise as a virucidal agent to prevent mucosal HIV transmission.

Accordo di Collaborazione n° 40C.47

ARYL-CARBOTHIOAMIDES WITH ANTI-HIV-1 ACTIVITY

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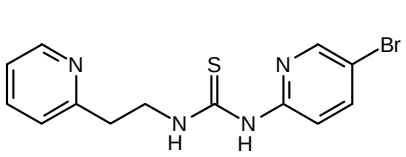
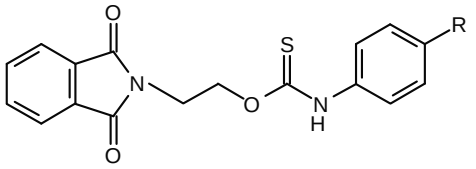
PETT derivatives are some of the most potent and selective non-nucleoside reverse transcriptase inhibitors (NNRTIs). Trovirdine is the lead compound of this class. As part of our program aimed at developing new NNRTIs endowed with activity against clinically relevant resistant mutants, we synthesized a series of trovirdine analogues characterized by aryl-carbothioamide groups.



TROVIRDINE



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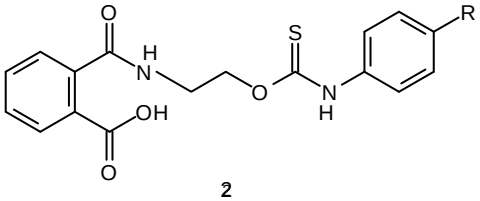

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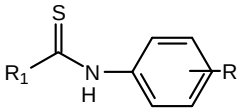

3

R = halogen, NO2 R1= heteroaryl

SAR studies suggest that the presence of an electronwithdrawing substituent in the para position of the phenyl ring of compounds 1, 2 and 3 is required for anti-activity (EC_{50} range = 0.2-0.006 μM). However, the highest potencies correlate with the presence of the phtalimidic group (1) rather than with that of substituents 2 and 3. Data obtained against HIV-1 strains carrying the major NNRTI mutations indicate that title compounds inhibit Y181C mutants but do not inhibit neither K103R nor K103N/Y181C mutants. Structural changes are in progress to optimize the antiviral activity against resistant mutants.

Accordo di Collaborazione n° 40C.47

VALIDATION OF A 40^{MER} OLIGO REPRODUCING THE HIV-1 U3 AND U5 3' ENDS AS SUBSTRATE FOR THE IN VITRO INTEGRASE REACTION.

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Retrovirus multiplication requires integration of a DNA copy of the viral RNA genome into a chromosome of the host cell. This essential step in the retrovirus growth cycle is accomplished by the viral-coded integrase (IN). In order to understand the mechanisms of the HIV-1 IN reaction and to identify potential inhibitors of this enzyme, several cell-free assays have been proposed which use recombinant enzyme (rIN) and a variety of synthetic substrates. Since none of these assays reproduces adequately the in vivo reaction, inhibitors identified in large screenings using these assays rarely prove active in cell based assays or targeted at the integration step.
Common substrate of all the above assays is a short dsDNA ($18\text{-}24^{mer}$) which reproduces the 3' end of the U5 region of the proviral DNA. In our opinion, its inadequacy can be ascribed to the fact that, in vivo, the HIV-1 IN processes the 3' ends of both U5 and U3 regions simultaneously. Therefore, with the aim of improving the in vitro reaction, we designed a longer oligonucleotide (40^{mer}) containing the 3' ends of both U5 and U3 regions.
The HIV-1 rIN proved able to perform its 3'-processing activity on both ends of the 40^{mer} oligo. Interestingly, the U5 end was processed with 2-fold efficiency with respect to the U3 end, and with the same efficiency of the 21^{mer} U5. The product of the above reaction was then used as substrate for the rIN strand-transfer reaction. The HIV-1 rIN was able to covalently link the latter to an acceptor substrate (another 40^{mer} oligo functioning as surrogate of the cellular DNA). Once again, the 40^{mer} oligo was used as substrate 4-fold more efficiently than the 21^{mer} oligo.
It remains to be seen whether assembly of rIN with the substrates mimicking the viral and cellular DNA will also be more efficient when the 40^{mer} oligo is used, and whether the assay will gain in predictivity for anti-IN activity of the screened drugs. Nevertheless, the intermediate objective of significantly improving the rIN assay sensitivity was reached.

Accordo di Collaborazione n° 40C.47

EFFECT OF β-L-2'-DEOXYNUCLEOSIDES AGAINST HIV AND HBV REPLICATION

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Co-infection with HBV and HIV is a relatively common problem since transmission risk factors are similar. As the survival of patients with HIV has improved, the importance of morbidity due to HBV infection has increased. Even though interaction between the two viruses does not appear to be direct, the effects of the HIV infection on the immune function allow higher rates of HBV replication.
Discovery and development of new antiviral agents with dual efficacy – i.e. active against both HIV and HBV - may represent an important therapeutic objective. The rationale is twofold: firstly, both viruses have a viral polymerase with reverse transcriptase activity. Secondly, drugs active against both viruses may help to decrease toxicity and to solve compliance problems.
Recently, the attention has been focused on a new generation of nucleoside analogues with the unnatural β-L configuration. The leading compounds of this class is β-L-2'-3'-dideoxy-3'-thiacytidine (3TC), which has been approved by FDA for the treatment of AIDS as Lamivudine and, actually, is the world's first oral antiviral treatment approved as Epivir for chronic HBV.
Data will be presented on the preclinical studies (anti-HBV and anti-HIV activity in vitro and animal models) carried out on three β-L-2'-deoxynucleosides: β-L-thymidine, β-L-2'-deoxycytidine and β-L-deoxyadenosine.

Accordo di collaborazione scientifica n. 40C.47

HIV-1 INTERACTIONS WITH HUMAN HEMATOPOIETIC PROGENITOR CELLS. RELEVANCE OF EXTRACELLULAR TAT PROTEIN IN THE PATHOGENESIS OF HIV-1 INFECTION.

La Placa,M[(a)] , Re,M.C.[(a)] , Gibellini,D. [(a)], Furlini,G.[(a)], Vignoli,M. [(a)], Ramazzotti,E. [(a)], Zauli,G.[(b)].

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The study of the interaction of HIV-1 antireceptor (gp120) with the membrane of human lymphomieloid precursor cells and/or human hematopoietic progenitor (CD34+) cells, has shown that gp120 interacts with the main (CXC-R4 and CCR5) HIV-1 coreceptors only in the presence of CD4 (1) and that a defined topological relationship between CD4 and co-receptors is very likely essential for a successful infection of susceptible cells (2). We have also shown that the response to HAART is not dramatically affected by the initial CD4-positive T-lymphocyte count (3).

The presence of extracellular Tat protein in serum-starved Jurkat cell cultures induces a significantly increased expression of a specific member of the bZIP-CREM family of transcription factors: namely, the inducible cAMP early repressor (ICER). The ability of ICER to attenuate the activation and degree of expression of several genes containing CRE domains, including IL-1 gene, may contribute to explaining the abnormalities in the T cell responses observed in the presence of extracellular Tat (4). We have also shown that Tat-protein protects CD4+ Jurkat lymphoblastoid cells from apoptosis mediated by TNF-related apoptosis-inducing ligand (TRAIL) (5) and the existence of a direct correlation between the presence of circulating antibodies against full-length Tat protein and/or some low molecular weight Tat-peptides, with low or undetectable viral load in HIV-1 seropositive patients (6).

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III Programma Nazionale di Ricerca sull'AIDS. Accordo di collaborazione N. 40C.48

ROLE OF P-TEFb KINASE COMPLEX IN TAT TRANS-ACTIVATION.

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A number of cellular factors that include P-TEFb, TIP30, the co-activators p300/CBP and PCAF have been found to interact with Tat and to potentate transcription from the HIV-1 LTR. The P-TEFb contains a catalytic subunit (Cdk9) which in association with a regulatory subunit (cyclin T) has the ability to phosphorylate the CTD of RNAPII. CyclinT1 appears to be a dedicated human-specific cofactor for Tat, as demonstrated by the inability of the mouse homologue to support Tat-mediated transcription. The current model for recruitment of P-TEFb to the LTR, predicts the formation of the Tat-P-TEFb complex which efficiently binds TAR, causing hyper-phosphorylation of the CTD, which enhances processivity of RNAPII to produce full-length mRNAs. Consequently, hyper-phosphorylation of the RNAPII CTD represents an important rate-limiting step in Tat-mediated activation.

In the last years we have carried out a number of comprehensive studies on the ability of the P-TEFb complex to regulate Tat activity in vivo. We demonstrated that Tat transactivation can be by-passed by direct targeting of Cdk9/CyclinT1 complex to HIV-1 LTR sequences (Majello et al. 1999). Moreover, we showed that the Cdk9-associated Cyclins T1 and T2 exert opposite effects on HIV-1 Tat activity (Napolitano et al. 1999), and more recently, that the transcriptional activity of P-TEFb complex in vivo requires the C-terminal domain of RNA polymerase (Napolitano et al. 2000). Finally, we have assessed the role of a novel described RNAPII CTD-phosphatase FCP1 on Tat activity. Because an increased level of CTD phosphorylation can also result from inhibition of CTD phosphatase, this class of nuclear phosphatases might represent potential Tat cellular cofactors. We found that FCP1 binds directly to Tat and over-expression of the FCP-1 phosphatase suppresses Tat-activated expression of the HIV-1 LTR. Thus, Tat has the ability to interact with proteins that can either phosphorylate or de-phosphorylate the CTD (Licciardo et al. 2000).

More recently, we have been studying the subcellular localization of the P-TEFb complex. We found that the state of cyclinT1/Cdk9 phosphorylation regulates the nuclear import of this complex, and reveal a novel control mechanism for the function of the P-TEFb complex (Napolitano et al. 2001, submitted). These findings will be instrumental for the proper designing of nuclear localized dominant-negative P-TEFb complex able to modulate Tat activity in infected cells.

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N°. Accordo di Collaborazione 40C.49

NOVEL STEALTH DELIVERY SYSTEMS FOR ODN DERIVATIVES TARGETING PROVIRAL DNA.

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Therapeutic application of antisense and antigene oligonucleotides (ODNs) is seriously limited by their poor biological stability and rapid in vivo elimination kinetics which require frequent administration of oligonucleotides for sustained efficacy. The enhancement of their bioavailability can be faced by their binding to specifically designed carrier systems like polymethylmethacrylate nanospheres, whose surface is functionalized with cationic groups able to reversibly bind the phosphate groups of the unmodified nucleic acids [1-6].
Three different series of polymeric nanospheres have been studied until now, differing in the lenghts and the amount of polyethylenglycol chains covalently bound to the surface, in order to avoid in vivo opsonisation and to mask the presence of the carrier to the RES. Surprisingly, increasing the length of the PEG chains, smaller particles are obtained with a narrow size distribution of the inner core and a widely expanded outer shell bearing both the positively charged groups and the PEG hydrophilic chains. Preliminary experiments indicate an higher loading ability of these nanospheres together with a slow and sustained release of model oligonucleotides in the presence of high ionic strength buffer.
Aim of the project is the delivery of a triplex-forming ODN (TFO), able to recognize and specifically bind the PPT segment of the integrated proviral HIV genome, covalently linked with daunorubicine derivatives [7], through the different types of the core-shell nanoparticles described. In vitro experiments in the presence of the first generation of nanospheres in HIV infected CEM cells did not show any relevant toxic effect. The inhibitory effect on the in vitro replication of viral DNA is currently under investigation.

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Accordo di Collaborazione ISS 40C.50

INDUCTION OF POTENT HIV-SPECIFIC T CELL RESTRICTED IMMUNITY BY GENETICALLY-MODIFIED DENDRITIC CELLS

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Background: HIV-specific T cell responses decline with time in patients treated with highly active antiretroviral therapies, and therapeutic immunization has the potential to boost these responses and control HIV.

Methods: We used a novel replication- and integration-defective HIV-1 vector to genetically modify dendritic cells (GMDC) in order to safely express most HIV antigens and induce T cell immunity. We introduced the vector into dendritic cells as a plasmid DNA using polyethylenimine as gene delivery system, thereby circumventing the problem of obtaining viral vector expression in the absence of integration.

Results: GMDC presented viral epitopes efficiently, secreted IL-12 and primed a high number of naïve T-cells capable of exerting vigorous HIV-specific cytotoxicity in vitro. In non-human primates, GMDC migrated into the draining lymph node at an unprecedented high rate and expressed the plasmid DNA. The animals presented a vigorous effector CTL response within 3 weeks, which later developed into a memory CTL response. Interestingly, antibodies did not accompany these CTL responses.

Conclusion: These data suggest that GMDC can raise a pure Th1 type of immune response in primates without any toxic side effects. Successful induction of HIV-specific cellular immunity by genetic immunization is expected to contribute to control virus replication in infected individuals.

N°. dell'Accordo di Collaborazione. 40.C.51

ANTI HUMAN CCR5 ANTIBODIES: A NEW APPROACH FOR HIV-1 PROTECTION

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Primary HIV infection is associated with CCR5-tropic HIV variants (R5-strains). Cells lacking surface CCR5 are resistant to the infection by R5-strains. Among exposed seronegatives (ESNs), two kinds of individuals lacking surface CCR5 have been described so far, i) homozygotes for a defective CCR5 gene and ii) individuals producing anti-CCR5 antibodies which cause antigen down-regulation and a CCR5-minus phenotype. A single conformed extracellular domain (corresponding to a 13-mer peptide) of CCR5 is recognized by ESN antibodies.

We have cloned and produced human recombinant monoclonal antibodies Fab fragments reactive to CCR5 starting from biological cellular clones of EBV transformed cell lines derived from ESN with anti CCR5 antibodies. Fifty bacterial clones producing antibodies to CCR5 were selected from a human combinatorial antibody library constructed in a phage-display vector by a panning procedure against a CCR5 peptide recognised by antibodies. Preliminary data shows that the human monoclonal antibodies recognise CCR5 and inhibit HIV viral entry.

The region recognised by anti CCR5 antibodies is identical in sequence to its mice homolog. In order to verify the possibility to induce and reproduce infection-protecting anti-CCR5 antibodies in individuals at high risk of HIV infection, we have generated chimeric immunogens containing the relevant CCR5 peptide in Flock House Virus (FHV) capsid protein. In a mouse model, the chimeric immunogens should induce a specific and vigorous humoral immune response to CCR5.

N°. dell'Accordo di Collaborazione 40C52

HIV-SUPPRESSIVE CHEMOKINES AND CHEMOKINE ANALOGUES

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The discovery in 1995 of the HIV-inhibitory activity of selected chemokines has opened new perspectives for the development of effective anti-HIV therapies and vaccines. We have conducted a detailed structure-function analysis of RANTES, the most effective anti-HIV chemokine that binds CCR5, by introducing specific mutations into the wild-type (WT) gene and by expressing the mutated proteins in a recombinant baculovirus system. Our results demonstrate that non-conservative mutations at the N-terminus induced a significant loss of function with regard both to the ability to activate chemokine receptor-mediated signaling and chemotaxis, and to the antiviral activity. However, other mutations resulted in an enhanced antiviral activity associated with a reduced ability to activate the CCR3 and CCR5 receptors. These biological features may be desirable for the development of new therapeutic molecules. Another critical functional region of RANTES was identified as the N-loop. Analysis by alanine scanning of the recombinant chemokine and by peptide scanning of the entire mature protein conclusively demonstrated that the N-loop region contains critical determinants for the antiviral activity of RANTES, suggesting that this region is directly involved in the primary interaction with the CCR5 receptor. Of importance, the data obtained with synthetic peptides documented that the antiviral and signaling activities of RANTES can be completely uncoupled. In contrast with the alterations of the N-terminus and N-loop, mutations introduced into the 40s' loop domain, which contains several positively charged residues, failed to disrupt the antiviral and receptor-activation functions. Altogether, these data indicate that both the N-terminal and the N-loop domains are essential for the functional activity of RANTES, although the former is dispensible for the antiviral activity. The formal dissociation of the antiviral and receptor-activating functions of RANTES may have relevance for potential therapeutic approaches aimed at blocking the entry of HIV into cells.

Accordo di Collaborazione n. 40C.53

MACROPHAGES AS A RESERVOIR FOR CONTINUOUS VIRAL INFECTION IN COMBINATION-ANTIRETROVIRAL THERAPY IN THE MOUSE.

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Different experiments were performed to evaluate the capacity of classical inhibitors of reverse transcriptase belonging to the class of nucleoside analogues to protect the macrophage compartment. In particular the efficacy of the administration of single drugs and their combination with a system able to selectively protect macrophages has been studied. The best combination of two nucleoside analogues selected were AZT and DDI. Reduced glutathione (GSH) was also administered encapsulated into autologous erythrocytes modified to favour macrophage targeting. LP-BM5 infected mice were treated with AZT and DDI for the duration of the experiments and with week-interval intraperitoneal GSH-loaded erythrocytes. After 9 weeks of infection the mice of all experimental groups were sacrified ad proviral DNA content was quantified in lymphoid organs, brain and in macrophage cells isolated from peritoneal cavity and from bone marrow. Preliminary results show that the combination of the nucleoside analogues tested are more effective in protecting lymphoid organs than single drugs from LP-BM5 infection. On the contrary they are not able to efficiently protect the macrophage compartment. The additional administration of GSH-loaded erythrocytes to AZT treatment produce 60% inhibition of proviral DNA content in brain of infected animals. After 9 weeks of treatment with AZT plus DDI, the proviral DNA content is undetectable in the brain of 25% of animals. If we add GSH-loaded erythrocytes to AZT and DDI, the proviral DNA content is undetectable in the brain of 80% of mice. Similar figures were obtained analyzing the proviral DNA content in the peritoneal macrophages.
These data suggest that macrophages are potential reservoirs of virus contributing to brain infection and persistence in the body cavities. To date most efforts have been concentrated on the elimination of CD4+T memory infected cells. The results presented suggest the importance of a system able to selectively protect and/or eliminate the infected macrophage compartments.

Accordo di collaborazione N° 40C.54

CD38: MECHANISMS OF HIV-1 INHIBITION

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CD38 is a surface molecule acting as an ectoenzyme involved in the regulation of intracytoplasmic Ca^{2+} concentration and is a surface receptor regulating activation and differentiation. The transfection of CD38 is reported to inhibit the replication of X4 and R5/X4 HIV-1 primary isolates in the MT-4 human lymphoid cell line and the attachment of X4 and R5 strains to murine lymphoid cells expressing human CD4 (SR.hCD4). A possible inference is that CD38 interferes somehow with gp120/CD4 binding. To test the specificity of this effect, we evaluated the interference of CD38 with gp120/CD4 binding in comparison to the effects of other molecules capable of lateral association with CD4 might inhibit. Therefore, human CD59 or CD95 (both laterally associated with CD4), or human CD31 (not laterally associated with CD4) were transfected into the SR.hCD4 clone. The binding of gp120 was evaluated by immunofluorescence techniques. Results confirmed that CD38, but not CD59, CD95 or CD31, affected binding of gp120 to human CD4 (Fig. 3 A). This CD38-related inhibition was confirmed also using a $CD38^+$ transfectant derived from the human $CD38^-$ MT-4 cell line. Inside-out signaling does not apparently intervene in the CD38-mediated down-modulation of gp120 binding to CD4, in that the down-modulation was observed at 4°C, at which signal transduction is minimized.

Then, we evaluated whether the inhibitory effects observed might be due to a discrete amino-acidic sequence of CD38. Given that 1) the sequence GPGTTK of CD38 (amino acids 52-57) is reminiscent of the GPG/TTK crest of the V3 loop of HIV-1 MN gp120, and that 2) V3 peptides have been shown to favor gp120/CD4 binding, a likely hypothesis is that the V3-like portion of CD38 might counteract the effects of the V3 loop. The results obtained indicate that a peptide containing amino acids 51-74 of CD38 significantly inhibits the replication of several laboratory strains and primary isolates from different HIV-1 subtypes, implying that it affects a conserved site on gp120. Instead, a peptide containing amino acids 52-57 of CD38 stimulated the replication of HIV-1 IIIB similarly to a V3-derived peptide. The involvement of these domains in the anti-HIV-1 effects of the native CD38 glycoprotein is under analysis by using truncated forms of the molecule. These findings, on one side, delineate a participation of CD38 in the pathophysiology of HIV-1 infection; on the other side, the functional properties of the 51-74 peptide represent a potential lead for the development of inhibitors of HIV-1 entry.

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HIV SPECIFIC CD4 RESPONSES AND VACCINE DEVELOPMENT

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The development of an effective HIV vaccine is highly desirable as a prophylactic and therapeutic tool. Unfortunately the protective mechanisms are not well established, HIV is a hypervariable pathogen and antigenicity of HIV proteins must be optimized. Since antibodies and CTL are likely to be beneficial, and their induction is T helper cell dependent, we focused on CD4 helper cells as essential components of an immune response. We report on three experimental systems that make use of mice, macaques and humans to investigate helper responses, as summarized below.

4. Mice were used to determine in vivo the epitopes of gp120 that provide optimal help for a V3 specific antibody response. This is a functional definition of helper activity, that is not simply inferred according to CD4 phenotype. Mice were primed i.p with soluble overlapping peptides of gp120 and subsequently boosted with a substimulatory dose of gp120 i.v. V3 specific antibodies were tested in sera. A limited number of peptides was able to prime for V3 antibodies, suggesting that this type of approach identifies peptides that provide functional help for B cells.
5. Our previous data showed that low doses of antigens bound by specific antibodies as immune complexes (IC) are taken up by APC and presented to specific CD4 cells with high efficiency. Thus we prepared SIV gp120 - IgG IC that was administered intrarectally to Rh. macaques. Due to the presence of FcR on rectal epithelium, this resulted in enhanced immunogenicity.
6. Since in vivo experiments cannot be performed in humans, we mimicked priming of human CD4 T cells by an immunogen by generating CD4 T cell lines from non immune individuals. Different preparations of gp120 derived from different strains were used for these assays. PBMC were cultured with gp for several days, expanded with IL2 and monthly restimulated with autologous APC, antigen and IL2. The most recent experiments showed that it is possible to produce gp120 specific CD4 lines from naive donors, that can be used for accurate epitope mapping to define T helper epitopes to be incuded in tailored vaccines.

In conclusion, our focus on CD4 T helper cells specific for retroviral antigens can take advantage of different experimental systems and is in keeping with the current views that T helper cells are crucial to control progression of infection and to mount desirable immune responses upon vaccination.

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AMPLICON VECTORS AS A NOVEL APPROACH TO HIV-1 VACCINE DEVELOPMENT

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Herpes simplex virus (HSV) are potential vectors for immunoprophylaxix against infectious diseases. There are three main types of HSV-based vectors, namely replication-incompetent recombinant viruses, replication-attenuated mutants and amplicons, which are helper-dependent vectors. Amplicons are promising tools for vaccine development because they combine the properties of both naked DNA vaccines (the genome of the amplicon vector is derived from a DNA plasmid, namely the amplicon plasmid) and of recombinant HSV viral vectors (the amplicon genome is packaged into a virion which is provided by the helper system). Although amplicons have not yet been tested as vaccine vectors, they show unique advantages over the other HSV vectors. Firstly, amplicon particles are absolutely apathogenic for infected cells as their genome is devoid of HSV-1 genes. Secondly, the repetitive character of the genome carried by the amplicon particle ensures the introduction of multiple copies of the transgene transcription unit per infected cell, which is likely to result in strong expression. Lastly, the pantropic properties of HSV-1 particles, which are conserved in amplicons, should allow these vectors to infect a large range of cells, including dendritic cells. Moreover, amplicons could also allow antigens to be presented by both MHC pathways during the same immunization protocol. Furthermore, this last property could be enhanced taking advantage of the trafficking properties of the HSV tegumentary protein VP22. VP22 can act as a carrier, allowing VP22-fused proteins to be also brought into adjacent cells. Should this properties of VP22 be equally effective in vivo, this protein could become a potent tool to target both MHC-I and II molecules. In order to test this hypothesis and to construct amplicon vectors for the development of a vaccine against HIV-1 we have chosen, as selected viral antigen, the regulatory protein Tat. We constructed the amplicon plasmids expressing Tat wild type (wt) and Tat_{22} transdominant negative mutant or the chimeric proteins VP22-Tat_{wt} and VP22-Tat_{22} and are currently testing their biological activity in vitro. The studies will be subsequently extended to the in vivo model.

Accordo di Collaborazione n.40C.58

REDOX REGULATION OF CHEMOKINE RECEPTOR EXPRESSION.[+]

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An increase in oxidative stress and a weakened antioxidant defense system has been reported in HIV-positive patients. We have shown that signals, such as LPS, IL-1 and TNF-α, also capable to induce production of reactive oxygen intermediates (ROI), induce in human monocytes a rapid downregulation of the Monocyte Chemotactic Protein-1 receptor (CCR2). Based on this, here we investigate the influence of antioxidants and/or ROI on the expression of chemokine receptors, including CCR5 and CXCR4 the main coreceptors for macrophage and T tropic HIV-1 strains respectively.

In human monocytes, the antioxidant pyrrolidine dithiocarbamate (PDTC) rapidly inhibited CCR2 (95%-100% of inhibition) and CCR5 (77%-100% of inhibition) mRNA expression, by strongly decreasing transcript stability. CCR2 half life was decreased from 1.5 h to 45 min; CCR5 half life from 2 h to 70 min. This inhibitory activity included also CXCR4, but not CXCR2 receptor and, although to a lesser extent, was shared by the antioxidants N-Acetyl-L-cysteine (NAC) and β-mercaptoethanol. In contrast, the ROI-generating system Xanthine/Xanthine Oxidase (X/XO) increased CCR5 and CXCR4 mRNA expression and counteracted the inhibitory effect of PDTC. Accordingly, H_2O_2 and the glutathione (GSH) depleting drug Buthionine Sulfoximine (BSO) increased to different extent CCR2, CCR5 and CXCR4 mRNA expression. The PDTC-mediated inhibition of CCR5 and CXCR4 mRNA expression was associated with decreased chemotactic responsiveness (>90% of inhibition) and with a marked inhibition of surface receptor expression. In contrast, X/XO opposed the LPS and TNF-α-mediated inhibition of CCR5 and CXCR4 mRNA expression and increased both the CCR5 surface expression and the cells migration (three fold) in response to MIP-1β. These results suggest that the redox status of cells is a crucial determinant in the regulation of the chemokine system and that activation of chemokine system during oxidation may have a potential impact in HIV infection. In particular, we propose that during cell oxidation combination of the observed increase of the NF-κB dependent HIV-1 LTR activity with the increased expression of chemokine receptors may drastically favor HIV-1 infection and replication, particularly in consideration of the lower glutathione levels observed in HIV patients.

In conclusion, our data suggest that ROI have an important role in maintaining high expression of certain chemokine receptors and provide a new mechanism that supports the concept that targeting ROI with antioxidants might be useful in the therapy of HIV infection.

Accordo di collaborazione n. 40C.59

ROLE OF HUMAN FC⊕RI$^+$ CELLS IN HIV-1 INFECTION

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HIV-1 glycoprotein (gp) 120 from different clades (MN, SF2, LAV and CM) is a potent stimulus for IL-4 and IL-13 release from basophils and mast cells (Fc∑RI$^+$ cells) purified from subjects seronegative for antibodies (Abs) to HIV-1 and HIV-2. IL-4 mRNA was increased after stimulation by gp120, whereas IFN-© mRNA was not detected in any of the basophil preparations. There was a correlation between the maximum gp120- and anti-IgE-induced IL-4 release from basophils. Removal of IgE from basophils abolished the release of IL-4 and IL-13 in response to gp120. Three human V_H3^+ monoclonal IgM inhibited gp120-induced secretion of IL-4 from basophils, whereas V_H6^+ monoclonal IgM did not inhibit the release of IL-4 induced by gp120. Incubation of basophils and mast cells with synthetic peptides of the $gp120_{MN}$ core motif prevented cytokine release induced by $gp120_{MN}$. These results indicate that gp120 acts as a viral superantigen, interacting with the V_H3 region of IgE to induce cytokine release from human Fc∑RI$^+$ cells. Human Fc∑RI$^+$ cells express the chemokine receptor CCR3, which binds the chemokines eotaxin and RANTES. HIV-1 Tat protein is a potent chemoattractant for basophils and lung mast cells obtained from healthy individuals seronegative for Abs to HIV-1 and HIV-2. Tat protein induced a rapid and transient Ca^{2+} influx in basophils and mast cells, analogous to ß-chemokines. The chemotactic activity of Tat protein was blocked by preincubation of FcεRI$^+$ cells with anti-CCR3 Ab. Tat protein or eotaxin desensitized basophils to a subsequent challenge with the autologous or the heterologous stimulus. Preincubation of Fc∑RI$^+$ cells with Tat protein up-regulated the level of CCR3 mRNA and the surface expression of the CCR3 receptor, a co-receptor of several strains of HIV-1. Our results indicate that gp120, which acts as a viral superantigen, interacts with the V_H3 region of IgE to induce the release of IL-4 and IL-13 from human FcεRI$^+$ cells. Tat protein is the first identified HIV-1-encoded ®-chemokine homolog that influences the directional migation of human FcεRI$^+$ cells and the expression of surface receptor CCR3 on these cells.

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NEW INHIBITORS OF THE HIV-1 ASPARTIC PROTEASE: COMPUTER-AIDED DESIGN, SYNTHESIS AND ACTIVITY EVALUATION

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Inhibitors of HIV-1 aspartic protease (AP), which plays an essential role in viral maturation, are currently used as components of drug cocktails employed in the highly active antiretroviral therapy (HAART) of AIDS. Most of these compounds are transition state analogs with IC_{50} in the nanomolar or subnanomolar range.

Viral resistance with any of these drugs is a significant problem. Thus new HIV-1 AP inhibitors with different resistance profiles are still actively being pursued. We have developed a novel, integrated method for the design, synthesis and testing of peptidomimetic and nonpeptidic inhibitors of HIV-1 AP, privileging simplicity and flexibility of the synthetic approach. Our method is based on the modular assembly of appropriate flanking residues onto presynthesised, nonhydrolisable, diaminodiol cores or monohydroxy ethylene cores.

The inhibitors are modelled in the HIV-1 AP active site on a Silicon Graphics workstation starting from known active inhibitors (e.g. A76928 or ABT-538). After geometry optimisation, relative complexation energies between AP and inhibitors ΔE_{compl} are calulated [1,2] and used to qualitatively predict the inhibition potency of candidate structures. Peptidomimetics are synthesised from Boc-monoprotected, dihydroxy- or monohydroxy- ethylene central mudules of defined configuration with Phe side-chains in positions P1 and P1'(i.e. Boc-NH-Phe-ψ-Phe-NH_2) [2,3], employing as flanking units, P_n either proteic or nonproteic aminoacids or carboxylic acids such as D-α-(2-thienyl)glycine (Dtg) or kynurenic acid (Kyn) or phenoxyacetic acid (Poa). IC_{50} values are determined with recombinant wild-type HIV-1 AP and a fluorogenic substrate [2]. Octanol/water partition constants $P_{o/w}$ are predicted with the KOWINN programme (v1.65, Syracuse Research Centre); in general, log $P_{o/w}$ between 2 and 5 are thought to be appropriate for uptake and penetration of cell membranes by HIV-1 AP inhibitors [2]. The methodology employed i) avoids the need for diastereoisomer separation thanks to the stereoselective synthesis of the central diolic core, and ii) offers the possibility of independently varying the number and type of P_n, P_n' residues flanking the central unit on either side.

Of the over thirty hexameric, pentameric or tetrameric pseudoC_2-symmetric peptidomimetics produced several showed IC_{50} ≤10 nM. Multi-variable regression analyses for correlating log(IC_{50}) and calculated properties indicated that besides ΔE_{compl} also the bulkiness of the side chains and the torsional flexibility of the linkages between P_1/P_1' and P_2/P_2' may play a role in determining the binding properties of the inhibitors to the AP active site.

Three of the novel AP inhibitors were also tested on PHA-stimulated PBMC from healthy donors, infected with HTLV-IIIB (4000 $TCID_{50}/4x10^6$ PCMB). Inhibitors were dissolved in DMSO, diluted in culture medium and added to the cell cultures at concentrations ranging from 0.01 to 5 μM. Viral replication was analysed after 7 days by determining the amount of p24 antigen. The hexameric peptidomimetic AcTrp-Val-Phe-ψ-Phe-Val-TrpAc (mol. wt. 955 Da, IC_{50} = 6 nM, log $P_{o/w}$ 4.5) showed an EC_{50} of 2.3 μM, whereas the smaller pentameric inhibitor Kyn-Dtg-Phe-ψ-Phe-Poa (mol. wt. 707 Da, IC_{50} = 9 nM, log $P_{o/w}$ = 3.6) exhibited an EC_{50} of 0.5

μM. Finally, the negatively charged inhibitor AcTrp-Val-Phe-ψ-Phe-Glu-PheAc (mol. wt. 946, Da, IC_{50} = 9 nM, log $P_{o/w}$ 3.3), presumably a poorer passive permeant of cell membranes, at 5 μM inhibited HTLV proliferation by only 30%.

Having demonstrated the validity of the computational approach for designing inhibitors, and the possibility of rapidly assembling the best candidates, we endeavoured to design various non-peptidic inhibitors based on molecular structures such as hematophyline, anthraquinone, coumarine, and polyphenols, as well as on cyclic urea analogs. More than 30 structures were thus designed, some of which with extremely high predicted affinity (picomolar range). Furthermore, our methodology was applied to the analysis of the molecular factors underlying resistance to commercial inhibitors in drug-resistant, mutant AP.

Finally, we have used 473 carboxylic acids, 58 aminoacids, 37 aldehydes and a diaminodiol core as building blocks for constructing a series of in silico combinatorial libraries of pseudopentapeptidic HIV-1 AP inhibitors. First, a full library was generated, containing 89 billion hypothetical compounds. Then, appropriate filters of molecular and economic parameters were applied (molecular weight, volume, lipophilicity, interaction energies, cost of reagents), and the original full library was gradually reduced to 12,000 compounds, representing the potentially best combination of these parameters. Upon further reduction of this series of designed compound to a more manageable number and development of appropriate high-efficiency synthetic methodologies and high-throughput screening methods, the "filtered" combinatorial libraries should lead to a considerably high number of new AP inhibitors.

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DISPENSABILITY OF HCMV RIBONUCLEOTIDE REDUCTASE (RNR) HOMOLOG FOR VIRUS GROWTH IN VITRO , AND INDUCTION OF CELLULAR RNR

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Contrary to other DNA viruses, β-herpesviruses –including major AIDS opportunists cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6)– have lost a ribonucleotide reductase (RNR) small subunit (R2) gene. Further, the homolog of the large (R1) RNR subunit (pUL45 in hCMV) lacks most catalytic residues (Chee et al. 1990. Curr. Top. Microbiol. Immunol. 154:125-69). We have studied HCMV UL45 gene and protein product, to clarify how the virus raises an active RNR and thereby the dNTP pool essential to viral DNA replication.

Laboratory strains of HCMV or its mouse counterpart (MCMV) have recently been cloned into E. coli Bac vectors, and methods to mutagenize them by random transposon (Tn) insertion have been described (Borst et al. 1999. J. Virol. 73:8320-8329). We have exploited such a reverse genetics approach to check UL45 role in virus growth. UL45 Tn-mutations in hCMV, ad169 strain, did not affect virus growth on diploid fibroblasts, thus the gene is dispensable for hCMV replication in vitro. UL45 protein product is expressed late, after the burst of viral DNA replication. Cellular RNR expression in infected cells was investigated in parallel. Cellular R2 has been known to act as the limiting factor for RNR activity, as it is S-phase restricted in cycling cells. In G_0 fibroblasts, the R2 transcript was shown indeed to be absent, but early after hCMV infection it was fully induced; induction depended on virus immediate-early genes, since it was abolished by UV irradiation of the virions. Thus, hCMV has evolved a replication strategy relying on cellular RNR induction. What is UL45 for? In transfected HEK-293 cells, pUL45 undergoes ubiquitination, sticks to insoluble cytoskeleton, and both insolubilizes and causes the phosphorylation of a cotransfected R2 subunit, which is cytosolic by itself. In yeast two-hybrid system pUL45 strongly dimerizes, and binds the cellular R2 as efficiently as the cellular R1 subunit. In vitro, pUL45 efficiently inhibits human RNR. These properties may be related to the most recent finding that M45, the MCMV UL45 homolog, is essential for growth in endothelial cells, as its ablation induces apoptosis (Brune et al. 2001. Science, in press). R2 protein is part of S-phase checkpoints in yeast and mammals, and excess dNTP (dATP) activate the apoptosome through Apaf1. pUL45 could therefore have been diverted to an antiapoptotic function in some cell types (endothelium), by quenching excess R2/dNTPs at late (postreplicative) infection times.

These results are also relevant to antiviral therapy. The finding that HCMV and presumably all β-herpesviruses strictly depend on cellular RNR suggests that both traditional anti-RNR drugs (hydroxyurea) and the highly specific anti-RNR peptidomimetics currently under study as antiproliferative drugs can exert a pronounced anti-β-herpesviral action in the context of antiretroviral combinatorial therapies. Further, pUL45 is the first natural protein inhibitor of mammalian RNR described to date. Dissection of the RNR (R2)-pUL45 binding mechanism may prompt the design of novel anti-RNR drugs targeting distinct enzyme interfaces.

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A CELLULAR UBIQUITIN HYDROLASE MAY MEDIATE MULTIPLE NEF EFFECTS ON ENDOCYTOSIS

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Immunodeficiency viruses express a nonstructural protein, Nef, required for pathogenicity and exhibiting pleiotropic effects in vitro and in vivo: virion infectivity enhancement, interactions with signalling pathways, CD4/MHC-I downregulation via endocytosis, enhancement of surface expression of effector cytokines (TNF and LIGHT 1, 2).

Among a series of Nef interaction partners identified through a yeast two-hybrid system (Y2HS) screening (3, 4), we are analyzing hFaf, the product of the human gene homologous to Drosophila melanogaster fat facets. hFaf is a large (2547 aa) protein, including an ubiquitin hydrolase domain close to the C-terminus. Mammalian Fafs are essential during development of the CNS and epithelia (5). In polarized epithelial cells they are peripheral membrane proteins localizing at the cadherin belt, where they interact with AF-6 and β-catenin (6) In D. melanogaster, Faf is essential for the pathway that limits to eight the photoreceptors of the eye ommatidia, and genetically interacts with liquid facets, coding for the homolog of mammalian Epsin, a major clathrin coated pit regulator (7). Fafs might bind the similar armadillo repeat and ENTH domains beared by β-catenin and Epsin, respectively (8). The hFaf cDNA tract isolated in our Y2HS screening covers the 3'-terminal portion encoding aa 2058-2547, downstream from the deubiquitinating enzyme domain. The Nef-Faf(C-terminus) interaction as measured in Y2HS assays is very strong and specific. In Nef, part of the N-terminal solvent-exposed loop and of the alpha-beta structural core (not the polyproline helix-II needed for association with SH3 domains) is required for the interaction. Rabbit antibodies raised against the mouse Faf (fat facets of the mouse, Fam) cross-react against hFaf, and have allowed to analyze hFaf tissue and subcellular distribution. In western blot hFaf is detected in cell lines infectable by HIV-1 and PBLs. Immunofluorescence analysis has shown that hFaf localizes to the peripheral cell membrane not only in polarized cells, but also in T-cell lines. Overexpressing a mutated hFaf (deubiquitinating enzyme off) supresses Nef-induced CD4 dowregulation. We plan now to: (i) confirm Nef-hFaf interaction in HIV-1-infected cells; (ii) analyze the consequences (for progeny virus infectivity and surface protein turnover) of overexpressing wt/dominant negative hFaf in HIV-1 infected cells. A mutational analysis of hFaf nonezymatic domains is also appealing, as they are necessarily involved in subcellular targeting and physical interactions with other proteins. A number of reports had suggested intersections between HIV-1 biology and ubiquitination: (i) HIV-1 Gag is ubiquitinated; (ii) proteasome inhibitors enhance HIV-1 replication; (iii) Nef binds a proteasome subunit (3). The hypothesis we favour, supported by Drosophila genetics, is that Fafs act as positive regulators of endocytosis by deubiquitinating Epsin or other endocytic machinery components. By recruiting hFaf Nef might thus influence the surface half-life of many proteins at once, which would resolve the conundrum of how it can exert opposite effects (up- or downregulation) on a subset of unrelated surface molecules.

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POSSIBLE HCV INVOLVEMENT IN ACUTE MENINGORADICULITIS/POLYRADICULITIS OF HIV-1 COINFECTED PATIENTS

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Acute or chronic polyneuroradiculopathy has been associated in both early and advanced HIV-1 infection, and have been attributed to a wide spectrum of infectious agents. Syndromes mimicking Guillain-Barré Syndrome (GBS) have been described early during HIV-1 infection. We have performed virological and immunological investigations in a 42-year-old HIV-1 infected male patient who had HCV coinfection. Clinical presentation occurred two weeks after a mild episode of fever and asthenia with rapidly progressing sensorimotor symptoms, EMG/ENG evidence of sensory and motor nerve damage, and CSF evidence of meningoradiculitis with mild pleocytosis, oligoclonal bands but normal protein concentrations. The clinical course was rapidly improvng within one month after plasmapheresis. Quantitative analysis of HIV-1 and HCV viral load in paired plasma/CSF samples revealed detectable amounts of HCV-RNA in CSF at the onset, and a subsequent decrease below quantification limits after plasmaheresis. Conversely, HIV-1-RNA in the CSF was persistently high also after plasmapheresis, even if symptoms were improving. Comparative analysis of HIV-1 specific CTL activity on CSF-derived CD3+CD8+ populations and fresh PBMC showed lack of virus-specific CTLs in the CSF while they were present in PBMC. Thus, both a cellular immune response against HIV-1 antigens and direct HIV-1 mediated injury were unikely to be involved in the pathogenesis of the disease. HCV infection, either by direct invasion and replication or through immune-mediated mechanisms, seemed a more likely cause for the disease in this patient. This case report and the results from the immune function and virological investigations suggest that in some HIV-1 infected patients the pathogenesis of GBS or acute polyradiculitis could be associated with HCV coinfection, rather than attributed to HIV-1 as currently proposed.

N° dell'Accordo di Collaborazione: 40C.63.

DIFFERENTIAL DISAPPEARANCE OF INHIBITORY NK RECEPTORS DURING HAART AND POSSIBLE IMPAIRMENT OF HIV-1-SPECIFIC CD8+ CTL.

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Antiretroviral treatment (HAART) is associated with a decrease in viral replication to undetectable levels and with an increase in CD4+T-lymphocytes. Residual HIV-1 replication occurs, together with incomplete recovery of cytolytic CD8+ T-lymphocyte (CTL) numbers and function. We sought to determine whether expression of HLA-class I-specific inhibitory NK receptors (iNKRs) on CTLs of patients successfully treated with HAART for 24months could be involved, at least in part, in residual CTL functional inhibition.

We analyzed by two color cytofluorometry the expression of 6 different iNKRs, including p58.1, p58.2, p70, p140, CD94/NKG2A, LIR1/ILT2, on $CD3^+CD8^+$ lymphocytes of 8 patients with successful long-term suppression of viral replication before and after 3, 6 and 24 months of HAART. Healthy subjects were comparatively analyzed. HIV-1-specific cytolytic activity was determined after 24 months of HAART in the presence and absence of iNKR-masking.

No significant reduction of iNKRs expressed on CD8+ T-cells was observed after 6 months of successful HAART. After 24 months, p58.1, p58.2, p70, p140, CD94/NKG2A expressing $CD8^+$ T-lymphocytes returned to levels undistinguishable from healthy controls. Expression of p70 and p140 was negatively correlated with the increasing CD4+ numbers. After 2 years, a significantly increased proportion of $CD8^+$CTL expressed LIR1/ILT2. Functional analysis of freshly separated cells revealed that the disruption of the interaction between LIR1/ILT2 and HLA-class I could partially restore HIV-1-specific lysis.

During HAART the decrease in $CD3^+CD8^+iNKR^+$ cells is considerably slower than expected. In some patients functional impairment due to LIR1/ILT2 expression may persist even after 24 months of successful HAART. During HAART the decrease in $CD3^+CD8^+iNKR^+$ cells.

N° dell'Accordo di Collaborazione: 40C.63.

EVOLUTION OF HIV-1 env GENE AND HOST SELECTIVE CONSTRAINTS IN SEROCONVERTERS

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The natural history of HIV-1 infection is linked to a marked increase in virus diversity over time and the analysis of HIV-1 variation in subjects at different stage of disease have shown that the heterogeneity of viral species is directly associated with a prolonged survival of patients. The outcome of HIV-1 infection in recent infected individuals is positively or negatively related to the low or high standpoint of HIV-1 activity after acute infection. However, only few data are available regarding HIV-1 evolution in primary infection in both untreated and treated seroconvertes (SC). We addressed the relation among HIV-1 activity and virus evolution within subjects in drug-naive individuals who seroconverted for HIV-1 showing either low or high indexes of HIV-1 replication after acute infection compared to those who have undergone to highly active antiretroviral therapy (HAART).
HIV-1 variation over time was studied by analysing C2V3C3 sequences of gp120 after RNA extraction, RT-PCR amplification and sequencing of 8 to 15 clones at different time-points (time of seroconversion and 1 year late) for a total of 205 clones. Genetic analyses were performed using specific phylogeny softwares (DNAstar, Clustal 1.7, Mega 1.02, Phylip 3.573).
Preliminary data were obtained in 9 SC, 4 of whom were treated with HAART at seroconversion. Among the subjects who were naive for antiretroviral therapy two patients shoved a Ka/Ks positive ratio in C2V3C3 region (1.08 and 1.1, respectively). However, when the V3 alone was considered only 1 patient who had low and very low indexes of HIV-1 replication at seroconversion and 1 year later maintained a positive Ka/Ks ratio (3.2). In contrast, naive patients with high values of HIV-1 viremia both at time 0 and at 1 year showed a Ka/Ks ratio <1. Regarding the subjects treated with HAART at seroconversion 1 patient had a positive Ka/Ks ratio. This seroconverter experienced a marked control of virus replication and the reversion of the HIV-1 phenotype from a SI to an NSI variant. Of the remaining 3 treated patients none had a positive value of Ka/Ks ratio either when the replication rate was undetectable or low.
These findings support that a positive selective pressure on V3 loop of env gene parallels a marked autonomous control of HIV-1 replication both at baseline and 1 year after seroconversion even in the absence of therapy. Intrapatients variation analysis gave similar results indicating that host-mediated selective forces act as constraints of intra-host HIV-1 evolution. Our data suggest that HIV-1 env gene evolution is driven by host-specific selective constraints. Both immune mediated responses and HIV-1 coreceptor usage may serve as such forces and need to be further investigated in individual hosts.

No. dell'Accordo di Collaborazione: 40C.64

SOLID PHASE SYNTHESIS OF 2,5,6-TRISUBSTITUTED-4(3*H*)-PYRIMIDINONES TARGETING HIV-1 RT

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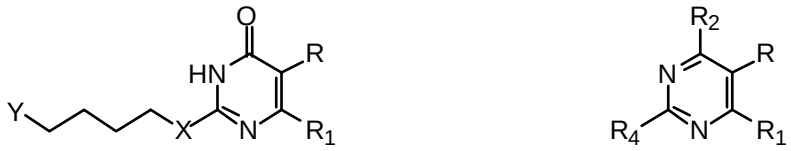
To meet an increasing demand of novel and diverse small molecular-weight compounds necessary for screening, combinatorial or parallel chemistry are currently in the focus for the creation of novel and diverse compound collections. Solid Phase Synthesis (SPS) is playing a decisive role in the ongoing development of combinatorial chemistry, mainly because it offers high synthetic flexibility (excess reagents can be used to drive reactions to completion; impurities and excess reagents can be removed by simple washing of the solid phase), as well as the possibility of automatization.
In the last few years some uracil and pyrimidinone derivatives substituted at C-5 and C-6 positions have emerged in the field of antiviral chemotherapy.[1,2] As a part of an ongoing research program on new anti HIV-1 agents, we decided to set up an SPS method for 2,5,6-trisubstituted-4(3*H*)-pyrimidinones which would allow for the maximisation of the number and chemical diversity of these compounds. Molecular modelling studies of our group[3] demonstrated that compounds of general structure **1** (Figure 1) would interact effectively with the target enzyme. The SPS methods we developed allows the synthesis of the target compounds in an easy and profitable way, showing good potential for the preparation of the combinatorial libraries of small molecules **2** (Figure 1), bearing different substituents and functionalities.
Synthesises and biological evaluation of the new compounds in enzyme assays against recombinant HIV-1 RTs will be reported in poster session.



1 **2**

Figure 1

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Accordo di Collaborazione N° 40C.65

SYNTHESIS OF 3'-MODIFIED NUCLEOSIDES AS POTENTIAL ANTI-HIV-1 AGENTS

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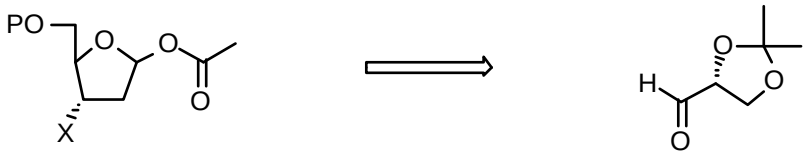
The HIV multifunctional enzime Reverse Transcriptase (RT) is an important target for the treatment of AIDS and the nucleoside (i.e. AZT, ddC, d4T, 3TC) and non-nucleoside (i.e. nevirapine) RT inhibitors are among the presently approved therapies for HIV. The nucleoside RT inhibitors belong to 3'-deoxynucleosides class and all of them show the same mechanism of action, i.e. following intracellular phosphorylation to their 5'-triphosphate form, they serve as chain terminators and/or inhibitors in the viral reverse transcription reaction. In view of their importance and high bioactivity against HIV, our attention has been focused on the synthesis of 2',3'-dideoxynucleosides, having a bulky substituent in 3' position. Our synthetic purpose is the coupling of some pyrimidine bases with a new sugar of appropriate configuration, already synthesized in our laboratories. The sugar moiety is obtained from a chiral precursor and the nucleoside synthesis is performed in CH_3CN at room temperature, using the appropriate silylated bases and TMSOTf as a Lewis acid.[1] This route affords the target compounds in high yield, as a/b anomers mixtures (1/3) that can be separated *via* chromatography on silica gel.



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Accordo di Collaborazione N° 40C.65

HIGHLY EFFICIENT TRANSDUCTION OF HEMATOPOIETIC STEM CELLS AND ANALYSIS OF THE REQUIREMENT FOR CYTOKINES BY IMPROVED LENTIVIRAL VECTORS

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N°. dell'Accordo di Collaborazione. 40C.66

Hematopoietic stem cells (HSC) have proven difficult to transduce with traditional retroviral vectors due to a requirement for cell division to allow integration into the host cell genome. As HSC are largely quiescent, they must be induced into cycle for successful retroviral transduction. This requires several days of in vitro manipulation of the cells, which can be detrimental to their subsequent long-term repopulating ability. Lentiviral vectors, however, have the ability to integrate into the genome of nondividing cells. Successful transduction of extended long term culture initiating cells (LTC-IC) and NOD/SCID mouse repopulating cells (SRC) by lentiviral vectors has been reported by several groups, but efficiencies have been variable (10-20%) and a detailed analysis of the requirement for cytokines during transduction has not been done.

We have advanced the performance of lentiviral vectors by introducing a new design that improves the efficiency of gene transfer. We found that structural elements within the pol gene of HIV-1 are required in cis for nuclear translocation of the vector genome. We restored these sequence elements into the backbone of a self-inactivating lentiviral. The new vector transduced to much higher efficiency several types of human primary cells, including lymphocytes, macrophages, and human cord blood-derived CD34+ cells assayed for long-term in vivo repopulation of SCID/NOD mice (SRC, SCID/NOD Repopulating Cells). (Follenzi, Ailles, Bakovic, Geuna and Naldini. Nature Genetics, 25, 217-222, 2000).

We then optimized the conditions of transduction of SRC. Human CD34+ cells were incubated with the new vector carrying an expression cassette for GFP driven by the promoter of the human PGK gene at multiplicity of infection (MOI) ranging from 5 to 500 for 24 hours in serum-free media either with or without a cocktail of cytokines (IL-6, 20 ng/ml; Flt-3L, 100 ng/ml; SCF, 100 ng/ml; TPO, 20 ng/ml). This cytokine combination was previously shown to allow maintenance and some expansion of SRC ex vivo. We then seeded some of the transduced cells in clonogenic assays and injected the rest of them intravenously into sublethally irradiated NOD/SCID mice. GFP expression in cultured CFC two weeks later showed that a high frequency of cells was transduced independent from the cytokines (78% and 86% without and with cytokines, respectively, $p=0.5$). Engraftment levels, as determined by percentage of human CD45+ cells in the bone marrow of mice between 6 and 12 weeks post-injection, were not significantly different between the two groups ($34 \pm 14\%$ and $20 \pm 8\%$ without and with cytokines, respectively, $p=0.35$) and were not affected by exposure to the vector. Limiting dilution analysis showed no difference in the frequency of SRC between the two groups. However, a statistically significant difference was found in the transduction efficiency of SRC. Mice repopulated by cells tranduced in the presence of cytokines had $75 \pm 5\%$ ($n=10$) of GFP+ cells, while mice repopulated by cells transduced in the absence of cytokines had $44 \pm 12\%$ ($n=7$) ($p=0.01$). Because 24 hours is too short a time to induce the majority of cells into cycle, the improvement in transduction efficiency may result from activation of G_0 cells into G_1, as it was found for human T-lymphocytes. In addition, secondary transplants have been performed, and clonal analysis of engrafted populations will be done to prove self-renewal of SRC in the bone marrow of the primary mice. In conclusion, SRC were transduced to very high efficiency within 24 hours, both in the absence and in the presence of a cytokine cocktail, and neither of these protocols had detrimental effects on the ability of the cells to engraft in NOD/SCID mice. While transduction efficiency was lower in the absence of cytokines, both protocols yielded previously unattained levels of gene transfer into SRC that could be useful for clinical purposes.

ANALYSIS OF THE ROLE OF IFN-γ AND IL-12 SIGNALING PATHWAYS INPROTECTION OF T CELLS FROM HIV-1 INFECTION

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The role of IFN-γ and IL-12 in HIV-1 infection of human T lymphocytes was investigated by evaluating cytokine and β-chemokine secretion, Fas-L and HIV co-receptor expression and HIV replication in T cells from patients with inherited IFN-γR1 ($GR^{-/-}$) and IL-12Rβ1 ($12R^{-/-}$) deficiencies. PHA-derived T cell clones and T lymphocytes from deficient patients showed a normal IL-2 and IL-10 production and moderate ability to produce IFN-γ that was abolished when T cell clones from $12R^{-/-}$ and $GR^{-/-}$ patients were generated in the presence of anti-IFN-γR1 or anti-IL-12 mAb respectively. IL-4 production was increased in $12R^{-/-}$ T cell clones only. RANTES and, to a lesser extent, MIP-1β production was strongly decreased in $CD4^+$ T cell clones from both types of deficient patients. By contrast, MIP-1α production was reduced in $CD4^+$ clones from $12R^{-/-}$ patients only. $GR^{-/-}$ $CD8^+$ clones displayed intact secretion of all three β-chemokines, whereas $12R^{-/-}$ clones displayed a reduced secretion of MIP-1α and MIP-1β. As a correlate of RANTES and MIP-1β secretion inhibition, compared to $CD4^+$ T cells from healthy donors, those from both types of patients displayed higher CCR5 but unaffected CXCR4 surface expression. This CCR5 increased expression was probably the outcome of defective RANTES-mediated internalization since the ligand-independent recycling of CCR5 was not affected in deficient $CD4^+$ T cells. A parallel marked defect in Fas-L surface expression was also observed in CD4+ T cells from deficient patients. Both laboratory-adapted and primary isolates of R5-HIV strains replicates more effectively in $GR^{-/-}$ and $12R^{-/-}$ T cells than in healthy ones and the replication of Bal strain in T cells from healthy donors was inhibited by IL-12. By contrast, replication of the X4- HIV IIIB strain is observed in $12R^{-/-}$ T cells only, probably due to an increase in IL-4 production in these cells that enhances CXCR4 expression. These data as a whole provide new evidence that both IFN-γ and IL-12 play a pivotal role in controlling IFN-γ and β-chemokine secretion by T cells and thereby protect $CD4^+$ T lymphocytes from R5 HIV-1 infection and replication. The control of Fas-L expression by IFN-γ and IL-12 suggests that these two signals may play a critical role in regulating both the effector cytotoxic functions against HIV infected cells and their activation-induced apoptosis. The role of IL-12 and IFN-γ in regulating the susceptibility to infection by X4- HIV strains is being currently investigated.

Contributo ISS N. 40C.67.

PATHOGENESIS, PREVENTION AND THERAPY OF HIV-1 INFECTION

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Our project deals with different aspects of HIV-1 infection and pathogenesis. In particular, it focuses on virus-cell interactions and novel therapeutic approaches.
A number of efforts have been made in order to identify viral and/or cellular factors which might promote the pathogenesis of HIV-1 infection. Herpes simplex viruses (HSV) have been suggested to interact with HIV-1 at molecular and cellular level, affecting AIDS progression. In particular, we established a human $CD4^+$ T cell line chronically infected by HSV-1 (CEM_{HSV}) to study molecular interactions occurring between HIV-1 and HSV-1 (Calistri et al., 1999). Superinfection of CEM_{HSV} with HIV-1 led to a double chronic infection of these cells ($CEM_{HSV/HIV}$). $CEM_{HSV/HIV}$ cells produced HIV-1 virions pseudotyped by HSV-1 envelope, which were able to infect CD4 negative cells. Vero, Cf2-Th and 293T cell lines were productively infected by pseudotyped HIV-1, even at very low multiplicity of infection. The infection was sensitive to HSV-1 neutralizing antibodies and to AZT treatment. In addition, our data seem to indicate that the HIV-1 env gene is partially dispensable (Calistri et al., submitted). A chronic carrier state of the two viruses in lymphocytes as well as HIV-1 pseudotyping might be relevant to AIDS pathogenesis and could explain the presence of an elevated HIV-1 load in the absence of circulating $CD4^+$ cells during late stages of the disease.
In another line of research the two hybrid system has been utilized to identify cellular proteins which interact with HIV-1 Tat and could mediate its interaction with the cellular transcription factor Sp1. In particular, our study focused on interactions of TATA-binding protein (TBP), TBP-associated factor 55 (TAF55), Cyclin T and Cyclin-dependent kinase 9 (Cdk9) with Tat and/or Sp1. Our data confirmed interaction of TBP with Tat but not with Sp1. On the contrary, TAF55 didn't interact either with Tat or with Sp1. We also confirmed that Tat interacts with Cyclin T to form the P-TEFb complex and that such an interaction is not direct but mediated by Cdk9. Finally, none of the investigated proteins forms a ternary complex with Tat and Sp1. These results could contribute to understand funtional interactions between Tat protein and cellular factors.
As for vaccine development, we are producing chimeric toxins based on the non-toxic B subunit of E. coli enterotoxin (EtxB) fused to HIV-1 immunodominant epitopes. The latter will contain T and B epitopes of Gag, Env, Nef and Tat, together with signals for endoproteolytic cleavage and localization of epitope to endoplasmic reticulum. The chimera design will exploit the intrinsic immune-adjuvant activity and mucosal tropism of EtxB. It will also take advantage of EtxB ability to direct bound oligopeptides to specific intracellular compartments after vesicular trafficking and proteolytic release of peptides from acidic endosomes/lisosomes, as recently demonstrated by our group (Marcello et al., 1994; Loregian et al., 1999; Loregian et al., 2000).
In the search for new antiviral compounds, we recently evaluated a series of 6-aminoquinolones for their in vitro activity against HIV-1. A molecule (WM5) exhibited high antiviral activity in de novo infected human lymphoblastoid cells (Cecchetti et al., 2000). While proviral DNA formation and integrase activity were not affected, a slight inhibition of HIV-1 protease has been noticed (Parolin et al., submitted). In addition, we developed an integrated methodology for the design and synthesis of HIV-1 aspartic protease diaminodiol inhibitors (Tossi et al., 2000). This approach is based on a computational method that predicts the potential ihibitory activity of designed structures in terms of calculated enzyme-inhibitor complexation energies. This is combined with a versatile synthetic strategy that couples a high degree of stereochemical control in the central diamonodiol module with complete flexibility in the choice of side chains in the core and in flanking residues.

N°. dell'Accordo di Collaborazione = 40C.68

GLUCOSYLCERAMIDE INHIBITIONAFFECTS CELL GROWTH AND ADHESION BY MODULATING RHO A GENE EXPRESSION

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Treatment of human cells, expressing CD4 and various chemokine receptors, with the glucosylceramide synthase inhibitor 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), blocked target membrane glycosphingolipid (GSL) biosynthesis and reduced the susceptibility of cells to infection and fusion mediated by envelope glycoproteins from a variety of human immunodeficiency virus type (HIV-1) isolates that utilize CXCR4 and/or CCR5. PPMP treatment of the cell lines did not significantly change the cell surface expression of CD4, CXCR4, and/or CCR5, nor did it alter the chemokine receptor association with CD4. These data are consistent with the notion that a limited number of specific GSL species serve as crucial elements; In order to directly address the role of GLS in HIV infection we transfected with an expression vector containing a 700 bp segment of the human glucosylceramide synthase cDNA in antisense orientation, identifying three clones exhibiting a stable decrease in glucosylceramide synthase activity (up to 65% in comparison to control cells). In keeping with reduced ceramide utilisation for glycosphingolipid synthesis, antisense clones displayed higher levels of endogenous ceramide in comparison to control cells. The antisense derived clones exhibited a significative decline in growth, when compared to Neo controls associated with a dramatic loss of cell-cell and cell-substratum adhesion. In attempt to define the molecular mechanisms by which inhibition of ceramide glucosylation causes these effects, the expression of 1200 human genes in CHP-100-derived clones was studied. The inhibition of glucosylceramide synthase expression induced significant changes in the expression of cell-cycle and adhesion-associated genes: in particular the RhoA transcript was markedly downregulated in glucosylceramide synthase antisense clones compared to control cells, a result that coincided with a marked decrease of RhoA protein expression. The possibility that dysregulation of RhoA function may provide a key mechanism by which inhibition of ceramide glucosylation affects cell adhesion and morphology was supported by the observation that treatment of glucosylceramide synthase antisense clones with cytotoxic necrotizing factor type 1 restored the phenotype of control cells. Treatment of CHP-100 cells with short chain ceramide also elicited downregulation of Rho A expression; the same effect was observed for D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol. These results strongly indicate that inhibition of glucosylceramide synthesis may impinge on the transduction mechanism driving cell-cell and cell-substrate adhesion via ceramide-mediated Rho downregulation.

N°. dell'Accordo di Collaborazione. 40C.69

THE RHO-FAMILY GTP EXCHANGE FACTOR VAV COUNTERACTS CD4-MEDIATED APOPTOTIC PROGRAMS BY REGULATING BAX EXPRESSION

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Our observations that the engagement of CD4 by soluble ligands such as HIV-gp120 and Leu3a separately from TCR activates in $CD4^+$ human memory T cells the expression of the proapoptotic protein Bax independently of the antiapoptotic protein Bcl-2 (J Immunol. 2000,164:5078) while the ligation of CD28 rescues these cells from apoptosis, prompted us to analyze the molecular mechanisms that allow CD4 to control the balance between survival and apoptotic signals. Indeed a such analysis could have clear implications in the design of new therapeutical strategies able not only to abrogate HIV-induced apoptosis but also to activate it in triple therapy treated patients.

To address this issue, we verified whether the stimulation of CD4 alone could influence Bax expression and if the GTP/GDP exchange factor Vav, that transduces the activation signals from CD28, could interfer with the apoptotic signals from CD4. As experimental system we used the CD4+ Jurkat T cell line (clone J20), stable transfected or not with Vav construct. CD4 triggering was mediated by anti-CD4 mAb, Leu3a. Bax expression was evaluated by western blot and flow cytometry and mitochondrial damage by JC-1 staining. Apoptotic fhenomena were evaluated by flow cytometric analysis.

Our data showing that anti-Bax oligonucleotide treatment inhibited anti-CD4 mithocondrial damage in T cells stimulated with the anti-CD4 alone, gave us the confirmation that the ability of CD4 to regulate Bax expression was responsible of apoptosis induction. The same analysis were performed in Jurkat T cells stable trasfected with Vav. Interestingly, the overexpression of Vav abrogated both the increase of Bax as well as the apoptotic phenomena induced by anti-CD4 treatment.

In conclusion, we present a clear evidence of the ability of CD4 to regulate Bax expression and of Vav to transduce counteractive signals. Overall, these data suggest a novel immunoregulatory function of CD4 and Vav useful to fight HIV infection.

Accordo di collaborazione n.40C.70.

HIV-1 NEF DOWNREGULATES LIPID RAFTS AND INDUCES A DEFECTIVE ACTIVATION OF $CA2^+$ PATHWAY WITHOUT AFFECTING ERK/JNK CASCADES

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Although accumulated data suggest that HIV-1 Nef protein influences T cell activation by interferring with T cell receptor machinery, the mechanisms are not yet completely understood. Since recent evidences indicate that T-cell receptor triggering and T-cell activation are dynamic processes, which involve various aspects of T cell organization, we hypothesized that Nef could interact differentially with the cell structures which regulate the signaling cascades that ultimately induce the transcription of genes controlling either the activation and/or the inhibition of T cell proliferation.

With the aim to define the mechanisms by which Nef interfers with TCR-mediated activation pathways we firstly investigated the effect of Nef on MAP kinases and NFAT, NFkB, AP-1 and Fos transcription factors. To reach our aim, we used Jurkat cell lines (clone J20), transiently transfected with CD8-Nef and CD8 constructs, activated or not by murine fibroblasts expressing MHC class II and B7.1 human molecules in the presence of Staphilococcus enterotoxin E. MAP kinase activity was measured by kinase assays and NFAT, NFkB, AP-1 and Fos activation by luciferase. Our data demonstrate that the engagement of TCR in T cells transfected with Nef induced a significant decrease of NFAT without modifying NFkB, AP-1 and Fos. Moreover, the addition of two important regulators of the $Ca2^+$ pathway, ionomycine and constitutively active calcineurin mutant, resulted in a restorative effect on NFAT activation, while PMA, an activator of MAP kinase, failed to influence NFAT expression. A further confirmation of the failure of Nef to influence JKN cascade derives from kinase assays performed in TCR-activated T cells expressing or not Nef. All cells gave comparable results. All together these data strongly suggest that $Ca2^+$ and not JKN pathways are modified in Nef-expressing $CD4^+$ T cells.

The differential effect of Nef on the above reported activation pathways prompted us to verify whether the intracellular presence of Nef could interfere with the membrane microdomains, commonly referred to as lipid rafts, that controlling the assembly of signaling molecules at the TCR synapse, play a critical role in TCR signaling. To do that, we next quantified in Nef expressing cells, the correct reorganization of membrane microdomain mediated by TCR engagement. The analysis of raft expression was evaluated by GM1 ganglioside staining using cholera toxin B. The data showing a 50% decrease of GM1 ganglioside molecules on cell membrane confirmed our hypothesis.

In conclusion, our data suggest that Nef acting at the level of rafts, proposed to function as platforms for TCR-mediated transduction signals, could modify raft-associated proteins not only quantitatively but also qualitatively. Likely consequences of this effect could be a differential recruitment of signaling proteins by TCR and costimulatory molecules that result in a defective activation of $Ca2^+$ and not JKN pathways.

Accordo di collaborazione n.40C.70

UNUSUAL ROLE OF A BASIC DOMAIN OF HIV-1 REV AS A SPECIFICITY DETERMINANT FOR THE PHOSPHORYLATION BY THE ACIDOPHYLIC PROTEIN KINASE CK2.

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Protein kinase CK2 is a multifunctional Ser/Thr-specific protein kinase characterized by high constitutive activity in most organisms and tissues. It is generally isolated as a stable heterotetramer composed by two catalytic (α and/or α') and two regulatory (β) subunits. Besides an exceptional pleiotropy with more than 200 protein substrates already identified, its most remarkable feature appears the preference for multiple acidic amino acids in the proximity of the phosphorylation sites of the substrates, the one at position n+3 playing a crucial role as specificity determinant. These negative charges are in electrostatic contact with a network of basic residues located on the surface of the catalytic site of α subunit. Consequently, β subunit was demonstrated not to play a rigorous control on CK2 site specificity while it confers stability and higher activity to the catalytic subunit.

In perfect agreement with the acidophilic nature of CK2 the phosphorylation sites of HIV-1 Rev transactivator protein were firstly identified at Ser5 and Ser8 [Meggio et al., Biochem. Biophys. Res. Commun. 226, 547-554, 1996]. In fact, in this case not only the minimun consensus sequence of CK2 is fulfilled (Ser/Thr-X-X-Glu/Asp/SerP), but it is also improved by the presence of three additional acidic residues at positions n-1, n+1 and n+2. However, the mechanism of Rev binding and phosphorylation by CK2 displays some unique features never observed with most other substrates. In particular we have found that: i) the phosphorylation of Rev is fully dependent on the regulatory β subunit of CK2; ii) the integrity of an acidic stretch of β subunit normally down-regulating CK2 activity is crucially required; iii) polyamines and polycationic compounds, known stimulators of CK2 activity, efficiently inhibit Rev phosphorylation; iv) alterations by deletion or mutations within helix-loop-helix basic motif of Rev strongly reduce its phosphorylation. Altogether these findings demonstrate that other distinct regions of Rev, also required for its transactivator's biological activity, might contribute to the optimal binding of the substrate and, more generally, suggest a direct involvement of the regulatory β subunit in the control of site specificity of CK2.

Project N. 40C.71

DEVELOPMENT OF FELINE IMMUNODEFICIENCY VIRUS TAT-MUTANTS: IN VITRO AND IN VIVO CHARACTERIZATION AND USE AS ATTENUATED VACCINES

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The feline immunodeficiency virus (FIV) ORF-A encodes a transactivating protein which shares functional and biochemical properties with HIV-1 Tat. It has been demonstrated that ORF-A inactivation prevents FIV replication in primary lymphoblasts but not in fibroblasts (Waters et al., Virology 215: 10-16, 1996). Based on this observation, we reasoned that FIV variants rendered irreversibly incapable of replicating in lymphocytes but still capable of growing in other cell types might present an attenuated phenotype and prove useful immunogens.

Development and growth characteristics of 11 tat-mutant FIV clones were described in a previous report. In brief, they were produced by inserting several stop codons and deletions of various size in the ORF-A of p34TF10, which contains the whole genome of FIV-Petaluma (FIV-Pet). Here, we first completed the study of the cell tropism and stability in vitro of the tat-mutant, then studied three clones for stability, cell tropism and immunogenicity in vivo.

Eight clones proved replication-competent in fibroblastoid cell cultures, and six of these replicated moderately in monocyte-derived macrophages (MDM), whereas all were unable to replicate in lymphoid cell lines and primary lymphoblasts.Three representative mutants and one similar clone with intact ORF-A were inoculated subcutaneously into groups of SPF cats. The animals were monitored for plasma viremia, proviral load in purified peripheral blood lymphocytes and MDM, infectious virus producing cells, T-lymphocyte cell counts, and antiviral immune responses for 7 months. Results showed that the ORF-A mutants established a low-grade infection as compared to control virus and, were preferentially monocytotropic. The mutants were also stable, except one which was present in infected cats as multiple variants since the first month post-inoculation (p.i.) but did not revert to the wild-type ORF-A. At seven months p.i., three animals/group and five uninfected controls were challenged intravenously with fully virulent FIV-Pet. The cats preinfected with the three tat-mutants appeared to resist superinfection as judged from persistingly low plasma viremia and provirus loads during nine months of follow-up, whereas control cats were readily infected starting from one month post-challenge. These findings indicate that preinfection with tat-mutant FIV might induce resistance to fully virulent FIV, that and ORF-A deletion may represent a convenient and safe way of attenuating FIV. For more solid conclusions, we must however await for sequence analysis of the virus samples reisolated from preinfected and challenged cats to be completed.

Accordo di Collaborazione N. 40C.72

Th0/Th2-BIASED REPLICATION OF R5X4 DUALTROPIC HIV-1 PRIMARY ISOLATES AND THEIR SUSCEPTIBILITY TO INHIBITION BY SINGLE CHEMOKINE CO-RECEPTOR LIGANDS.

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We have previously reported that both laboratory-adapted and primary monotropic R5 HIV-1 strains possess a replicative advantage in comparison to X4 viruses in that they are capable of spreading in sub-optimally stimulated T cells, including Th1, Th2, and Th0 cord-blood derived cell lines (CB lines). This property is conferred by gp120 Env, but is not dependent from a restriction in viral entry (E. Vicenzi et al., J. Virol., 73:7515, 1999). We have now observed that both R5X4 and R3X4 dualtropic primary isolates replicate efficiently in Th0 and Th2, but not in Th1 CB lines and T cell clones. This phenotype was not reversed by anti-cytokine neutralizing Ab towards IFN-g or IL-4.

In addition, we investigated the potential effect of chemokine-based intervention on the ability of these primary dualtropic HIV isolates to replicate in conventionally activated T cells (PHA blasts). RANTES strongly inhibited the replication of 2 out of 8 primary R5X4 viruses, whereas the CXCR4 antagonist AMD3100 efficiently suppressed the replication of other 2 HIV isolates. The remainder 4 viruses were partially inhibited by treatment with either RANTES or AMD3100. The potency of chemokine-mediated inhibition was influenced by PBMC donor variability, but it was usually independent from the levels of expression of CCR5 or CXCR4. Dual co-receptor usage was maintained by the viruses after two serial passages on U87.CD4 astrocytic cell lines expressing exclusively either CCR5 or CXCR4. Virus replication into U87.CD4-CXCR4 cells did not result in changes in the V3 region, but perturbed the dominant env V4 sequence. Of interest, double passage onto U87.CD4-CXCR4 cells determined the loss of susceptibility to RANTES inhibition. Our results indicate that blocking CCR5 may efficiently prevent the replication not only of R5 monotropic, but also of some dualtropic HIV-1 strains, whereas forced usage of CXCR4 may result in viral escape from CCR5-dependent inhibitory effects.

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RECOMBINANT CYANOVIRIN-N EXPRESSED ON THE SURFACE OF THE COMMENSAL GRAM-POSITIVE BACTERIUM STREPTOCOCCUS GORDONII BINDS HIV-1

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Streptococcus gordonii is a human commensal bacterium which we developed as a model system for expression of heterologous proteins and mucosal delivery of heterologous antigens. Recombinant S. gordonii stably colonize the mouse and the rat vaginal mucosa, and persist in the vagina of cynomolgous monkeys. We have shown that S. gordonii expressing a microbicidal scFv can be used to treat vaginal infection by Candida (Beninati et al., Nat. Biotechnol. 18, 1060-1064, 2000). To use transgenic commensal bacteria for vaginal delivery of the HIV-inactivating microbicide cyanovirin-N (CV-N), we constructed strains of S. gordonii which express CV-N on the bacterial surface, or secrete CV-N as a soluble protein. The S. gordonii-produced soluble CV-N was able to bind to soluble gp120, whereas whole recombinant bacteria expressing CV-N on the surface were shown to efficiently bind HIV-1. (supported in part by grants n.40C.74 from PNR-AIDS-1999 to GP)

N°. dell'Accordo di Collaborazione. 40c.74

ACTIVATION OF ENDOTHELIAL CELL MITOGEN ACTIVATED PROTEIN KINASE $ERK_{1/2}$ BY EXTRACELLULAR HIV-1 TAT PROTEIN

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Extracellular Tat protein, the transactivating factor of the human immunodeficiency virus type 1 (HIV-1), modulates gene expression, growth, and angiogenic activity in endothelial cells by interacting with the vascular endothelial growth factor (VEGF) receptor-2 (Flk-1/KDR) (1).

Recombinant Tat protein, produced as glutathione-S-transferase chimera (GST-Tat), activates mitogen-activated protein kinase (MAPK) $ERK_{1/2}$ in human, murine, and bovine endothelial cells whereas GST is ineffective. In bovine aortic endothelial cells, GST-Tat and the 165 amino acid VEGF isoform ($VEGF_{165}$) induce transient $ERK_{1/2}$ phosphorylation with similar potency and kinetics. The synthetic peptide Tat(41-60), but not peptides Tat(1-21) and Tat(71-86), causes $ERK_{1/2}$ phosphorylation, thus implicating Tat/KDR interaction in the activation of this signalling pathway. Accordingly, GST-Tat induces $ERK_{1/2}$ phosphorylation in KDR-transfected porcine aortic endothelial cells (2) but not in parental cells. MAPK kinase inhibitors PD098059 and U0126 prevent $ERK_{1/2}$ phosphorylation by Tat. However, they do not affect the angiogenic activity exerted by Tat in the murine Matrigel plug and chick embryo chorioallantoic membrane assays. Blocking of MAPK kinase activity impairs instead the angiogenic response to $VEGF_{165}$ and to fibroblast growth factor-2 (FGF-2).

Our data demonstrate that $ERK_{1/2}$ activation following the interaction of HIV-1 Tat protein with endothelial cell Flk-1/KDR receptor does not represent an absolute requirement for a full angiogenic response to this growth factor that appears to utilize mechanism(s) at least in part distinct from those triggered by other prototypic angiogenic growth factors.

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PENTOSAN POLYSULFATE AS INHIBITOR OF EXTRACELLULAR HIV-1 TAT

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HIV-1 Tat protein, released from HIV infected cells, may acts as a pleiotropic heparin-binding growth factor (1). On this basis, extracellular Tat has been implicated in the pathogenesis of AIDS and of AIDS-associated pathologies and has been considered a target for anti-AIDS therapies aimed to block its interaction with target cells (2). We have recently demonstrated that cell-associated heparan sulfate proteoglycans act as receptors required for cellular interaction and biological activity of extracellular Tat (3).

Here we demonstrate that the heparin analogue pentosan polysulfate (PPS) inhibits the interaction of gluthatione-S-transferase (GST)-Tat protein with heparin immobilized to a BIAcore sensor chip. Competition experiments showed that Tat/PPS interaction occurs with high affinity (K_d = 9.0 nM). Also, GST-Tat prevents the binding of ^{3}H-heparin to GST-Tat immobilized to gluthatione-agarose beads. In vitro, PPS inhibits GST-Tat internalization and, consequently, HIV-1 long terminal repeat (LTR) transactivation in HL3T1 cells. Also, PPS inhibits cell-surface interaction and mitogenic activity of GST-Tat in murine adenocarcinoma T53 Tat-less cells. In all the assays, PPS exerts its Tat-antagonist activity with an ID_{50} equal to approximately 1.0 nM. This value is close to that of the K_d of Tat-PPS interaction, thus indicating that an extracellular sequestration is the mechanism by which PPS inhibits Tat biological activities. In vivo, PPS inhibits the neovascularization induced by GST-Tat or by Tat-overexpressing T53 cells in the chick embryo chorioallantoic membrane.

In conclusion, PPS binds Tat protein with high affinity and inhibits its cell surface interaction, internalization, and biological activity in vitro and in vivo. PPS may represent a prototypic molecule for the development of novel Tat antagonists with therapeutic implications in AIDS and AIDS-associated pathologies, including Kaposi's sarcoma.

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RECOMBINANT SIMIAN IMMUNODEFICIENCY VIRUS EXPRESSING A PROTEOLYSIS-RESISTANT INHIBITOR OF NF-κB IS POTENTLY ATTENUATED .

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Simian immunodeficiency viruses (SIV) deleted of accessory genes, such as nef, vpr and vpx, are live-attenuated viruses and confer a protective immunity against virulent SIV strains. However, the attenuation by gene deletion is not sufficient to prevent the emergence of escape mutants causing AIDS. We have recently developped a novel attenuation strategy based on the addition of transcriptional inhibitors to the viral genome. A recombinant HIV-1 expressing IκB-αS32/36A, a proteolysis resistant inhibitor of NF-κB, was shown to be stably and potently attenuated in cell culture (Quinto et al. 1999 J. Biol. Chem. 274:17567-17572). In order to develop an animal model of SIV attenuation, we have analysed the effect of IκB-αS32/36A on SIVmac239 transcription and replication. The SIV transcription and production was significantly reduced by IκB-αS32/36A in transient expression assays. In particular, the tat 2-mediated transactivation of SIVLTR was inhibited by IκB-αS32/36A, and enhanced by p50/p65 NF-κB complexes. As observed in the case of HIV-1, a recombinant SIVmac239 expressing IκB-αS32/36A was highly and stably attenuated in CEMx174 as well as monkey's PBMC cultures. Based on these results, a group of four rhesus monkeys was infected with SIV expressing IκB-αS32/36A either in sense or anti-sense orientation, here referred to as SIVIκB-αS32/36A or SIV-anti-sense, respectively. A peak of viremia in the range of 10^4 viral RNA copies Eq/ml was observed at 3 weeks post-infection in all the animals, followed by a viral clearance to undetectable levels. However, while the monkeys infected with the control SIV-anti-sense virus experienced a rebound of viremia up to 10^6 viral RNA copies Eq/ml, viremia was undetectable in one animal and occasionally detected in a second monkey in SIV-IκB-αS32/36A-infected monkeys.

Taken together these results indicate that: a) IκB-αS32/36A can potently interfere with the tat-mediated transactivation of HIV/SIVLTR; b) NF-κB factors play an essential role in HIV/SIV in vivo infection.

N°. dell'Accordo di Collaborazione: 40C.77

HAART, SERUM LEPTIN AND CD4 RECONSTITUTION: A PRELIMINARY STUDY.

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Leptin is an adipocyte-derived hormone structurally related to the long-chain helical cytokine family. Recent evidences have shown that this hormone regulates T-cell homeostasis by affecting thymocyte surviving and proliferation rate of naive lymphocytes. In murine models, Leptin is involved also in the induction of the pro-inflammatory response. To investigate the role of this hormone in HIV-1 infection and immunoreconstitution upon HAART, we analysed serum Leptin and CD4+ T lymphocyte counts in HIV+ children during HAART. Serum leptin positively correlated with CD4+ T lymphocyte number before treatment. After two years follow up, a significant increase in CD4+ T cells and serum Leptin were observed in the majority of the patients. Of note, in children not showing an increase in CD4 count, serum Leptin remained unchanged. Our findings reveal a novel link between CD4+ T-lymphocytes and serum Leptin in HIV-1 infection..

Accordo di Collaborazione N° 40C.78

ROLE OF T LYMPHOCYTES IN THE PROTECTION AND IN THE IMMUNOPATHOLOGY OF HIV INFECTION

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The research of the last year was focalized on two main topics:
1.Better replication of both X4- and R5-tropic HIV-1 strains in Th2 cells, despite the different expression of CXCR4 and CCR5, being predominantly expressed by Th2 and Th1 cells, respectively.
2. HIV-1 entry and replication in different human thymocytes subpopulations
Regarding the first topic, Th1- or Th2-oriented memory, as well as Th1- or Th2-polarized naive, T cells were infected in vitro, in parallel cultures, with both an X4- and R5-tropic HIV-1 strain (IIIB and BaL, respectively) and assessed for their profile of cytokine production, CXCR4 and CCR5 receptors expression and HIV-1 p24 Ag production. Higher p24 Ag production was found in CCR5-negative Th2-like than in CCR5-positive Th1-like memory T cells using both HIV-1IIIB and BaL strains. By contrast, p24 Ag production was higher in Th1-polarized activated naive T cells in the first four days after infection with HIV-1BaL. However, it became comparable or even lower than in Th2- polarized populations later during infection or when the cells were infected with HIV-1BaL after secondary stimulation. The higher levels of p24 Ag production by Th1-polarized naive T cells soon after infection reflected a higher virus entry, as assessed by the single round infection assay using the HIV-CAT R5-tropic virus that contains the YU2 Env protein. The limitation of virus spread in the Th1-polarized populations, despite the initial higher level of T-cell entry of R5-tropic strains, was due to the ability of Th1 cells to produce amounts of β-chemokines higher than Th2 cells. In fact, an inverse correlation between p24 Ag production and the release of CCR5-binding chemokines RANTES, MIP-1α, and MIP-1β was observed in both Th1-polarized naive and Th1-like memory activated T cells. Moreover, infection with HIV-1BaL strain of Th1-polarized T cells in the presence of a mixture of anti-RANTES, anti-MIP-1 α, and anti-MIP-1 β neutralizing antibodies resulted in a significant increase of HIV-1 expression. These findings suggest that Th1-type responses may favor $CD4^+$ T-cell infection by R5-tropic HIV-1 strains, but HIV-1 spread in Th1 cells is limited by their ability to produce CCR5-binding chemokines.
Regarding the second main topic of the project, really recent data, obtained in our laboratory, showed the expression of the CD30, a member of the TNF superfamily, by a small CD4+CD8+ thymocytes subpopulation and of the CD30L, the natural ligand of the CD30, by medullary epithelial cells in the human thymus. Even if the physiological role of the CD30-CD30L interaction in human thymus is still to be explained, several evidences suggest its role in the thymus negative selection. As some years ago we showed a crucial role of the CD30-CD30L interaction for HIV-1 replication in mature T cells, we wondered if: 1) the CD30+ thymocytes were more susceptible to HIV infection and replication; 2) the CD30-CD30L interaction could enhance the HIV-1 replication also in immature T cells. To do this, we infected in vitro, using both HIV-1IIIB (X4-tropic) and HIV-1BaL (R5-tropic), unfractioned, CD30- and CD30+ thymocytes purified from normal postnatal thymus specimens obtained from 10 children, aged between 5 days and 3 years, who underwent corrective cardiac surgery. At the moment, the results indicate a statistically higher HIV-1 replication, resulting in a higher p24 Ag production, for both HIV-1 strains in CD30+ sorted thymocytes in comparison to unfractioned and CD30- populations. It is of great interest that sorting out the CD30L positive cells from the CD30+ fractions the p24 productions were similar to those obtained in CD30- populations. The same results were obtained when the CD30+ fractions were infected an cultured in the presence of an anti-CD30L neutralizing monoclonal antibody. Even if these results are still preliminar, it could be of great interest to understand the reason why the CD30-CD30L interaction, that normally leads to thymocytes death, results in thymocytes activation after HIV-1 infection.
Titolo: "Ruolo dei linfociti T nella protezione e nella immunopatologia dell'infezione da HIV1"
Responsabile Scientifico: Prof. Sergio ROMAGNANI

No. Contributo: 40C.79

IN VITRO ANTI-HIV-1 INHIBITORY EFFECT OF COMBINATION OF MAXIMALLY SUPPRESSIVE CONCENTRATIONS OF CCR5- AND CXCR4-INHIBITORS.

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Antagonists of chemokine receptors CCR5 and CXCR4 are potential antretroviral agents. We studied the combined effects of a derivative of SDF-1β, Met-SDF-1β, and a modified form of RANTES, AOP-RANTES.
Inhibitory effects were tested with or without the two fusion inhibitors at virus inhibitory concentrations of 95% or 99% (IC95 or IC99). The viral isolates examined in the present study were derived from two patients with primary HIV-1 infection (PHI) before the start of any antiretroviral treatment: one (RM) was CCR5-tropic and the other (DK) was CXCR4-tropic. Experiments were conducted utilizing single or combined drugs against infection with RM or DK viruses or against mixed infection (50 : 50) with these two viruses. Studies were conducted in peripheral blood mononuclear cells (PBMC) or in U87MG-CD4 CCR5 or CXCR4 cell lines.
AOP-RANTES inhibited R5 viruses but not X4 viruses, whereas Met-SDF-1β inhibited X4 viruses, but not R5 viruses when used in the appropriate cells at IC95 or IC99 concentrations. Combination of AOP-RANTES and Met-SDF-1β inhibited dual infections with R5 and X4 viruses (95-99 %), whereas single drugs suppressed dual infections less well (32-61 %). Sequencing studies confirmed the outgrowth of single strain virus in subcultures when only one drug was used, e.g. R5 virus when Met-SDF-1β was used and X4 virus when AOP-RANTES was used. Subsequent plasmid cloning showed a higher frequency of DNA integration of the CCR5+ isolates in dual HIV-1 infections in the absence of drugs, as well as the maintenance of HIV-1 nucleic acid even in the presence of both dual-target inhibitors.
Our experiments suggest that use of combined inhibitors of R5 and X4 viruses may be useful to inhibit mixed infections mediated by viruses with a different tropism. Our study found the proof of principle for dual-receptor attack against HIV-1 attachment and entry.

N°. dell'Accordo di Collaborazione: 40C.80

THE IκB KINASE IKK IS THE MOLECULAR TARGET FOR ANTI-RETROVIRAL CYCLOPENTENONE PROSTANOIDS.

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The IκB kinase (IKK) complex, containing a regulatory (IKKγ) and two catalytic (IKKα and IKKβ) subunits, is a key enzyme in signalling the activation of the cellular transcription factor NF-κB. IKK activated in response to pro-inflammatory cytokines phosphorylates the NF-κB inhibitory proteins IκB, triggering their conjugation with ubiquitin and subsequent degradation. Freed NF-κB dimers translocate to the nucleus and induce the transcription of a variety of cellular as well as viral genes. NF-κB is a critical regulator of the immediate early pathogen response, playing an important role in promoting inflammation and viral gene expression, and is involved in promoting the progression of AIDS by enhancing HIV-1 transcription. We have previously shown that cyclopentenone prostanoids (cyPG), including PGA_1, PGJ_2 and $15dPGJ_2$, inhibit HIV-1 replication blocking viral RNA transcription by activating a cytoprotective cellular response (1-3). We have now identified the molecular target of cyclopentenones, and have shown that reactive cyPG are potent inhibitors of IKK activation induced by TNFα and IL-1 in human lymphoblastoid cells (4). CyPG inhibit IKK by direct modification binding to a cysteine residue at position 179 in the activation loop of the IKKβ subunit. The fact that high levels of endogenous cyPG obtained after transfection of human cells with expression vectors for enzymes ($cPLA_2$, COX-2 and PGD synthase) implicated in the biosynthesis of $15dPGJ_2$ resulted in the complete inhibition of TNFα-induced IKK activation, suggests the possibility that cyPG could be physiological inhibitors of NF-κB (4). Finally, we have shown that stimulation of IKK leading to transactivation of HIV-1 transcription is obtained during infection with herpes simplex and influenza viruses also in the absence of pro-inflammatory stimuli. Inhibition of IKKβ activation by cyPG was found to prevent virus-induced HIV-1 transcription. The results emphasize the role of IKK in the positive regulation of HIV-1 transcription and indicate that treatment with cyPG or different IKK inhibitors could be beneficial in preventing transactivation of HIV-1 transcription during viral infection and inflammatory disorders.

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THE HIV-1 TAT PROTEIN ASSOCIATES WITH E2F4, A TRANSCRIPTION FACTOR INVOLVED IN THE CELL CYCLE REGULATED GENE EXPRESSION

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The HIV-1 Tat protein plays an important role in the regulation of viral transcription and replication as well as in the development of AIDS-related diseases. Tat is directly interferes with several cellular processes by deregulating the expression of cellular genes (G. Scala et al. J. Exp. Med. 179:961-971, 1994), and by physical interacting with cellular proteins (C. Ambrosino et al. J. Biol. Chem. 272: 14483-14492, 1997). To gain further insights into the Tat-mediated functions, a lymphoblastoid cell line cDNA library was screening by using the two-hybrid system to identify cellular proteins interacting with Tat in vivo. To this end, Tat cDNA was cloned in frame with the DNA binding domain of the yeast transcription factor Gal 4 in pAS plasmid and co-expressed in Y190 yeast strain with the cDNA library cloned downstream the activation domain of Gal4. Several Tat interacting proteins were identified; among them E2F-4, a transcription factor involved in the regulation of cell cycle and in the expression of apoptotic genes, was selected for further studies. Two-Hybrid studies performed with plasmids expressing either the full length, or discrete domains of HIV-1 Tat protein and E2F-4 fused to the appropriate GAL4 domain showed that the cysteine rich domain of both proteins was involved in the physical interaction. The in vitro interaction between Tat and E2F-4 was verified by GST pull down experiments performed with the fusion protein GST-Tat and whole cellular extracts prepared from a lymphocyte T cell line (Jurkat cells) or E2F-4 in vitro translated. The Tat/E2F-4 interaction was confirmed in vivo by co-immunoprecipitation assays performed with nuclear and whole cellular extracts prepared from Jurkat transiently expressing the Flag-Tat protein. Super shift assays revealed DNA binding complexes formed by endogenous E2F-4 and Tat. Moreover, the viral protein promoted the nuclear binding activity of E2F-4. The functional relevance of this interaction was confirmed by co-expression of both genes in mammalian cells. We verified that HIV-1 Tat protein specifically promoted the activity of a E2F-regulated promoter cloned upstream to the cat indicator gene. Moreover, E2F-4 cooperated with Tat in inducing a substantial HIV-1 transcription. The role of Tat in cell cycle regulation was investigated by cell synchronization experiments performed with cells expressing a GFP-Tat fusion protein. These experiments showed a clear S phase deregulation in Tat expressing cells. The results shed some light on the role of Tat in HIV-1 transcription and replication, and may help to clarify the relationship between HIV replication and cell cycle regulation.

N°. dell'Accordo di Collaborazione:40C.82

VIROLOGIC AND CLINICAL OUTCOMES OF RHESUS MACAQUES VACCINATED WITH PHAGE-DISPLAYED HIV-1 EPITOPES AND SUBSEQUENTLY INFECTED WITH SHIV-89.6PD.

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To identify HIV-specific epitopes, we screened random peptide libraries (RPL) displayed on phages by using HIV-positive sera. By several criteria, these peptides behaved as antigenic mimics of conformational B-cell epitopes generated in vivo in the course of the natural HIV-1 infection. Consistent with these findings, sera of monkeys infected with SHIVs carrying envelopes from different primary isolates, such as DH12, 89.6 and 89.6P, also recognized the pool of HIV-specific epitopes (G. Scala et al., J.Immunol 1999, 162: 6155). When injected in a group of five Rhesus macaques with QS21 adjuvant, a pool of five epitopes induced an antibody response specific for each of the single epitopes and this response cross-reacted with HIV-1 envelope proteins. The mimotope-immunized animals, together with a control group of four monkeys immunized with wild-type phages and a group of three naïve animals, where challenged iv. with 60 AID_{50} of SHIV-89.6PD. During six months of observation, monkeys in the control groups experienced high peaks of viremia with substantial viral load thereafter, an irreversible decline of CD4 T cells and AIDS-like syndromes at six weeks post-challenge that required euthanasia. The mimotope-immunized monkeys were unprotected from primary infection; however, four out of five animals showed reduced levels of peak viremia with low or undetectable levels thereafter, and a sustained number of CD4+ T cells ranging between 30% and 60% of the pre-challenge levels. The four protected monkeys elicited a strong anamnestic antibody response against the challenge viral strain in the absence of AIDS-related diseases. The results suggest that phage-displayed epitopes may serve as a new approach in designing HIV-1 vaccines.

N°. dell'Accordo di Collaborazione:40C.83

MOLECULAR AND BIOLOGICAL EVOLUTION OF HIV-1 DURING DISEASE PROGRESSION OF INFECTED CHILDREN.

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Determination of the co-receptor usage of HIV-1 *in vivo* is relevant both to the prediction of disease progression and to the precise understanding of the dynamic process of virus evolution in infected subjects. The aim of our project was to study biologic and molecular evolution of HIV-1 during disease progression in children infected by their seropositive mother.

Between 2 and 8 sequential HIV-1 primary isolates derived from 9 children with slow or fast disease progression were studied. The biological phenotype of PBMC-derived isolates was determined in U87.CD4 cell lines expressing the chemokine receptors CXCR4, CCR5, CCR3, CCR2, and CCR1. Plasma-derived viruses were tested with Tropism Recombination Test (TRT), which relies on homologous recombination of RT-PCR amplified V1-V3 *env* sequences and allows the parallel genotypic and phenotypic analysis of the recombinants. Amino acid sequences of the V3 *env* region were obtained after cloning.

The first viral isolate close after birth from all children, except one, was of a R5 phenotype as determined in U87 CD4 cells. Later on and independently from the rate of disease progression, X4 viruses emerged in 7 out of 9 cases. Viral clones spanning V1-V3 env region, amplified from plasma of 3 children with a switch in viral phenotype, and tested in TRT, revealed that the R5X4 viral population was always composed of mixtures of monotropic, and dualtropic viral variants. Interstingly, the R5 population persisted after the emergence of X4 and dualtropic viruses in all tested patients. The amino acid sequences of the V3 loop of the clones obtained from the plasma of these children showed a marked increase of variability during time. Interestingly, X4 and R5X4 viruses of the same patient displayed identical V3 amino acid sequences, indicating that changes in this region are necessary for the use of CXCR4 but the overall context of the V1-V3 region is important to preserve the capacity to use CCR5. The phylogenetic analysis of the V3 region confirmed that R5X4 viral sequences are more related to X4 sequences than to R5 sequences.

In conclusion, viral phenotypic variation increases during disease progression, with frequent emergence of X4 monotropic or dualtropic viruses. Persistence of R5 viruses indicates that the environmental conditions favoring X4 and dualtropic virus replication *in vivo* are not necessarily more restrictive for R5 viruses. Moreover, our findings call attention to the need for further studies on the whole envelope gp120 since the interaction of several env domains may influence co-receptor utilization.

N. Accordo Collaborazione: 40C.84.

PATHOGENESIS OF PULMONARY INVOLVEMENT DURING HIV INFECTION

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The presence of HIV in the lung may cause an interstitial lung disease (ILD) characterized by an infiltration of HIV-specific CD8+ cytotoxic T lymphocytes (CTL) in the pulmonary interstitium and alveolar spaces. Although present at all stages of HIV infection, lymphocytic alveolitis is most pronounced in patients with early-middle stage disease. Alveolar macrophages (AMs) are not bystander cells in the development of CTL alveolitis, since they release a broad array of mediators of inflammation, such as cytokines (IL-15), proinflammatory cytokines (TNF-alpha, IL-6), and growth factors (GM-CSF) which favor CTL response. According to the major lines of our research project, the following results were recently obtained using the cells retrieved from the bronchoalveolar lavage (BAL) of HIV-infected patients.

We asked whether differences in the pulmonary production of chemoattractant molecules contribute to recruit specific T-cell subsets during HIV-associated ILD. We have shown that T lymphocytes purified from BAL of 29 patients with HIV infection are ultimately IFN-gamma producing cells. We found that CXCR3 and IL-15 receptor chains are expressed at the protein and mRNA level by BAL T cells. Immunohistochemical examination of lung biopsies showed that areas of pulmonary inflammation were infiltrated by T cells expressing CXCR3 and IL-15Ralpha. We have also demonstrated that lung CTL may show an overexpression of CCR6, CCR7 and CCR8. AMs purified from the BAL of patients with T-cell alveolitis but not from normal subjects expressed the IFN-gamma-inducible cytokines CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC) and IL-15. Concerning the CC chemokines, we demonstrated that AMs show an heterogeneous expression of CCL4 (MIP-1beta), CCL5 (RANTES) and CCL20 (MIP3alpha). Immunohistologic studies showed that ligands of CXCR3 and IL-15 were mainly expressed by infiltrating macrophages. From a functional point of view, purified BAL macrophages in vitro secreted definite levels of CXCR3 ligands capable of inducing chemotaxis of the CXCR3+ T-cell line 300-19; furthermore, they secreted IL-15 which was capable of inducing T-cell migration in chemiotaxis assay per se and inhibited T-cell apoptosis of lung T cells. IFN-gamma definitively upregulated the release of both CXCR3 ligands and IL-15 by alveolar macrophages. Finally, stricking levels of CXCR3 ligands and IL-15 were demonstrated in the fluid component of the BAL of patients with T-cell alveolitis. Taken together our data suggest that the dominant intraalveolar expression of IFN-gamma, which takes place in the lung during HIV-associated ILD, up-regulates the interactions between CXCR3 ligands, IL-15 and related receptors in the pulmonary milieu, favouring the trafficking of T cells involved in the pathogenesis of HIV-associated T-cell alveolitis.

Research grant: 40C.85

A SUBSET OF HLA CLASS I MOLECULES BINDS TO HIV-1 TRANSMEMBRANE GLYCOPROTEIN GP41 CONDITIONING VIRUS ENTRY

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HIV-1 entry is conditioned by sequential interactions of gp120 with CD4 and a chemokine receptor. We hypothesized that also gp41 needs to interact with a cellular protein to complete fusion. We demonstrated that a recombinant gp41 and a gp41-derived peptide (aa 616-642) specifically bind to HLA-C and we co-precipitated rgp41 with HLA-Cw4 using two HLA-C-reactive mAbs. Using a panel of transfected cells derived from the class I-negative B-cell line 721.221 (resistant to HIV infection), we demonstrated that both infection and fusion are dependent on the presence of HLA class I molecules on the surface of recipient cells. The infection was inhibited by an HLA-Cw4-restricted peptide, but not by mutated peptides, confirming that the HLA class I-dependent step is viral entry. These data provide evidence that the binding between gp41 and HLA-C is required for HIV infection.

N°. dell'Accordo di Collaborazione: 40C.86

NOVEL GENE PRODUCTS MODULATED BY HIV-1-TAT IN HUMAN ENDOTHELIAL CELLS: POSSIBLE ROLE IN THE PATHOGENESIS OF AIDS-KAPOSI'S SARCOMA

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The Tat protein of HIV, which enhances HIV-1 transcription and also affects strategic host genes, can function as a cytokine in the activation of endothelial cells. Moreover, HIV-1 Tat plays a role in the pathogenesis of Kaposi's sarcoma (KS), a highly vascularized skin lesion characterized by marked endothelial proliferation and migration, resulting in the formation of new capillaries. It is noteworthy that HIV-1 Tat is angiogenic in vivo and that Tat-transgenic mice develop highly vascularized lesions which closely resemble KS (1-4). Since HIV-1 Tat affects endothelial cell function (5), we assumed that the isolation of differentially expressed genes in Tat-treated endothelial cells would yield insights into the molecular mechanisms contributing to endothelial dysfunction in AIDS-associated Kaposi's sarcoma. By RNA fingerprinting, we isolated several transcripts modulated by HIV-1 Tat and focused our attention on two of them denominated Endothelial Differentiation Factor (EDF)-1 and WHSC2.
EDF-1, which is involved in the repression of endothelial differentiation, is downregulated by exposure to HIV-1 Tat of endothelial cells (6). Moreover, it is expressed at much lower levels in Kaposi's spindle cells than in endothelial or smooth muscle cells. EDF-1 encodes a basic intracellular protein of 148 amino acids which has an IQ motif, a conserved region of about 20 residues which contains a calmodulin binding domain and a protein kinase C (PKC) phosphorylation site. Indeed, human EDF-1 interacts with calmodulin and is phosphorylated by PKC both in vitro and in vivo. It is noteworthy that these two events are mutually exclusive (7). On the basis of the high homology of EDF-1 with Multiprotein Bridging Factor-1, a transcriptional coactivator which binds TATA Binding Protein (TBP), we have also demonstrated that EDF-1 interacts with the TATA Binding protein (TBP) in vitro and in human endothelial cells (7). We therefore hypothesize that EDF-1 serves two main functions in endothelial cells: i) to bind CaM in the cytosol at physiologic concentrations of Ca^{2+}; and ii) to act in the nucleus as a transcriptional coactivator through its binding to TBP.
WHSC2 is downregulated by HIV-1-Tat, whereas it is not modulated by angiogenic and pro-differentiative molecules. WHSC2 encodes a basic polypeptide of 528 amino acids with a molecular weight of 57 kDa. WHSC2 has two nuclear translocation sequences which actively mediate its transport to the nucleus, as shown in whsc2-GFP transfected NIH 3T3 cells (8). We also found a helix-loop-helix (HLH) motif in region 130-185. Since members of the HLH family control differentiation and cell cycle progression, we hypothesize that WHSC2 may function as a transcriptional repressor.

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Contributo 40C.87

NEUROLOGICAL SYMPTOMS DURING PRIMARY HIV INFECTION CORRELATE WITH HIGH LEVELS OF HIV-RNA IN CEREBROSPINAL FLUID.

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Neurological involvement occurs in about 40% of patients with primary HIV infection (PHI). Clinical manifestations may range from mild signs to a severe picture of meningoencephalitis. This analysis involves 22 patients with diagnosed symptomatic PHI. Correlation between plasma and CSF HIV-RNA, and between plasma and CSF HIV-RNA were analyzed. A covariance analysis, using neurological symptoms as the grouping variable, was then performed. Neurologic symptoms were present in 11 patients, ranging between severe and persistent headache to clinical signs suggestive of meningitis. We found a strong relationship between neurological symptoms and CSF viral load. The mean CSF HIV-RNA was 4.12 log in the neurological symptoms group and 2.58 log in patients without neurological symptoms ($p<0.00001$).
Our results indicate that plasma viral load alone does not correlate or predict the CNS involvement. This features indicate that HIV-1 RNA can be detected almost always in CSF in acutely infected patients and are a strong argument in favor of a local production of HIV-1. Moreover, an early treatment including drugs with high penetration in the central nervous system must be considered in patients with PHI.
Further studies aimed at investigating the tropism and coreceptor usage of HIV strains isolated from CSF are presently ongoing.

Grant n° 40C.88

EXPANSION OF RARE $CD8^+CD28^-CD11B^-$ T CELLS WITH IMPAIRED EFFECTOR FUNCTIONS IN HIV-INFECTED PATIENTS.

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The decline in the number of $CD4^+$ T cells in HIV-1-infected (HIV^+) patients is known to be related to the increased number of $CD8^+CD28^-$ T cells. We have shown that $CD8^+CD28^-$ T cells from HIV^+ patients have impaired in their capability to interact with human endothelial cells. This is due to the dramatic expansion, within this subset, of rare $CD11b^-$ cells lacking cell-cell adhesion functions. In 50 HIV^+ patients, 19.5% ± 6.5% of all T cells were $CD8^+CD28^-CD11b^-$, whereas only 0.8% ± 0.4% of all T cells from healthy donors showed this uncommon phenotype. This population is peculiar to HIV infection and was found to have impaired cytotoxic properties. $CD8^+CD28^-CD11b^-$ T cells, which lack the ability to interact with endothelial cells, are likely to accumulate and persist in circulation. Lack of cell-cell adhesion and impaired cytolytic functions favor the hypothesis of a role of $CD8^+CD28^-CD11b^-$ T cells in the development of immunodeficiency. Further studies on the biological properties of this lymphocytes have shown that they possess some characteristic associated with effector function. $CD8^+CD28^-CD11b^-$ T cells, as their $CD11b^+$ counterpart, gave no rise to development of lymphoblasts as well as they did not proliferate after mitogenic stimulation. Evaluation of their spontaneous cytokine pattern have shown that $CD8^+CD28^-CD11b^-$ T cells are capable to produce high amounts of pro-inflammatory cytokines but not of IL-2 and IL-4. Given that the $CD8^+CD28^-CD11b^-$ T cells displayed a strongly reduced cytolytic activity, we performed experiments to assess whether these cells contained perforin granules. Flow cytometric analysis at single cell level on freshly isolated $CD8^+$ lymphocytes displayed a strongly reduced perforin expression in terms of mean fluorescence intensity. Taken together these data suggest that $CD8^+CD28^-CD11b^-$ T cells might be end-stage and/or aberrant differentiated effector cells.
In a cross-sectional study we have previously found a highly significant negative correlation between the percentage of $CD4^+$ T cells and the percentage of $CD8^+CD28^-CD11b^-$ lymphocytes ($\rho = -0.82$), which was lacking for $CD8^+CD28^-CD11b^+$ cells ($\rho = 0.10$). In a longitudinal study, preliminary data obtained with patients treated with HAART over a 24 month follow-up showed an opposite trend among $CD4^+$ and $CD8^+CD28^-CD11b^-$ T cells. The increase in percentage of $CD4^+$ cells is always concomitant with a decline in the percentage of $CD8^+CD28^-CD11b^-$ lymphocytes. Thus, the decline in circulating $CD4^+$ T cells seems to be closely related to the presence of $CD8^+CD28^-CD11b^-$ T cells. Evaluation on the significance of these data and their correlation with the changes in the viral and clinical parameters, which occurred during the treatment, is ongoing.

N°. 40C.90. dell'accordo di collaborazione.

LPS INDUCES THE SECRETION OF MACROPHAGES-DERIVED SOLUBLE FACTOR(S) ABLE TO SUPPRESS THE REPLICATION OF PRIMARY X4 HIV-1 ISOLATES IN MACROPHAGES AND T LYMPHOCYTES

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We have shown that bacterial lipopolysaccharide (LPS) protects primary human monocyte-derived macrophages (MDM) from productive infection by R5 HIV-1 isolates largely through the release of RANTES, MIP-1α and MIP-1β. The finding that CXCR4 is a functional coreceptor for HIV-1 infection of macrophages prompted us to investigate the effects of bacterial LPS on MDM infected with X4 HIV-1 isolates.
Our results show that LPS was able to inhibit infection of MDM by X4 HIV-1 isolates through direct and marked downregulation of both CD4 and CXCR4. Furthermore, soluble factor(s) released upon LPS treatment (LPS-conditioned supernatants) neutralize infection with CXCR4-dependent viruses in macrophages as well as T lymphocytes.
Infection of both cell types appear to be blocked mainly at the level of entry and is independent by the only known natural ligand for CXCR4, because macrophages, unstimulated or LPS-treated, did not secrete SDF-1. Inhibition of HIV-1 entry was unrelated to the release of IFN-α\β. Indeed, unlike LPS-conditioned supernatants, IFN-α did not reduce proviral DNA levels at early times post-infection; moreover, depletion of IFN-α\β from LPS-conditioned supernatants did not neutralize their inhibitory potential. Finally, we didn't detect any HIV-1 inhibitory activity using macrophage-derived chemokine (MDC).
Overall these results strongly point to the existence of additional soluble HIV-1 suppressive factor(s) so far uncharacterized.

Accordo di collaborazione N° 40C.91

EVOLUTION OF THE NEF GENE IN A COHORT OF HEMOPHILIACS WITH PROGRESSING AND NON-PROGRESSING HIV INFECTION.

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We have previously characterized the nef gene variants in a group of 7 LTNP and 8 progressors, all belonging to the same cohort of infected hemophiliacs in a cross-section study (Virology 259:349-368, 1999). A longitudinal analysis of the nef gene evolution was, however, important to determine its potential variations and relationship to HIV disease progression since in the meanwhile (between 1995 and 2000) 3 of 7 LTNP have progressed to AIDS (late progressors, LP). Cloning and sequencing from both virion RNA and peripheral blood mononuclear cells (PBMC)-associated HIV DNA have been carried out from blood samples withdrawn three years after the previous report. Defective nef sequences still coexisted with full-length nef open reading frames (ORF) in 1 out of 4 LTNP, and in the 3 LP. Four out of 8 progressors were lost at follow up, whereas 3 of the 4 remainder progressors had nef defective sequences mixed with intact Nef. Therefore, the proportion of disrupted nef sequences within each individual appeared to be higher in progressors and LT (ranging from 10% to 90%) as compared to LTNP (ranging from 0 to 10%) suggesting that viral replication could determine the accumulation of variants with defective Nef. In-frame small deletions including the deletion of two alanines at position 49-50, that determined a defective replicative capacity of the laboratory-adapted LAI/IIIB isolate were maintained in all clones from 1 LTNP and 2 progressors.

Since Nef causes downmodulation of CD4 from the cell surface, a Nef allele from each individual was selected as the predominant RNA species, and cloned in an expression vector under the control of the beta-actin promoter. Co-transfection of Nef-expressing vector with a plasmid expressing CD4 was carried out in COS cells and CD4 modulation was tested by Facs analysis. Three out of 11 Nef alleles derived from 2 LTNP and 1 LP were less efficient (approximately 50%) in the downmodulation of CD4 as compared to Nef from the standard LAI/IIIB isolate. The Nef amino acid (aa) sequences of these 3 alleles were compared to the LAI/IIIB Nef sequence. No single aa position could predict the diminished efficiency in CD4 downmodulation, whereas we interpret these finding as an indicator that the aa changes observed in the Nef allele might affect the overall protein conformation and influence its biological effects.

N°. dell'Accordo di Collaborazione 40C.92

VACCINATION OF CATS AGAINST FELINE IMMUNODEFICIENCY VIRUS (FIV) BY INOCULATION OF PLASMID DNA ENCODING THE VIRAL PROTEINS ENV AND REV

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This study intends to evaluate the efficacy of a vaccination procedure using plasmid DNA encoding the Env and Rev proteins of FIV. To this end, groups of animals immunized according to a protocol that entails repeated and combined i.m. inoculations of cardiotoxin and plasmids coding for the viral proteins were monitored immunologically over time, and resistance to challenge with homologous virus is being evaluated.

FIV-specific antibody response were evaluated by Western Blot utilizing virus purified by ultracentrifugation as antigen. This method, which is sufficiently sensitive to detect the presence of virus-specific antibodies in infected cats, has given negative results for all the sera samples collected at 6, 16 and 20 weeks since the start of the protocol. This result does not imply immunization failure; previous findings obtained by our group and other researchers did not find correlation between presence of virus-specific antibodies and protection against the viral infection in cats vaccinated with a DNA plasmid encoding a partially deleted FIV.

To evaluate cell-mediated response, we plan to use a recently developed TaqMan-PCR method for analysing cytokine expression pattern. This assay proved reliable for measuring feline TNF alpha and IFN gamma expression of Con-A or whole FIV stimulated PBMC obtained from infected and uninfected cats. This method is being set up for measuring other cytokines such as IL-6, IL-18 and IL-16.

In reference to the challenge, animals will be inoculated i.v. with the FIV-Pet isolate cultured in the feline T lymphocyte line FL4 and titered in vivo. The course of the infection will be evaluated from the 2^{nd} to the 24^{th} week post-challenge by measuring plasma viremia and PBMC proviral load with RT-PCR-/PCR-TaqMan. The number of infected PBL will be calculated by means of viral isolation with the feline lymphoid cell line MBM. The course of disease will be followed by evaluation of standard chemical-clinical parameters, and analysis of circulating $CD4^+$ and $CD8^+$ T-cells. The results obtained will enable us to establish the potentialities of DNA vaccination in the feline model in comparison with the conventional procedure of vaccination.

Accordo di Collaborazione 40C. 93

NATURAL KILLING OF AUTOLOGOUS DENDRITIC CELLS IN INHIBITED BY HIV-1 TAT THROUGH THE BLOCK OF CAMKII.

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An impaired clearance of infected dendritic cells (DC) by natural killer (NK) cells may be critical in early HIV-1 infection. Objective of this study is the definition of the role played by HIV-1 Tat protein in this process.
Design: The ability of NK cells to lyse autologous DC or HIV-1 infected cells and the molecular mechanism(s) underlying this killing were investigated in the presence or absence of synthetic Tat or Tat-derived peptides.
The killing of DC and of chronically HIV-1 infected cells by NK cells was assessed by a 4-h 51 chromium release assay. In addition the following biochemical steps, critical for the delivery of the lethal hit, were examined: effector-target cell adhesion, lytic enzyme release, extracellular calcium influx and activation of the calcium-calmodulin kinase II (CAMKII).
Binding to DC induces a calcium influx in NK cells, followed by the release of perforin and granzymes, leading to DC killing. The activation of CAMKII is required for this process, as DC lysis is strongly reduced in the presence of CAMKII inhibitors. NK cell mediated killing of DC and of HIV-1 infected cells is impaired by treatment with exogenous HIV-1 Tat, that blocks calcium influx and degramulation and impairs CAMKII activation in NK cells.
These data provide evidence that Tat contributes to the impairment of autologous DC killing by NK cells. As DC represent an important reservoir of HIV-1, Tat-mediated inhibition of their killing may favour the persistence of the virus.

N. 40C.94

The National research program on AIDS

(Extramural research projects)

Project

OPPORTUNISTIC INFECTIONS AND TUBERCULOSIS

Scientific Coordinator: **Antonio CASSONE**

Projects financed N° 34

MODULATION OF TELOMERASE ACTIVITY OF NORMAL LYMPHOCYTES BY HIV-TAT OR ANTI-HIV AGENTS

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Telomerase is a ribonucleoprotein which elongates and/or maintains telomeres by adding TTAGGG tandem repeat sequences, thus protecting chromosomes from degradation and rearrangements. Telomerase activity is associated with cell immortalization and malignant progression. Most of the normal somatic cells express very low or undetectable levels of telomerase activity and show decreasing telomere lengths at each cell division. However, telomerase activity increases in normal lymphocytes following antigenic stimulation, probably to prevent excessive telomeric loss and senescence in expanding clones during the generation of immune reponses. Recent studies show that Terminal Restriction Fragments (TRF) of telomeres of CD4+ cells in advanced HIV-infected patients are shorter than those of uninfected subjects. This suggests that a decline of telomerase activity could occur in lymphocytes of AIDS patients, leading to impairment of the proliferative capacity of different immunocompetent cell clones. Aim of the present studies has been to evaluate the possible influence of HIV-associated products and anti-HIV agents on stimulation-induced telomerase activity in non adherent mononuclear cells (NA-MNC) obtained from peripheral blood of healthy donors. Our results showed that exposure of cells to high concentrations (up to 1000 ng/ml) of soluble recombinant HIV-Tat protein reduce telomerase activity of NA-MNC stimulated by recombinant antibodies to CD3/CD28 membrane antigens. On the other hand, Tat concentrations less than 1 ng/ml did not impair telomerase function. Expression of catalytic sub-unit of the enzyme (i.e. hTERT) has been considered the rate limiting factor for telomerase activity. Therefore, studies have been performed on a possible modulation of htert mRNA mediated by soluble Tat. It was found that high concentrations of the factor (i.e. 100-1000 ng/ml) reduced the expression of htert mRNA. In contrast, soluble Tat up-regulated the levels of htert mRNA at lower concentrations. Moreover, in accordance with these results, high concentrations of recombinant Tat down-regulated the binding of nuclear factors to the E-Box region of htert gene promoter: The region is a consensus binding sequence for c-Myc/Max complexes, responsible of 60-70% of htert gene transcription. These findings suggest a potential mechanism by which the HIV product could contribute (directly or indirectly) to the decline of telomerase activity.

Studies on the possible influence of anti HIV-agents on telomerase activity have been focused on Saquinavir, a protease inhibitor playing a pivotal role in HAART , and on Paclitaxel, a microtubule-stabilizing drug that has recently been found to be effective in the treatment of patients with advanced HIV-associated Kaposi sarcoma. NA-MNC were stimulated in a non selective way with PHA or with αCD3 monoclonal antibody. Treatment with Saquinavir was able to up-regulate telomerase activity. The increased levels of telomerase activity observed in Saquinavir-treated cells were accompanied by up-regulation of htert mRNA and increased levels of binding of nuclear factors to the E-Box of htert promoter. On the other hand, treatment of PHA-stimulated NA-MNC with Paclitaxel led to a concentration-dependent reduction of telomerase activity. This effect was accompanied by reduced levels of htert mRNA and of the binding of nuclear factors to the SP-1 specific binding region of htert promoter. In conclusion, the present results suggest that the effects of therapeutic agents used against HIV infection or AIDS-related diseases on telomerase activity of immunocompetent cells should be taken into proper consideration. In particular, greatly attractive appears to be the "anti-senescence" activity of Saquinavir, which would allow adequate clonal expansion of immunocompetent cells during long-term therapy against HIV infection

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BACILLUS CALMETTE-GUERIN (BCG) DOWN-REGULATES INDUCTION OF GROUP 1 CD1 MOLECULES BY GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

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A number of recently published studies support the hypothesis that T cell responses against non-peptide antigens presented by CD1 molecules expressed on cytokine-activated monocytes (CAM) can play an important role in host resistance against mycobacterial infections. On the other hand, host sensitization with BCG, antigenically related to M. tuberculosis, and expected to induce either MHC-restricted and CD1-restricted T cell responses, does not provide full protection against mycobacterial diseases. Previous studies performed by Stenger et al. (J.Immunol. 161: 3582-88, 1998) showed that exposure of GM-CSF-pretreated CAM to a virulent strain of M. tuberculosis is able to down-regulate severely the expression of CD1b molecules. Therefore, the present research program has been designed to test whether BCG infection would influence and possibly down-regulate the induction phase of group 1 CD1molecules (i.e. CD1a, CD1b and CD1c) that are elicited by GM-CSF on monocytes. Adherent mononuclear cells (AMNC) were obtained from peripheral blood of healthy donors, and exposed ($2x10^6$ cells/flask) to graded amounts of BCG organisms at 37 °C in 5% CO2 atmosphere for 4 h. The "multiplicity of infection" (MOI, i.e. the ratio between the amount of BCG organisms, expressed in terms of CFU_{BCG}, and the number of AMNC) ranged from 0.25 to 10. Thereafter BCG was removed by careful washing and AMNC were incubated with GM-CSF (200 IU/ml). On day 3 of culture, flow cytometric analysis with anti-CD1 monoclonal antibodies showed that: (a) the expression of CD1 molecules was very high (i.e. more than 65%) in MNC not exposed to BCG infection; (b) membrane CD1a, CD1b and CD1c levels were low in BCG-infected targets, and a progressive decline of the expression of these molecules occurred when increasing MOI were used. In particular at MOI of 1, CD1b expression (in terms of percentage of CD1b-positive cells) was found to be consistently inhibited (i.e. by 35% to 75%); (c) inhibition of CD1b by BCG was also observed both at the levels of specific mRNA transcripts and CD1b protein, as evidenced by Northern blot and Western blot analysis respectively; (d) BCG alone was found to promote a slight increase of the expression of all antigen-presenting molecules on target cells (i.e. 15-20% positive cells) not subjected to cytokine stimulation, with respect to that detectable in non-infected AMNC not treated with GM-CSF (i.e. 2-5% positive cells). Attempts have been made to revert BCG-induced down-regulation of CD1 expression, using not only very high concentrations of GM-CSF, but also GM-CSF associated with IL-4. The results point out clearly that extremely high concentrations of GM-CSF (i.e. 2000 IU/ml) or cytokine combination of GM-CSF + IL-4 did not influence BCG-mediated down-regulation of CD1 expression. In conclusion the present findings have potential implications for understanding the unsatisfactory results obtained with BCG vaccination and the nature of the immune response elicited by BCG in humans. They also suggest new strategies that could be important for the development of better vaccines for the prevention of tuberculosis, especially in patients with potential immunodeficience, such as HIV-positive subjects. (Supported by Italian National Institutes of Health, contract # 50c.1)

PCP OCCURRING IN HIV PTS UNDER HAART: ASSESSING QUALITY AND NOT ONLY QUANTITY OF IMMUNORECONSTITUTION.

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Background: The beneficial effect of HAART in reducing opportunistic events during AIDS is currently attributed to viral suppression and T cell reconstitution especially when using powerful drug like HIV PI. We also determined that aspecific antipneumopcystic effect is exerted by classic HIV PI (IDV, RTV, NFV, SQV) in vitro at concentrations clinically achievable in vivo, thus possibly contributing to the positive outcome. Additional confirmatory data of antipneumocystic activity of PI have been obtained by testing increasing concentrations of APV, a newer PI known to be effective also against Giardia. Observational studies suggested that PCP prophylaxis can be safely discontinued when CD4 count arises over 200. We however observed 2 PCP events occurring under such conditions: their in vitro immunoprolypherative (IP) response to aspecific and specific P.carinii antigens (Pc Ag) was studied in comparison to control groups.

Methods: PBMC samples from 65 Pts (HIV positive and healthy donors) were obtained, freshly separated by Ficoll and studied for their ability to prolypherate in presence of MED, FLU, PHA, Env and PcAg prepared from almost pure (less than 1% contaminants) Pc trophozoites grown in spinner flasks. To inoculate confluent HEL 299 on Cytodex beads mantained in continuous rotation, omogenate of lungs from immunosuprressed, transtracheally infected rats were used. Results were evaluated after stratification of pts enrolled according to CD4 counts, occurrence and type of HAART and opportunistic events.

Results: 2 pts under HAART with CD4 count>250 (269 and 286 respectively) who developed PCP showed elevated VL and decreased IP answer to Pc Ag, similar to that from AIDS presenter, naive pts with PCP and CD4<200. Immunoreconstituted pts (who started HAART with CD4<200 but with cd>200 when tested) showed statistically different answer to Pc antigens compared to AIDS presenters with PCP and their IP was similar to asymptomatic naive HIV pt with CD4 >200. All healthy donors had strong IP to Pc Ag.

Conclusions: Rat derived, highly purified Pneumocystis carinii trophozoites from spinner flasks are adeguate as Pc specific antigen to be used in IP testing. Despite CD4 count >250 the supposed T clonal reconstitution was not achieved in HAART pts who developed PCP. Careful evaluation of quality and not only quantity of CD4 should be verified in a larger cohort of immunoreconstituted pts before definetively stating that prophylaxis can be safely discontinued. An IP test with specific P.carinii antigen could be considered for doubtful situations, i.e. pt starting HAART with low CD4 counts when reaching CD4>200, for a safer clinical decision.

Grant 50C.2.

P.CARINII ITS TYPING: DOUBTFUL EVIDENCE OF GENOTYPE-RELATED VIRULENCE AND POSSIBLE QUASI SPECIES PATTERN DURING SINGLE EPISODES

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Background: Since Internal Transcribed Spacers (ITSs) locus, a single copy gene, has been identified as first choice for P.carinii genotyping, controversial results have been presented on possible relationship between certain genotypes and clinical virulence .

Methods: 112 BALs morphologically positive for P.carinii and non invasive samples from 112 pts with PCP, collected from 1994 to 1999 in II Infect. Dis. ward were analyzed by ITSs nested PCR followed by TSO or direct sequencing. Clinical and biochemical parameters such as arterial pO2, serum LDH, degree of radiologic involvement, occurrence of lung or systemic co-pathologies, occurrence and type of previous PCP prophylaxis, final outcome, respiratory support and use of corticosteroids as side therapy were scored in order to detect possible genotype-related virulence.

Results: more than 21 ITSs genotypes were detected, with new ITS2 type (named u) discovered in addition to those already described by Lee (J.Clin. Microbiol., 98). The more frequentely isolates were Eg and Kf. Single infection (about 70%) was predominant over coinfection with 2 or more genotypes. The TSO system did not allow careful description of coinfections, so that direct sequencing of multiple clones is the only tool to reach this aim. Genotypes detected in blood or in gargling during single episodes only partially corresponded to that detected in BAL .

Conclusions: Varieties of copathologies, the occurrence of drug intolerance to first choice therapy, lacking information on DHPS gene mutation (recently indicated as related to drug-resistence) complicated the final evaluation of final clinical outcome (true microrganism virulence or resistence?). Analysis of genotypes found in BAL and other respiratory or blood isolates during a single PCP episode showed that also in single infection there was not always correspondent to genotypes found in BAL and garglings: since ITSs gene is present as a single copy, a quasispecies infection due to eterogeneous P. carinii population may be postulated. Although Eg genotype (corresponding to the Miller's reported more virulent isolate) was frequently associate to the need of aggressive respiratory support, the multivariate analysis did not confirm a statistically relevant association. The wide number of ITSs variants prevented to attribute true virulence features to certain genotypes although the large sample of AIDS pts with PCP tested.

Grant 50C.2.

EVIDENCE FOR AN EARLY, DIRECT ANTICANDIDAL EFFECT OF HIV-PROTEASE INHIBITOR CONTAINING THERAPEUTIC REGIMENS IN SUBJECTS WITH AIDS.

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Background: Subjects with AIDS undergoing highly active antiretroviral therapy (HAART) which include HIV-protease inhibitors (PI), experience a marked decline in incidence and severity of oral-esophageal candidiasis, which is currently attributed to restoration of specific immunity. We and others have recently shown, both in vitro and in experimental model of mucosal candidiasis, that HIV-PI exert direct anticandidal activity through inhibition of Candida aspartyl proteases (Sap), a well established virulence trait of this fungus.
Objectives: We compared 10 naive patients undergoing HAART with PI with an equal number of naive subjects receiving the non-nucleoside reverse-trascriptase inhibitors (NNRTI). The objectives of the study were: i) isolation of virulent (high Sap producers) C. albicans; ii) determination of Sap salivar concentration; iii) peripheral blood lymphocyte proliferation to Candida antigens, at baseline, and at 2, 4, 12 and 24 weeks from initiation of therapy. The two groups of subjects did not significantly differ at baseline either in CD4+ cell number or HIV viremia values.
Results: After only 2 weeks of treatment the PI-treated subjects, but not those in the NNRTI-treated group, experienced a marked reduction in the number of high Sap strains isolated from the oral cavity ($p = 0.02$; χ^2 for trend), coupled with a lower amount of the enzyme in the saliva ($p = 0.006$). No changes were observed in the number of subjects with lymphoproliferative response to Candida antigen and no difference between the two groups concerning HIV viremia or CD4+ cells were found. After 12 weeks, no Candida species at all was isolated and no Sap was present in the PI-treated group whereas the same number of fungal isolates and a comparable amount of Sap were detected in the NNRTI-treated group as at the baseline. Overall, statistical analysis up to 24 weeks of treatment demonstrated, in PI-treated subjects, a significant reduction of Candida isolation ($p = 0.01$) and no detection of salivar Sap. These results were associated with a significant HIV viremia reduction but not with a significant increase of CD4+ cell number and no recovery of lymphoproliferative responses to Candida. The events associated with Candida virulence were not found in the NNRTI-treated group, which, conversely, enjoyed similar if not superior increase in CD4+ cell number and comparable HIV viremia decrease.
Conclusion: Altogether our data strongly support the concept that a direct anticandidal activity of PI is clinically expressed, and that, at least, part of the early and sustained anti-Candida benefit of PI-containing regimens is due to Sap inhibition by PI.

N° dell'accordo di collaborazione: 50C3

ANALYSIS OF THE MECHANISMS AND OF THE ROLE OF MYCOBACTERIUM TUBERCULOSIS-INDUCED APOPTOSIS IN MONOCYTES/MACROPHAGES

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The level of macrophage apoptosis is higher in tuberculosis HIV-infected than in HIV-uninfected patients and is directly related to the amount of mycobacteria (Placido R. et al. J. Pathol. 1997; 181: 31). In this research project we investigated both the mechanisms and the role of monocyte/macrophage apoptosis induced by high doses of the virulent strain MTB H37Rv. In this context, we demonstrated that MTB-induced apoptosis of monocytes/macrophages is a very early event occurring already after 1 hour from the exposure with high doses of MTB and is associated to a selective down-regulation of CD14 (Santucci M.B. et al. J. Infect. Dis. 2000; 181:1506). Moreover, as caspases are important mediators of apoptosis, we analysed caspase-1 involvement, considering its role in both inflammatory processes and in pathogen-induced apoptosis (Zychlinsky A. et al. J. Clin. Invest. 1997; 100, 493). In particular, we performed MTB infection in the presence of a caspase inhibitor (YVAD-CMK, selected for high specificity with caspase-1). Results showed a 50% inhibition of MTB-induced apoptosis after treatment with 50 μM of inhibitor. Furthermore, the levels of IL-1β have been determined in the supernatant of apoptotic monocytes/macrophages and a strong increase of cytokine production was observed. Such production decreased after inhibitor treatment, in a dose dependent manner. Finally, in order to analyse the role of MTB-induced apoptosis, mycobacterial viability was assessed by colony forming unit assay. Results showed no statistically significant decrease of mycobacterial viability. Altogether these results suggest that apoptotic pathway triggered by high doses of MTB is associated to pro-inflammatory cytokine production and to pathogen survival.

N° dell'Accordo di Collaborazione: 50C.4

DISCORDANT VIROLOGICAL AND IMMUNOLOGICAL RESPONSES TO HIGHLY ACTIVE ANTIRETROVIRAL THERAPY: PREVALENCE AND PREDICTIVITY FOR LONG-TERM OUTCOME.

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Objective: To study the frequency of discordant virological and immunological responses in HIV-positive patients on their first HAART regimen, and their predictivity of long-term outcome.
Design: Observational clinical study.
Methods: 298 patients were grouped according to their 3-month virological (undetectable HIV-RNA) and immunological ($\geq$70 cells/μL) response: group 1= responders; group 2= virological failures; group 3= immunological failures;, group 4= complete failures. The end-points of virological and immunological outcome were analyzed by means of intention-to-treat multivariate analyses by Cox model.
Results: After three months, 119 patients (39.9%) were classified in group 1, 32 (10.8%) in group 2, 87 (29.2%) in group 3, and 60 (20.1%) in group 4. During a median follow-up of 873 days (90-1597), only 13 patients (4.4%) experienced AIDS-defining events. The patients in group 1 (responders) showed a persistent increase in CD4+ counts reaching a median of 513 cells/μL and a decrease in HIV-RNA levels reaching a median of 1.90 $\log_{10}$ at the 30th month. The virological failures (group 2) showed a similar immunological course to that observed in group 1, with a median CD4+ count of 327/μ at the 30th month; however, their HIV-RNA levels did not change from those recorded at baseline (from 4.84 $\log_{10}$ copies/mL at baseline to 4.63 $\log_{10}$ copies/mL at the 30th month). The patients in groups 3 and 4 (immunological failures and complete failures) showed a delayed increase of their CD4 counts, that indeed reached a median of respectively 418 and 280 cells/μL at the 30th month; the median from HIV-RNA copy levels was 1.90 $\log_{10}$ in group 3 and 4.00 $\log_{10}$ in group 4.
Sustained virological success, i.e. at least two consecutive HIV-RNA measurements below 80 copies/mL after 12 months, was obtained in 93/236 (39.4%) patients, and sustained virological failure , i.e. at least two consecutive HIV-RNA measurements above 1,000 copies/mL after 12 months, in 103/236 (43.6%) patients. Only belonging to the 3-months groups was an independent predictor of a sustained virological success: having group 1 (success) as reference, both patients of group 2 (virological failure) and group 4 (failure) showed a lower probability of reaching this end-point (HR 0.15 –95%CI: 0.1-0.4- and HR 0.24 -95%CI: 0.1-0.5-). As refers to the second virological end-point, younger patients showed a higher risk of sustained virological failure (age >34 years: HR 0.66 –95%CI 0.4-1.0- vs age $\leq$ 34 years); further, virological failure occurred with higher probability in patients showing virological failure at the third month (groups 2 and 4) as compared to patients with a 3 month-success (group 2: HR: 6.30 -95%CI 3.4-11.5 and group 4: HR 5.16 –95%CI 3.0-9.0 vs group 1).
A sustained immunological success, i.e. two consecutive CD4+ cell counts measurements above 500 cells/μL after 12 months, occurred in 84/236 (35.6%) patients; it was reached less frequently by patients with AIDS at baseline (HR 0.48, 95%CI: 0.3-0.9 vs non AIDS). Patients with immunological failure at third month (i.e. those belonging to groups 3 and 4) showed a worse success compared to group 1 (group 3: HR 0.39, 95%CI 0.2-0.7 and group 4:HR 0.19, 95%CI 0.1-0.5 vs group 1). Sustained immunological failure, i.e. two consecutive CD4+ cell counts measurements below 200 cells/μL after 12 months, occurred in 58/236 (24.6%); it was reached more frequently by males compared to females (HR 2.11, 95%CI: 1.0-4.3) and by intravenous drug addicts groups (homosexuals: HR 0.15, 95%CI 0.1-0.4 ad heterosexuals HR 0.40, 95%CI 0.2-0.7 vs IVDU). All the 3 month groups showed a higher risk of immunological failure compared to group 1 patients (group 2: HR 3.28, 95%CI: 1.4-7.7, group 3 HR 2.53, 95%CI 1.2-5.5, group 4 HR 3.92, 95%CI 1.9-8.2 vs group 1).
Conclusions: a disconnection between a short-term virological and immunoglogical response does not always persist in the long-term follow-up. Even if patients failing virologically at 3 month have less chances of virological response in the long-term follow-up, at least a part of them do achieve persistent immunological success despite virological failure.

AIDS Project 50C.5

CHANGES IN THE DEMOGRAPHIC, IMMUNOLOGICAL AND CLINICAL CHARACTERISTICS IN A COHORT OF 437 AIDS-PRESENTER PATIENTS IN A 15 YEARS' PERIOD

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Objective: To study the incidence of AIDS-presenters and to define the groups at higher risk of such occurrence.
Design: Observational clinical study.
Methods: all the HIV-positive patients cared at the Institute of Infectious and Tropical Diseases, University of Milan, have been included. AIDS-presenters were defined as those subjects with a diagnosis of HIV-seropositive status concomitant (within 1 month) to the diagnosis of AIDS. Demographic (sex, age, risk factors for HIV, country of birth, level of scholarship) and clinical (CD4+ counts, AIDS-defining diseases, minor diseases occurring before the diagnosis of HIV/AIDS) variables have been collected. The observational period has been divided into four groups: <1988, 1988-1993, 1994-1996, 1997-1999. Statistical analyses included the Chi-square test and the incidence estimates.
Results: while the incidence of AIDS in non presenters has dramatically decreased in the last period (from 86/1,000 p-y in 1994-1996 to 40/1,000 p-y in 1997-1999) the incidence of AIDS in presenters has slightly increased (from 17 to 20/1,000 p-y). As a consequence, up to one third of the diagnoses of AIDS (n=230) in the last period do occur in AIDS-presenters. In 1997-1999 AIDS-presenters were with higher frequency homosexuals, subjects older than 35 years, with a lower level of scholarship and more frequently born in non-community countries as compared to the previous observational periods. Further, the diagnosis of MAC as index-disease has increased while the diagnosis of esophageal candidiasis has decreased according to the observational periods. Among females, it as been observed a sharp increase of heterosexually infected ones', that represent the 85% of AIDS-presenters females in 1997-1999. Among men, sexually (either hetero- or homo-) infected ones represent the 70% of AIDS-presenters in 1997-1999. A total of 169/437 (38.7%) subjects were older than 35 years, most of them infected through sexual routes. In up to 126 subjects (28.8%) HIV seropositivity could be suspected before the occurrence of AIDS on the basis of occurring hallmark diseases (particularly sexually transmitted diseases in 46 and cutaneous lesions in 22).
Conclusions: while in the last years the incidence of AIDS in subjects known to be HIV-infected has dramatically decreased, an increase of the incidence of subjects with a diagnosis HIV concomitant to AIDS was observed. In particular, subjects infected by sexual route and older than 35 years are at higher risk of being unaware of their HIV-seropositive status. In up to 30% of these subjects several hallmark diseases could have been considered as conditions leading to a screening for HIV.

AIDS Project 50C.5

TWELVE MONTHS FOLLOW-UP ANALYSIS OF IMMUNOPHENOTYPE IN LONG-TERM HAART TREATED HIV-1 INFECTED PATIENTS SHOWING PERSISTENT DISCREPANCIES BETWEEN VIROLOGICAL AND IMMUNOLOGICAL RESPONSE

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Most of the patients on an antiretroviral regimen including protease inhibitors (PI) experience a dramatic reduction in HIV plasma viral load and a rapid increase in the CD4 lymphocyte cell count during the first weeks of treatment Altough the control of virus replication is thought to be essential for immune restoration, recent reports have described paradoxical discrepancies between virological and immunological responses in 20-30% of subjects receiving HAART Aim of the present study was to evaluate immunological status in long term HAART treated HIV-1 infected patients persistently showing discrepancies between virological and immunological response.
Forty-three subjects with advanced HIV-1 infection who had been receiving HAART for at least 12 months were enrolled (T_1) in a cross-sectional study of lymphocyte surface phenotypes. The inclusion criteria were: CD4<100/microL and plasma HIV-RNA>10000 cp/ml before PI administration (T_0). Three groups were defined at T_1 on the basis of their response to HAART: i) responders: steady viremia levels <1000 cp/ml for at least six months before the analysis and a sustained CD4>400/microL; ii) immunological non-responders: steady HIV-RNA <1000 cp/ml and a CD4 count of never more than 200 cells/microL; and iii) virological non-responders: patients with steady HIV-RNA>10000 cp/ml and a sustained CD4>300 cells/microL. Twelve never treated LTNPs were evaluated as control.
The 43 patients receiving HAART underwent a further FACS evaluation after 12 months follow-up (T_{12}). Results are shown in the following table:

	Responders n=13	Immunological non Responders n=21	Virological non Responders n=9	LTNP n=12
CD4+ T_0	18 (5-66)	30 (2-90)	10 (2-96)	NA
CD4+ T_1	635 (410-917)	151 (40-200)	436 (330-660)	780 (510-1050)
CD4+ T_{12}	607 (409-861)	186 (34-438)	441 (368-766)	NA
CD45RA+CD62L+/CD4+ T_1	47.4 (29.2-84.6)	24.6 (4.4-57.7)	43.0 (19.4-63.1)	48.9 (18.9-60.2)
CD45RA+CD62L+/CD4+ T_{12}	43.6 (26.7-81.5)	29.1 (7.2-49.5)	43.6 (19.1-72.7)	NA
CD4+CD7+/CD4+ T_1	88.5 (83.3-92)	76.9 (45.4-86.8)	83,9 (72.8-90.3)	87.5 (61.9-93.7)
CD4+CD7+/CD4+ T_{12}	91.5 (77.7-95.3)	83.6 (67.1-91.6)	88.7 (83.1-93.5)	NA
CD8+CD38+/CD8 T_1	24.6 (2.2-36.7)	31.0 (3.0-68.5)	39.0 (26.4-75.3)	31.6 (17.1-36.2)
CD8+CD38+/CD8 T_{12}	10.7 (5.1-48.3)	13.4 (4.5-57.2)	18.9 (16.3-47.5)	NA

The median increase in CD4 was statistically significant in all the three HAART groups between T_0 and T_1 ($p<0,01$). The immunological non-responders showed a further significant increase between T_1 and T_{12} ($p<0,05$).
At T_1 the responders, virological non-responders and LTNPs all had significantly higher percentages both of the naive subset (CD45RA+CD62L+) and of the type-1 cytokine-producing cells (CD4+CD7+) than the immunological non-responders ($p<0,01$).
The virological non-responders had the highest, and the responders the lowest percentages of activated CD8+ lymphocytes (CD38+ and HLA-DR+), but there was no statistical difference between the two groups.
At T_{12} analyses both the responders and the virological non-responders maintained significantly higher percentages of naive CD4 cells and of Th0/Th1 CD4 lymphocytes (CD7+) than the immunological non-responders ($p<0,01$). A slight (albeit still non-significant) increase in the percentage of naive CD4 cells among the immunological non-responders was observed between T_1 and T_{12}. There was a decrease in the median percentages of activated CD8+ lymphocytes (CD8+CD38+) in all three groups receiving HAART.
Even when started during advanced disease, HAART may restore a stable immune condition similar to that observed in LTNPs regardless of the absence of complete viral suppression. Conversely, the patients whose CD4+ cells counts do not significantly increase, despite suppressed viral replication, have an immune status similar to that observed in patients with advanced infection. The twelfth month follow-up analysis showed that these patients seem to behave as extremely slow responders rather than true immunological non responders.

Accordo n° 50C.5 - (30C.32)

CIDOFOVIR ADDED TO HAART IMPROVES VIROLOGIC AND CLINICAL OUTCOME IN AIDS-ASSOCIATED PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY.

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Objectives: To analyse the virologic and clinical efficacy of cidofovir combined with highly active antiretroviral therapy (HAART) in AIDS-related progressive multifocal leukoencepalopathy (PML).
Design: Multicentre observational study of consecutive HIV+ patients with histologically or virologically-proven PML. Group A: 26 patients treated with HAART, group B: 14 patients treated with HAART plus cidofovir 5 mg/kg iv per week for the first 2 weeks, every other week thereafter. JC virus DNA was quantified in cerebrospinal fluid (CSF) by PCR.
Results: Baseline virologic, immunologic and clinical characteristics were homogeneous between the groups. In one case (7%) cidofovir was discontinued because of severe proteinuria. There was no significant difference in HIV-RNA responses and changes in the number of CD4+ cells between group A and B. After two months of therapy, 5 of 12 (42%) tested patients from group A and 7 of 8 (87%) from group B reached undetectable JC virus DNA in the CSF (Chi square P=.04); moreover, 24% of group A and 57% of group B patients showed neurological improvement or stability (P=.038). One-year cumulative probability of survival was 0.67 with cidofovir and 0.31 without (log rank P=.01). Variables independently associated with longer survival were the use of cidofovir, HAART prior to the onset of PML, a baseline JC virus DNA load in CSF <4.7 $\log_{10}$ copies/ml, and a baseline Karnofsky performance status ≥ 60.
Conclusions: In AIDS-related PML, cidofovir added to HAART is associated with a more effective control of JCV replication, with improved neurological outcome and survival compared to HAART alone.

Accordo di Collaborazione n. 50C.6

INTERACTIONS OF HHV-8 REGULATORY GENES ORF50 AND ORF57 WITH HIV TAT.

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Accordo di collaborazione: n. 50C.7

Human herpesvirus 8 (HHV-8) is a lymphotropic virus associated to several AIDS-related neoplasms. Two ORFs play a critical role in the regulation of viral replication: ORF50, encoding an immediate-early transcriptional activator, and ORF57, encoding a postranscriptional regulator. We analyzed their effects on the activation of HIV-1 LTR. ORF50 interacted synergically with tat, inducing a 10-fold enhancement of HIV-1 LTR transactivation. This effect occurred both in BCBL-1 cells, latently infected with HHV-8 and in HL3T1 cells, an epithelial cell line non permissive to HHV-8 infection. Also ORF57 enhanced tat-induced transactivation of HIV-1 LTR, but only in BCBL-1 cells, suggesting that its action was likely mediated by the induction of other viral functions. When both ORFs were expressed, the enhancement of transactivation induced by ORF50 was partially inhibited. These data show that HIV and HHV-8 can interact in a reciprocal mode of action: besides the influence exerted by HIV-1 upon HHV-8 reactivation and replication, also a direct effect of HHV-8 upon HIV-1 is possible. The findings show that ORF57 can modulate ORF50 activity and that ORF50 may render biologically active small amounts of tat. This is particularly relevant in the development of Kaposi's Sarcoma, due to the angiogenic properties of tat, which could be amplified by the presence of activated HHV-8. We are currently analyzing the functional relevance of these molecular effects by coinfection experiments, with the aim to understand which modifications are induced in the different cell types naturally infected by both viruses, with particular regard to lymphocytes, macrophages and endothelial cells.

OPEN, CONTROLLED, RANDOMIZED STUDY ON DISCONTINUATION OF SECONDARY PROPHYLAXIS FOR Pneumocystis Carinii PNEUMONIA IN PATIENTS WITH AIDS.

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Background: In individuals undergoing primary prophylaxis for Pneumocystis carinii pneumonia (PCP), whose CD4 count returned to levels exceeding 200 cells/mm3 as a result of Highly Active Antiretroviral Therapy (HAART), clinicians should consider the opportunity to interrupt prophylaxis.

Methods: In Italy, in January 1999, we began a multicenter (43 clinical centers) open controlled randomized trial on the effects of discontinuing PCP secondary prophylaxis among HIV-positive individuals whose CD4 count increased to > 200 as a result of HAART. Individuals were randomized to one of two arms: Arm A) discontinuing PCP prophylaxis, and Arm B), continuing prophylaxis. Participants in Arm A whose CD4 count decreased to <200 after discontinuation were newly offered prophylaxis. Main inclusion criteria were a previous episode of PCP and a CD4 count > 200 cells/mm3 for at least three months. Individuals with a previous diagnosis of toxoplasmic encephalitis were excluded. Signed informed consent was obtained.

Results: As of December 2000, 146 patients were enrolled (77 randomized to Arm A). The two arms were balanced for CD4, plasma HIV-RNA at enrolment, time between first CD4 > 200 cells/mm3 and enrolment, CDC stage, age, sex, and diagnosis of the previous episodes of PCP. Arms were also similar for immunologic, virologic, and clinical characteristics when beginning HAART and PCP-prophylaxis. During a median follow-up of 18.4 months for Arm A and 16.1 months for Arm B and a total of 108 and 86 person-years (p-y) for the 2 Arms, respectively, 2 events were observed, one definitive diagnosis and one presumptive diagnosis of PCP.

Conclusion: This study shows a very low risk of PCP for HAART patients whose CD4 returned to a level >200, even if they had a previous episode of PCP

Accordo di Collaborazione N°. 50C.8

CLONING AND PARTIAL CHARACTERIZATION OF AN ABC-TRANSPORTER GENE INVOLVED IN AZOLE RESISTANCE FROM CRYPTOCOCCUS NEOFORMANS

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Resistance to azole antifungal drugs was induced in a clinical isolate of Cryptococcus neoformans (CR22), susceptible to fluconazole (MIC 2 μg/ml), ketoconazole (MIC 0.0075 μg/ml), and itraconazole (MIC 0.03 μg/ml). The induced resistant strain (CR22.17) showed stable significant increased values of minimum inhibitory concentrations to the drugs listed above (MIC values of 32 μg/ml, 0.25 μg/ml and 1 μg/ml, respectively). The two strains (CR.22 and CR22.17) were analyzed by rhodamine 6G accumulation assay, that demonstrated a different accumulation of the substance. Therefore, their mRNAs were analyzed by Suppression Subtractive Hybridization to evaluate possible overexpression of genes involved in azole resistance. Several cDNA clones, differentially expressed in the CR.22.17 strain, were obtained. One of them, the c1H9 (238 bp), showed high nucleotide homology to the ATP Binding Cassette (ABC) Transporter genes of Penicillium digitatum, Candida albicans, Saccharomyces cerevisiae and Candida glabrata. Its overexpression was confirmed by Northern blot analysis. To generate the full length cDNA of the c1H9 clone, we performed both 3' and 5' rapid amplification of the cDNA ends (RACE). Sequencing of the 3' RACE (about 1.8 kb) was performed and compared with the GeneBank data base. High homology with the ABC transporter BMR1 of Botryotinia fuckeliana was found and with the genes listed above. Sequencing of the 5' RACE (about 3 kb) is in progress. Contemporaneously, thanks to the kind gift of John Perfect (Department of Microbiology, Duke University Medical Center Durham, North Carolina) we screened the C. neoformans genomic library and found the phage clone corresponding to the c1H9 cDNA. Sequencing of the phage clone and comparison with 3' RACE cDNA showed the presence of six introns in the 3' end of our gene. To demonstrate the possible involvement of this ABC transporter gene in the azole resistance in C. neoformans we will perform experiments of gene knockout and expression in a hyper-susceptible Saccharomyces cerevisiae host. Even if c1H9 was the clone of interest for the study design, other clones, differentially expressed, could be considered for further investigation with regard to the virulence (i.e. clone c1E1 SOD Cu, Zn or Mg?) or for a better understanding of up- and down-regulation of genes in Cryptococcus neoformans.

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N°. dell'Accordo di Collaborazione. 50C9

VIROLOGICAL AND MOLECULAR STUDY OF JC VIRUS IN PATIENTS WITH AND WITHOUT PROGRESSIVE MULIFOCAL LEUKOENCEPHALOPATHY AND WITH OTHER PATHOLOGIES.

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JC virus (JCV) infection is very common and it is usually acquired during the first two decades of life. After primary infection, the virus establishes latent infection in the kidney and may be reactivated in the course of immunosuppressive states. Different factors are probably involved in developing of serious pathologies as PML or cerebral tumors: in an attempt to give a contribution to understanding the mechanisms involved in evolution of JCV infection we have included in our project different parallel points. For these reasons we are continuing to investigate the possible role of viral factors, as particular rearrangement of regulatory region or the presence of different genotypes in PML development. Our data indicate that infection with JCV type 2 and dual infection with JCV type 1 and 2 are a significant risk factor for PML. To further validate these observations we are currently studying the distribution of the different JCV genotypes in Italian healthy population. We have tested 160 urine samples collected from individuals living in various Italian regions (56 from north, 53 from center, 51 from south Italy). The overall JCV prevalence was of 52.5%, without significant differences between the three Italian geographic areas. The nucleotide sequence analysis shows that the JCV genotypes 1a, 1b and 4 are more prevalent in Italy while JCV 2b and 2c have been detected, with a low frequency, only in northern Italian subjects.
Since polyomaviruses (JCV, BKV and SV40) are known for their capability to induce brain tumors in animals and have been suggested as a possible risk factor in the etiology of some types of human tumors, recently we are performing a study to verify the involvement of JCV in the pathogenesis of human brain tumors. Brain tumor tissue, obtained by biopsy, cerebrospinal fluid (CSF) and peripheral blood samples collected from 16 histologically diagnosed cases of tumors (meningiomas, glioblastomas, oligodendrogliomas, gangliocitomas and ependimomas) were investigated for the presence of a sequences belonging to the highly conserved large T (LT) antigen coding region by nested Polymerase Chain Reaction (n-PCR).
The molecular amplification followed by Restriction Fragment Length Polymorfism (RFLP), executed to discriminate between the viruses, indicate the presence of JCV, but not BK and SV40, in 3out of 7 cases of glioblastoma and in one case of meningioma, while the other types of tumors were negative. Moreover, JCV DNA was amplified in two CSF collected from patients with glioblastoma, one of whom was JCV positive also in the tumor tissue.
Sequence analysis of VP1 region amplified from two tissue samples (glioblastoma) and two CSF (glioblastoma) performed at this time permit us to detect four JCV type 1, including three 1a and one indiscriminate 1a/b. The transcription control region (TCR) of the two JCV type 1a showed a Mad-4 type rearrangements.
On the whole these results, although preliminary, support the possible involvement of JCV in human brain tumors and in particular in glioblastoma, and confirm the finding of JCV type 1, Mad-4 strain, as more relevant in human brain tumors.

Accordo di Collaborazione n. 50C10

POSSIBLE PATHOGENIC ROLE OF GENE fadD33 OF MYCOBACTERIUM TUBERCULOSIS H37Rv

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The possible pathogenic role of gene fadD33 of Mycobacterium tuberculosis H37Rv, encoding an acyl-CoA synthase, an enzyme predictively involved in lipid metabolism, under-expressed in the attenuated mutant M. tuberculosis H37Ra, has been investigated.
As a first step, we tested the distribution of fadD33 among mycobacteria. Hybridization experiments identified fadD33-specific sequences only in the species belonging to the M. tuberculosis complex, i.e., M. tuberculosis strain H37Rv and H37Ra, M. bovis, including the BCG strain, and M. microti, but not in several other mycobacterial species, including M. avium, M. intracellulare, M. scrofulaceum, M. kansasii, M. xenopi, M. malmoense, M. gordonae, M. marinum, M. simiae, M. fortuitum, M. smegmatis, and M. vaccae, thus indicating that fadD33 is associated with the most virulent mycobacteria.
The gene fadD33 of M. tuberculosis H37Rv was then cloned into the E. coli-Mycobacterium shuttle expression vector pROLHYG, which was electroporated into the the non-pathogenic, fast-growing bacterium M. smegmatis mc^2155. The transformed M. smegmatis bacteria expressing the fadD33 gene (M. smegmatis-fadD33) were used to investigate whether fadD33 might confer growth advantages to M. smegmatis in human and murine macrophages in vitro. For this purpose, cultures of phorbol myristate-acetate (PMA)-activated human THP-1 cells and cultures of PMA- or lypopolysaccharide (LPS)-activated mouse peritoneal cells were infected with M. smegmatis-fadD33 and M. smegmatis transformed with the vector without insert (control). In THP-1 cells, the numbers of intra- and extracellular CFUs of M. smegmatis-fadD33 were markedly lower (10-40 fold) than those of control strain at 24 and 48 hrs after cell infection. In PMA- or LPS-activated mouse peritoneal cells a similar reduction in the numbers of intracellular CFUs of M. smegmatis-fadD33 was observed at 48 hrs after infection. These data indicate that fadD33 plays a role in the mycobacterium-macrophage interaction, which might affect the pivotal role of macrophages in M. tuberculosis infection and disease.
M. smegmatis-fadD33 bacteria were then tested in vivo in the experimental infection of CD-1 mice. No differences in the splenic CFU counts of M. smegmatis-fadD33, as compared with animals infected with control strain, were found. The pROLHYG-fadD33 vector was then electroporated into the attenuated strain M. tuberculosis H37Ra and the complemented strain M. tuberculosis H37Ra-fadD33 was injected intravenously into BALB/c mice. It was found that at 21 and 42 days of infection the CFU counts of M. tuberculosis H37Ra-fadD33 in the liver were significantly higher than those of the attenuated strain M. tuberculosis H37Ra and did not differ from those of the virulent M. tuberculosis H37Rv.
In conclusion, fadD33 expression failed to confer a virulent phenotype to M. smegmatis in vivo, but complementation of fadD33 expression in the natural host M. tuberculosis H37Ra seems to restore virulence. In vivo experiments, that are in progress at moment, employing the virulent strain M. tuberculosis H37Rv in which the fadD33 gene has been disrupted, will likely provide conclusive evidence on the role of fadD33 in M. tuberculosis virulence.

Accordo di Collaborazione No. 50C.11

EVALUATION OF IS6110 TRANSPOSITION IN THE ATTENUATED MYCOBACTERIUM TUBERCULOSIS H37Ra

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Mycobacterium tuberculosis H37Rv and the attenuated strain H37Ra were used as a model to investigate the virulence properties of M. tuberculosis at the gene level. Transposition of the insertion element IS6110 is considered a major force in generating genome plasticity of the tubercle bacilli, including the strains H37Rv and H37Ra. By using Pvu II, a restriction enzyme that cleaves IS6110 once, and by probing for an IS6110-specific target sequence located to the right of the Pvu II site, we previously found that the strains H37Rv and H37Ra share 13 IS6110-positive restriction fragments and that one IS6110-positive restriction fragment of H37Rv of 5.1 kbp is replaced by four novel fragments ca. 1.12, 2.29, 3.03 and 4.90 kbp in H37Ra, thus demonstrating that novel insertions of the IS6110 element do exist in the attenuated strain H37Ra. In this and other studies, it has been hypothesized that the transpositions of IS6110 might have involved virulence genes.
To test whether transposition of the insertion element IS6110 might be involved in the loss of virulence of strain H37Ra, we determined the nucleotide sequence of a differential IS6110-positive restriction fragment detected in H37Ra, but not in H37Rv. The region flanking the 3' end of the IS6110 element showed partial sequence homology to internal sequences of M. tuberculosis H37Rv genes plcA, plcB, and plcC, each one coding for phospholipase C, a well-known bacterial virulence factor. PCR experiments, however, ruled out IS6110 insertional events in the plc locus of the H37Ra strain. A 100% homology was found between the IS6110-flanking region and an internal sequence of M. bovis plcD, a further phospholipase C gene that is truncated and partly lost in H37Rv in the so-called RvD2 deletion. This result indicates that the differential restriction fragment of H37Ra originally stems from the plcD gene interrupted by the insertion of the IS6110 element. To investigate whether the RvD2 region plays a role in mycobacterial virulence, 45 clinical isolates of M. tuberculosis were analyzed by Southern blot: the RvD2 deletion was demonstrated in 15 isolates; the entire RvD2 region, including the undisrupted plcD gene, was detected in 29 isolates, while only one isolate showed the RvD2 region in which the plcD gene was interrupted by an IS6110 insertion.
It is concluded that disruption of plcD gene and deletion of the RvD2 region by IS6110 insertion have no consequence for the virulence of M. tuberculosis, although the role of phospholipase C as a virulence factor of M. tuberculosis still remains debatable.

Accordo di Collaborazione No. 50C.11

DIFFERENTIAL RESCUE AND PROGNOSTIC IMPLICATIONS OF THE LYMPHOPROLIFERATIVE RESPONSE TO HUMAN CYTOMEGALOVIRUS (HCMV) IN AIDS PATIENTS WITH OR WITHOUT PRE-HAART HCMV DISEASE FOLLOWING LONG-TERM HAART.

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Objective of the study was to investigate the lymphoproliferative response to human cytomegalovirus (HCMV) and the rate of HCMV viremia in two groups of AIDS patients following long-term HAART: i) one (n=23) with <50 $CD4^+$ T cells/μl and no pre-HAART HCMV disease; and ii) the other one (n=18) with pre-HAART HCMV disease. Patients were prospectively monitored for $CD4^+$ T cell count, HIV RNA load, HCMV viremia and lymphoproliferative response to HCMV. A control group of 13 recently diagnosed treatment-naive AIDS patients with <100 $CD4^+$ T cells was investigated to resemble the pre-HAART era for occurrence of HCMV infection/disease. No response to HCMV was observed in treatment-naive patients either with or without disease. Recovery of the lymphoproliferative response to HCMV in the absence of HCMV viremia was observed in most patients of the two HAART-treated groups. However, a significantly higher rate of both non-responders and viremic patients was found in the group with HCMV disease prior to HAART. The $CD4^+$ T cell count correlated significantly with the lymphoproliferative response to HCMV in both groups of patients, whereas HIV load did not. However, some discrepancies between high $CD4^+$ T cell count and low or absent lymphoproliferative response (sometimes associated to HCMV viremia) were observed in a fair number of patients of both groups. The lymphoproliferative response was stable in most patients of both groups during a 6-18 month follow-up following long-term HAART. HCMV retinitis appeared in a single patient with no pre-HAART disease during first months of treatment concomitantly with $CD4^+$ T cells <100/μl, no lymphoproliferative response to HCMV and HCMV viremia. On the contrary, anti-HCMV therapy could be safely discontinued in 8 patients with pre-HAART HCMV retinitis showing $CD4^+$ T cells >150/μl, recovery of the HCMV lymphoproliferative response and no HCMV viremia, whereas it could not be discontinued in a patient with $CD4^+$ T cells >300/μl and no lymphoproliferative response to HCMV in the presence of HCMV viremia. In conclusion: i) pre-HAART HCMV disease hampers specific immune reconstitution; ii) although the clinical impact of the lymphoproliferative response to HCMV remains to be assessed, it seems reasonable that discontinuation of anti-HCMV therapy in patients with past HCMV disease be based on both the recovery of HCMV lymphoproliferative response and the absolute $CD4^+$ T cell count rise in the absence of HCMV viremia.

Accordo di Collaborazione 50C.12

TRANSMISSION TO LEUKOCYTES IS A MARKER OF PATHOGENICITY OF HUMAN CYTOMEGALOVIRUS STRAINS IN AIDS PATIENTS.

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In AIDS patients with disseminated human cytomegalovirus (HCMV) infection, HCMV strains are transmitted from endothelial cells to peripheral blood leukocytes and, namely, polymorphonuclear leukocytes (PMNL). This phenomenon, that is the basis of diagnostic assays for HCMV viremia in immunocompromised patients, has been recently reproduced in vitro (M.G. Revello et al., J. Clin. Invest. 101:2686-2692, 1998) and its mechanism clarified (G. Gerna et al., J. Virol. 74: 5629-5638, 2000). On the other hand, in in vitro cocultures of HCMV-infected human embryonic lung fibroblasts and PMNL, it has been shown that attenuated HCMV strains are not transmitted to PMNL. This lack of transmissibility has been initially proven for both the well known laboratory adapted HCMV strains (AD169, Towne, Davis, as well as Toledo) and four cell culture-adapted clinical HCMV isolates following a different number of passages in cell cultures. Subsequently, virus transmission to PMNL has been consistently confirmed for all of the 70 HCMV recent clinical isolates tested so far. Transmission included infectious virus, as well as viral DNA, immediate-early and late mRNAs, and viral proteins. The development of a HCMV vaccine for the prevention of HCMV disease is urgently needed. Since reliable markers of attenuation are not available at the moment, we propose our in vitro model of virus transmission to leukocytes as a potential marker of pathogenicity and dissemination of HCMV strains in humans. If a candidate vaccine is based on a live virus strain, then it seems reasonable to assume that this strain must be deprived of the property to be transferred to leukocytes cocultured with infected permissive cells, prior to being administered to humans. In fact, lack of pathogenicity in the presence of effective immunogenicity should represent a major prerequisite of a candidate live HCMV vaccine.

Accordo di Collaborazione 50C.12

CLINICAL AND IMMUNOLOGICAL BENEFIT OF ADJUVANT THERAPY WITH THALIDOMIDE IN THE TREATMENT OF TUBERCULOSIS DISEASE

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Objective. We analyzed the clinical and immunological role of adjuvant therapy with thalidomide in the treatment of tuberculosis disease, studying its in vivo effects in patients affected by tuberculosis.
Methods. We enrolled a consecutive series of patients with documented active tuberculosis. All subjects received a standard anti-tuberculosis treatment according to the WHO treatment guidelines. Patients with good clinical and microbiological responses were followed up without further therapeutic interventions. Patients with signs and symptoms of persistent disease, after at least 15 days of treatment, were randomized into two groups: the first one continued the therapy without changes or with an adjunctive anti-tuberculosis drug, and the second one added thalidomide (200 mg/die for 90 days). The effects of thalidomide on clinical and microbiological response, cytokine production, CD4+ T-cell counts and HIV-1 viral load were evaluated at days 0, 15, 30, 45, 60 and 90 of treatment.
Results. All patients treated with thalidomide showed a significant improvement in clinical conditions, including reduction of fever, increase in body weight, improvement of radiological features, and negativization of M. tuberculosis cultures after thalidomide introduction. Prior to thalidomide introduction, the immunological evaluation showed that the patients with poor outcome had low levels of TNF-α production. On the contrary, among the three different groups analyzed, type 1 (IL-2, INF-γ) and type 2 (IL-4, IL-10) cytokines analysis showed comparable levels. After the introduction of thalidomide we observed in all patients an increase in TNF-α levels. By contrast, those patients with poor outcome who were not designated to receive thalidomide continued to show clinical progression of the disease and low levels of TNF-α and type 1 cytokines. In the thalidomide group, a progressive increase in IL-2 and IFN-γ levels, without significant changes in IL-4 and IL-10 production was concomitantly observed. No significant changes in HIV viral load were detected after the introduction of thalidomide treatment.
Conclusion. The association of thalidomide to standard anti-tuberculosis therapy, in patients with a poor response to the treatment, resulted in an improvement of the clinical conditions and in a microbiological negativization of clinical samples with a restoration of the immunological parameters. This clinical evolution has been strictly correlated to a significant increase in TNF-α, IFN-γ and IL-2 levels.
An increase in TNF-α levels following thalidomide introduction could be explained by an activation of T-cell activity. Thalidomide's principal effect is likely to be in stimulating T lymphocytes, resulting in an increase in IL-2 and INF-γ production.

N° Accordo di collaborazione: 50C.13

WHO GETS HIV-ASSOCIATED TUBERCULOSIS IN THE ERA OF HAART? A PRELIMINARY ANALYSIS OF A STUDY OF TUBERCULOSIS TREATMENT OUTCOME.

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Background: HIV-patients who receive HAART have a markedly reduced risk of developing active tuberculosis (TB). Nevertheless, HIV-associated TB continues to occur even in the context of a widespread use of HAART. We analyzed characteristics of HIV-infected patients presenting with active TB in Italy in the HAART era.

Methods: A total of 211 HIV-infected persons diagnosed with active TB were recruited from 96 infectious diseases hospital units in Italy between 1 May 1999 and 1 September 2000 into an observational, prospective study aimed to evaluate tuberculosis treatment outcomes. We analyzed demographic and clinical characteristics of these patients at the time of TB diagnosis .

Results: Of the patients enrolled, 46 (22%) were diagnosed with HIV at the time of TB diagnosis or shortly before, 82 (39%) had a positive HIV test at least 3 months before TB but did not receive any antiretroviral treatment, and 83 (39%) had received HAART before TB diagnosis. Being foreign born and being in transmission categories other than IVDU, were independently associated with lack of awareness of HIV status. Among those aware of HIV status before TB, being foreign born was associated with lack of previous antiretroviral treatment. Patients with previous HAART, were significantly less immunosuppressed than other patients at the time of TB diagnosis (median CD4 236 cells/mmc vs 106 cells/mmc, $p< 0.001$; proportion with CD4>500/mmc 20.5% vs 5.5% $p<0.001$). Proportion of patients with "atypical" clinical presentation did not differ between previously HAART treated and untreated patients after adjustment for CD4 count.

Conclusion: The majority of TB cases in the last year occurred in HIV-infected persons who never received antiretrovirals. TB occurs at higher CD4 cell counts in previously treated patients. This may reflect an increased proportion of patients with minor levels of immunosuppression in the population of HAART treated individuals or, less likely, a defect in immune restoration.

Grant no. 50C.14

CYTOMEGALOVIRUS: A NEW POLYMORPHIC ENVELOPE GLYCOPROTEIN ABLE TO INDUCE A VIRUS-NEUTRALIZING IMMUNE RESPONSE

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Contract n. 50C.15

Human Cytomegalovirus (CMV) is a widespread human pathogen that is rarely pathogenic in immunocompetent subjects but becomes a major problem after transplantation and during the progression of AIDS. Unfortunately the control of this infectious agent is still problematic because only two drugs are available with resistant mutants often arising and it does not exist a vaccine. At this respect some attempts carried out using well known viral glycoproteins failed. For this reason the identification and the study of new envelope glycoproteins is essential.
We studied the product of ORF UL73, a putative HCMV transmembrane glycoprotein which is highly conserved among herpesviruses (EBV BLRF1; HHV6 U46, MCMV M73A, HSV-1, PrV and BHV UL49.5). Using a pUL73-specific antiserum raised against two synthetic epitopes of the protein, we demonstrated that pUL73 is a true-late protein expressed on the surface of infected cells (1). Immunoblot analysis showed that pUL73 is a glycoprotein component (gpUL73) of the viral particle which associates in a disulfide-linked high molecular complex with the product of UL100 (gM). The association of gN with gM seems necessary for the translocation of both glycoproteins to the cell surface (2). Furthemore, immunoelectron-microscopy revealed that gpUL73 localizes in the viral envelope and at least the N' terminus of the molecule seems to be exposed at the external surface (3).
As structural glycoproteins are often involved in virus attachment and penetration and are important modulators of the host immune-response, the immune response against gpUL73 is under investigation. Preliminary results indicate that antibodies raised against gpUL73 have a strong neutralizing activity on the virus and that more than 60% of the immune sera from patients have high antibody titers to the gN/gM complex (2).
Furthermore, sequence analysis of gN from viral strains from AIDS patients as well as from other patients showed that gpUL73 strongly diverges from that of laboratory adapted strain AD169, suggesting an important role of gN for in vivo pathogenesis. In particular, the N-terminal portion from clinical isolates is highly polymorphic and this finding allowed the identification of different gpUL73 genomic variants, as previously described for gB and pUL144 (4). The different genotypes do not seem to be randomly distributed, on the contrary we identified four distinct genotypes which do not correspond to gB genotypes. The role of gpUL73 polymorphism in virulence and/or cell tropism is under investigation.

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TOWARD THE IDENTIFICATION OF A NEW ANTIVIRAL STRATEGY: INHIBITION OF INTRANUCLEAR TRANSLOCATION OF ESSENTIAL GENE PRODUCTS

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Contract n. 50C.15

Human Cytomegalovirus (CMV) is a widespread human pathogen that is rarely pathogenic in immunocompetent subjects but becomes a major problem after transplantation and during the progression of AIDS. Unfortunately the control of this infectious agent is still problematic because it does not exist a vaccine and only two drugs are available with resistant mutants often arising. For this reason the identification of new drugs and new antiviral strategies are essential.
At this respect we aim at identifying peptides that bind specifically and with high affinity to nuclear localisation signals (NLS) present on the product of CMV UL44, ppUL44. Among identified peptides we expect to select peptides that can efficiently compete with cellular factors for binding ppUL44 NLS, and consequently interfere with nuclear localisation of ppUL44, since functional ppUL44 is essential for HCMV replication.
Our final goal is the inhibition of HCMV replication by preventing the localisation of this essential viral protein in the cell by retaining it in a non-functional site which may also target it for degradation
We have previously identified two groups of peptides from a bacterial random dodecapeptide expression library (Invitrogen) using chemically synthesised peptides mimicking the two functional NLS present on ppUL44. The sequences of the 31 identified peptides were aligned with known sequences of eukaryotic import factors and significant homologies were found in peptides of both groups.
We then set up an EIA to verify the affinity of the peptides to a recombinant ppUL44 (rppUL44) as expressed in bacteria. Purified rppUL44 was adsorbed to the surface of 96 wells EIA plates, and subsequently exposed to induced peptide expressing bacterial clones. Bacteria retained in the reaction wells after washing were revealed by means of a monoclonal antibody directed against a thioredoxin domain flanking all random peptides expressed at the bacterial surface. The amount of cells retained in each well, as compared to suitable controls was considered a measure of the affinity of the corresponding peptide to ppUL44 NLS. This study allowed the sorting of all identified peptides by a criterion of ppUL44 NLS affinity.
In parallel we have made a construct (pMB2000) that expresses the CMV DNA polymerase (ppUL54) in the eukaryotic expression vector pcDNA3 as a recombinant protein carrying a six histidine tag at the NH2 terminus (6HrppUL44). We have established an astrocytoma cell line permanently transfected with pMB2000 (U373-MB2000). When U373-MB2000 is infected with CMV, 6HrppUL44 is expressed both with the expected kinetics of expression and colocalises with ppUL44 in nuclear replicative centers.
We plan now to synthesise peptides with high affinity to ppUL44 NLSs and study their effect in nuclear translocation assays in partially permeabilised cells on ppUL44 localisation. In these studies we will use both a previously established cell line permanently expressing ppUL44, and the newly produced U373-MB2000 cell line.

EFFECTS OF CMV INFECTION ON CELL CYCLE AND DNA PRECURSORS BIOSYNTESIS

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Earlier studies revealed that CMV rapidly inhibits the growth of fibroblasts by blocking cell cycle at multiple time points, including the G1/S phase transition. Differential display analysis and DNA microarray assay demonstrated that HCMV infection before the onset of virus DNA synthesis caused changes of about 258 cellular mRNAs, some of which also induced by Interferons (IFN). Among these the Ifi200 genes appear to be increased at both mRNA and protein level upon infection with viable or UV-inactivated MCMV. Activity of a reporter gene driven by the Ifi204 promoter induced following virus infection showed that this increase was due to transcriptional activation. FACS analysis of infected MEF stably transfected with a p204-dominant negative mutant (p204dmMEF) revealed that they do not accumulate at the G1/S border in the same way as infected MEF transfected with the empty vector. Moreover, MCMV DNA synthesis is significantly delayed, due to retarded expression of viral genes, namely IE1 and DNA polymerase suggesting that MCMV may exploit the Ifi200 genes to regulate the cell cycle. In quiescent cells the reduction of ribonucleotide into deoxyribonucleotide (dNTP) and the biosynthesis of thymidylate (dTMP) are highly repressed. We have demonstrated that CMV infection of quiescent cells leads to the coordinated induction of the cellular enzymes *folylpolyglutamate synthetase* (FPGS), *dihydrofolate reductase* (DHFR), *dCMP deaminase* and *thymidylate synthetase* (TS) involved in dTMP synthesis through the stimulation of the relative gene promoters. More recently, we have shown that CMV replication depends on ribonucleotide reduction since it is prevented by hydroxyurea an inhibitor of the enzyme *ribonucleotide reductase* (RNR). CMV infection markedly induces both mRNA and protein corresponding to the cellular R2 subunit, whereas expression of the cellular R1 subunit is not up-regulated. The increase in R2 gene expression is due to an increase in its gene transcription. It was found that the viral gene M45 encoding a putative homologue of the R1 subunit is expressed and its product has a cytoplasmic localization. Meanwhile, we observed an expansion of the deoxyribonucleosides triphosphates pool between 24 and 48h after infection, whereas neither CDP reduction nor viral replication was inhibited by treatment with 10 mM thymidine. These findings indicate the induction of an RNR activity with an altered allosteric regulation compared to the cellular RNR following CMV infection, and suggest that the virus R1 homologue may complex with the induced cellular R2 protein to reconstitute a new RNR activity.

N°. dell'Accordo di Collaborazione. 50C. 16

ROLE OF HUMAN HERPESVIRUSES IN AIDS

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Several lines of clinical and experimental evidence indicate that selected human herpesviruses play an important pathogenic role in the course of HIV infection. In particular, HHV-6 has been suggested to act as a cofactor in the progression from the asymptomatic state to full-blown AIDS. To generate appropriate diagnostic tools for the identification of active (thus, clinically relevant) HHV-6 infection, we developed a quantitative real-time PCR system based on the TaqMan technology. This method is highly sensitive and reproducible, and displays a remarkable dynamic range, as it allows to detect between 1 and 10^6 viral genome equivalents in a single reaction. The use of this new method will be useful for large epidemiological surveys aimed at verifying the temporal association between active HHV-6 infection and progression to AIDS. A similar assay has also been developed for HHV-7 and HHV-8. Preliminary data indicate a strong association between the presence of HHV-8 plasma viremia and risk of Kaposi sarcoma. We have investigated the in vivo interactions between HHV-6 and HIV-1 using a chimeric SCID-hu thy/liv model; as we recently demonstrated, HHV-6 actively replicates in such mice causing a dramatic depletion of the engrafted human thymocytes. Coinfection with HHV-6 and HIV-1 resulted in the simultaneous replication of both viruses, although no evident interference or synergy was observed in this model. To better understand the pathogenic effects of HHV-6, we have used a newly developed fusion assay for investigating its cellular receptor. We identified such receptor as the CD46 glycoprotein, a ubiquitous complement regulatory protein that plays an essential role in protecting autologous cells from complement attack. Remarkably, HIV virions incorporate CD46 into their membrane, suggesting a potential direct interaction between HHV-6 and HIV virions in vivo. Preliminary data indicate that engagement of CD46 by the HHV-6 envelope represents in itself a virulence factor that could mediate some of the pathogenic effects of this virus, including suppression of cell-mediated immune responses and exposure of cells to complement attack.

Accordo di Collaborazione n. 50C.17

IMPAIRMENT OF THE T CELL RESPONSE TOWARDS CANDIDA ALBICANS ANTIGENS IN HIV INFECTION: ROLE OF CYTOKINES IN THE INDUCTION AND MAINTENANCE OF FUNGAL INFECTION.

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The first part of the study has been focused on the functional T cell response towards Ca antigens in seronegative patients with Chronic Vaginal Candidiasis (CVC). The cytokine production by PBMC under the M-sMP stimulation was initially evaluated Antigen induced a higher IL-4 production in healthy subjects than in CVC patients, mainly when low doses of stimulus were employed, whreas no difference were found in IFN-g production. This suggests a shift to Th1-like response in CVC patients. To confirm such initial result, M-sMP-specific T-cell lines were generated from PBMC of two healthy and two CVC women. T-cell clones (TCC) derived from T-cell lines were subsequently assessed for specificity to M-sMP and MP65 antigens as well as for cytokine production.
The great majority of TCC showed a CD3+CD4+CD8- phenotype. The clonal analysis has shown that TCC derived from CVC patients exhibited a higher IFN-γ production than their counterparts from healthy donors. When cathegorized according to their cytokine profiles (Th1, Th2 and Th0) the proportion of Th2 clones from CVC patients was significantly ($p<0.001$) than that observed in control clones. This finding confirms the presence of a Th1 shift of memory T cells specific for Ca antigens in the peripheral compartment of CVC patients. Furthermore, the proportion of TCC specific for M-sMP was significantly lower than their control counterparts. This depletion was almost exclusively due to the reduction of MP-65+ TCC, whereas that of MP-65-, M-sMP+ TCC was similar in the two groups of subjects. This could suggest that, during a chronic Ca infection, a recruitment of T cells specific for major Ca antigens occurs. To confirm this hypothesis, the comparison between the proportion and type of specificity of T cells infiltrating Ca lesions and circulating T cells in CVC patients is in progress.
In the second part of the project the study has been devoted to analyse the functional T cell response to Ca antigens in HIV seropositive patients.
Thirthy HIV-infected patients selected on the basis of some parameters (numbers of CD4+ T cell/l, viraemia, months of HAART and history for previous local or systemic candidiasis) were evaluated for their ability to proliferate to Ca antigens and to produce cytokines upon antigen-stimulation. The mitogenic indexes of T cells from HIV-infected individuals were significantly lower than those exhibited by control T cells at all doses employed (10 μg, 1 μg and 0.1 μg/ml). Moreover, T cells from HIV+ patients with previous systemic candidiasis showed a significantly lower proliferative response than those of patients without history of candidiasis. More importantly, T cells from all HIV-infected subjects produced significantly lower and, respectively, higher amounts of IL-4 and IFN-γ, thus suggesting that, similar to CVC, circulating Ca-specific T cells from seropositive people show a clearcut shift towards a Th1-like profile.Subsequently, 28 TCC (12 CD4+ and 16 CD8+) from infiltrating T cells of an oral biopsy from one HIV-infected subject were generated and analysed. Six out of 12 CD4+ TCC were specific for Ca antigens (one clone to MP65 and 5 to M-sMP), thus confirming a lesional accumulation of Ca-specific T cells. Of note all six Ca-specific TCC produced high levels of IL-4 (two of them did not produce IFN-γ), suggesting that local T cell response to Ca antigens is prevalently Th0/Th2-oriented. This finding, even though preliminary, suggest that, during chronic Ca infection, the recruitment from periphery to lesions of Ca-specific T cells with a type 2 phenotype may occur.

No. Contributo: 50C.18

CD4 RESPONSES AND T CELL REPERTOIRES SPECIFIC FOR OPPORTUNISTIC PATHOGENS DURING HIV INFECTION

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Opportunistic infections (OI) are an important cause of morbidity and mortality in HIV infected patients. Even though antiretroviral therapies have reduced the incidence of OI, patients who do not or cannot comply with therapy, or are infected by resistant HIV strains, suffer from OI. Most opportunistic pathogens (OP) that characterize AIDS are controlled in normal individuals by cell mediated immunity (specific CD4 cells or CD4 dependent specific CTL). Therefore the analysis of CD4 cells specific for relevant OP should be performed not only to monitor these cells by conventional proliferation assays, but also in terms of clonal heterogeneity and fine epitope specificity.
For these studies we focused on two representative OP, the fungus P. carinii (Pc) and virus CMV. T cell lines specific for these OP were obtained from normal donors and from HIV patients by repeated in vitro stimulation with antigens and APC, followed by IL2 expansion. The established lines specific for these pathogens were examined for specificity and clonal heterogeneity.
1. Pc specific CD4 lines were obtained from two asymptomatic patients in spite of lack of proliferative response to Pc. This showed that residual Pc specific cells were present, but undetectable. Yet they were sufficient to prevent clinically apparent Pc infection. The clonal diversity of these lines (assayed by TCR BV PCR and by spectratyping) was similar to that observed in normal, Pc responsive controls. This suggests that in the patients Pc specific CD4 cells were evenly lost from most of the clonal components of the specific repertoire, with no depletion selectively affecting given TCR BV gene families. It also suggests that in the case of immunoreconstitution (i.e. recovery of CD4 counts) these patients should be able to recover a clonally diverse repertoire from the residual CD4 cells.
2. In the case of CMV infection, we have confirmed that the antigen pp65 accounts for most of the proliferative responses to inactivated virions. CMV specific CD4 lines from most donors respond vigorously to pp65. Panels of overlapping peptides (pruduced by P. Neri et al, Univ. of Siena) are currenty being used to define epitope specificity. Clonal analysis of the CMV and of the pp65 specific lines showed that several TCR BV gene families are involved, but oftentimes single peaks are detected by spectratyping in the individual BV families. This observation suggests that upon sequencing of the TCR hypervariable regions of these oligo-monoclonal families, TCR-CDR3 specific primers can be synthesized to monitor the relevant clone by PCR during disease progression.
3. A similar approach to monitor CMV cellular immunity is currently being tested also on other immunocompromized patients who are subject to CMV complications, confirming that different disease groups can benefit from investigations originally performed on HIV patients.

Grant n. 50.C.19

THE RESPONSE OF γδ T CELLS TO MYCOBACTERIA TRIGGERS HIV EXPRESSION IN CHRONICALLY INFECTED U1 PROMONOCYTES.

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CD4- lymphocytes, involved in the "first line of defence" against pathogens, might play an increasingly more relevant role in HIV+ patients with CD4 lymphopenia. Intriguing candidates for this role are Vγ9Vδ2+T lymphocytes. These cells comprise an important fraction (1-5%) of circulating T lymphocytes all specifically recognising mycobacterial antigens. Upon recognition, γδ cells undergo clonal expansion and kill the infected antigen presenting cells. HIV+ subjects infected by mycobacteria have an higher risk to develop active tuberculosis. Conversely, the mycobacterial infection induces a stark increase of viral replication and of the viral burden and accelerates the clinical progression of the disease. The latter event is accompanied by an increased cellular activation, with massive secretion of soluble factors known to favour viral replication, including tumor necrosis factor-α and interferon-γ. We therefore verified whether chronically infected cells presenting mycobacterial antigens to γδ lymphocytes were indeed recruiting functional activities involved in the control of the infection by the opportunistic pathogen. The engagement of the γδ TcR resulted in the production of IFN-γ, IL-4, MIP1β, RANTES, IL-8, I-309 and TNF-α while IL-10, MCP-1, MDC and SDF-1, were not induced. Of interest, the presence of moieties able to engage the physiological ligand of the CD30 molecules on γδ T cells selectively influenced the pattern of soluble factors released, implicating an instructive role of the co-stimulatory molecules expressed by infected antigen presenting cells. We then verified the outcome of the presentation of increasing concentrations of soluble phospho-antigens (isopentenyl pyrophosphate) by the latently infected promonocytic U1 cells. At higher concentration of tubercular antigens, γδ cells released cytokines and chemokines and efficiently killed the antigen presenting U1 promonocytes. However, at lower (but still substantial – in the millimolar range) concentrations of IPP, the lytic efficiency was reduced. In these conditions, the recognition of tubercular antigens by γδ T cells resulted in the HIV replication in the surviving U1 cells. This event required the co-culture of γδ lymphocytes, U1 promonocytes and mycobacterial antigens. In the absence of tubercular antigens, γδ cells ignored the U1 cells. Accordingly, the lytic efficiency was comparable when U937 parental cells were used in the assay instead of U1 cells. The effect on the viral replication was due to the soluble factors released upon TcR activation: it was abolished by the simultaneous treatment with monoclonal antibodies capable to block the β chain of IFN-γ, receptor and the soluble TNF –α . The blockade of the Fas-Fas ligand interaction, which efficiently prevented the lysis of U1 cells, did not significantly impinge on the viral replication. These results identify in the control of γδ T cell activation a goal for the treatment of tuberculosis in HIV + patients.

N°. dell'Accordo di Collaborazione. 50C.20

THE VIRAL REPLICATIVE PROTEINS E2 FROM HPV-16 AND TAT FROM HIV-1 SHARE A COMMON CELLULAR PATHWAY FOR TRANSCRIPITONAL ACTIVATION

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Contract n. 50C.21

Invasive cervical carcinoma is a disease associated to oncogenic papillomavirus (HPV) infection that has been included among the AIDS defining conditions since 1993. Infection by human adeno-associated virus type 2 (AAV2) is a possible protective factor in the development of cervical carcinomas associated with HPV. The replicative proteins of AAV2 (Rep) have been implicated in the inhibition of papillomavirus replication and transforming activities. We observed that each of the four forms of AAV2 Rep inhibited the E1/E2-driven replication of oncogenic HPV16. Rep40, corresponding to the C-terminal domain of all Rep proteins, was inhibitory of both HPV DNA replication and HPV16 E2-mediated transactivation. Rep 40 specifically bound the N-terminal transactivation domain of HPV16 E2 both in vitro and in vivo. This interaction was found to specifically disrupt binding of E2 with the cellular transcriptional co-activator p300. Accordingly, the inhibitory effect of Rep on HPV16 E2 transactivation was rescued by overexpression of p300. These data point to a novel role of Rep in the down-regulation of papillomaviruses by inhibiting complex formation between the HPV16 E2 transcriptional activator and its cellular co-activator p300[1]

While performing these experiments we reasoned that recruitment of the transcriptional co-activator p300/CBP is a cellular pathway shared among different viral transactivators, including HIV-1 Tat. We observed that, similarly to what happened for E2, the interaction of Tat with p300/CBP was also disrupted by Rep. The latter interaction requires integrity of the basic domain of Tat and occurs through domains of p300 that we could demonstrate being overlapping with those of E2. Tat functionally recruits p300/CBP through the formation of multiple protein-protein interactions that involve Tat, p300/CBP and other cellular factor(s). In order to explore the interaction of Tat with cellular partners within the living cell we developed a strategy based on fluorescence energy transfer (FRET) and co-localisation by confocal microscopy. We observed that the localisation of p300/CBP and cyclin T1 does not necessary match that of Tat, rather Tat appears to be capable of recruiting these factors from PML nuclear bodies[2]. Interestingly, AAV Rep does not localise in these compartments and appears to accumulate at their periphery and might prevent trafficking between intra-nuclear compartments.

Key macromolecular interactions that mediate the function of viral replication factors represent a promising target for an effective antiviral therapy. For this purpose we developed a hybrid Rep protein carrying the trans-cellular trafficking domain of Tat. The recombinant protein was efficiently produced in E. coli, purified and shown to be functionally capable to access the target cells. Targeting of HPV-infected cells can be achieved by AAV vectors, which are being prepared, carrying the modified Rep proteins.

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ISOLATION AND DISRUPTION OF THE DIFFERENTIALLY EXPRESSED CA19B GENE AND ITS PHENOTYPIC CHARACTERIZATION.

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The ability of C. albicans to cause infection is related to its capacity to survive inside the macrophages. Indeed, C. albicans responds to the intracellular environment by initiating a specific genetic program whose expression permits the survival in the new environment. However, so far little it is known on how this fungus accomplish this.
Our aim is to identify, clone, and characterize differentially expressed genes of C. albicans during infection of macrophages, as to better understand the interaction between the fungus and macrophage. Total RNA was extracted from C. albicans yeast collected during the time course (T_{15}, T_{30}, T_{60} and T_{120}) of the macrophage infection. Using differential display reverse transcription-polymerase chain reaction (DDRT-PCR), originally developed by Liang and Pardee (1), we have isolated a gene CA19b, whose expression increases 30 min after infection. Northern analysis using CA19b as a probe, confirmed the differential expression of this cDNA during macrophage infection.
By using the PCR product in a BLASTN search on the Candida database (http://www-sequence.stanford.edu/group/candida/search.html), we identified homology with the contig5-2935. The study of the homologue region of this contig revealed the presence of an open reading frame of 2,678 base pairs encoding for a protein of 892 aminoacids. Searching Genbank database, CA19b coding sequence showed homology to the CAT family proteins that are mostly involved in the transport of acyl-CoA esters from peroxysomes to the mitochondrial matrix. Based on the contig sequence, we designed two primers to amplify by PCR the full-length gene (CA19b) from C. albicans wild type (SC5314). The genes was cloned and to study the phenotype of the disrupted strain we obtained a ca19b null mutant using the HisG-URA3-HisG technique (2). analysis of the phenotype is presently under study.

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N°. dell'Accordo di Collaborazione. 50C.22

COMPARATIVE EVALUATION OF TECHNIQUES AND INSTRUMENTATION FOR REAL TIME PCR

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Real time PCR is an amplification reaction, the outcome of which is evidenced in real time by the use of fluorescent molecules or probes. It's application as diagnostic tool presents a variety of advantages over standard PCR including the shorter time needed for obtaining a result, the possibility of evidencing directly the amplification product without the need of electrophoresis, the possibility to analyse multiple targets simultaneously, and the high reliability of target DNA quantification.
With the intent to set up correctly a protocol for the use of real time PCR as diagnostic toll for tuberculosis and the determination of antibiotic resistance in Mycobacterium tuberculosis we evaluated different instruments and methods for detection. The spectrofluorometric thermal cyclers evaluated in our study were supplied from Roche, Applied Biosystems (2 systems) e Biorad. The detection modes evaluated included the fluorescent dye SYBR Green I and hybridization probes marked with fluorophores and their respective quenchers (molecular beacon TaqMan and LC probe). The sensibility of the compared instruments was not significantly different and all were able to detect fluorescent probes. The time necessary for evidencing results varied from 20 minutes to 4 hours depending on the instrument. Some instruments gave the opportunity to follow the PCR reaction during amplification, which in some cases permitted to abbreviate obtainment of results and also gave the option to change some parameters, if PCR kinetics were found to be suboptimal. Not all instruments were found to be able to perform melting curves, which are essential for programming correct temperatures and for detection of possible contaminating unspecific amplification products using SYBR Green and for mutation detention by LC probe. Different levels of sensibility and specificity were evidenced for the distinct detection methods. In our hands SYBR green I was the most sensitive method followed by TaqMan, molecular beacons, and LC, while the most specific hybridisation probes were molecular beacons followed by LC and TaqMan.
The essential parameters identified were applied to the detection and quantification of Mycobacterium tuberculosis (SYBR Green) and for the determination of resistance to rifampicin (molecular beacon).

Project number N° 50C.23

HUMAN POLYOMAVIRUS JC REARRANGEMENT PATTERNS IN CEREBROSPINAL FLUID OF HIV-1 POSITIVE AND NEGATIVE SUBJECTS WITH OR WITHOUT PML.

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The human polyomavirus JC (JCV) is endemic in the population and the infection rate by adulthood is greater than 70%. The virus reaches its target organs by the hematogenous route and becomes latent in kidney and brain as well as in other organs where it can reactivate and duplicate under conditions of immunological impairment. Although the primary infection and reactivation are asymptomatic, in immunocompromised patients JCV can cause a fatal demyelinating brain disease, the progressive multifocal leukoencephalopathy (PML). In fact, HIV-1 infection of the central nervous system (CNS) induces a variety of clinical manifestations. Once rare, PML is now the most common of the HIV-1 associated CNS complications.
The diagnosis of PML is based mainly on the clinical presentation, the detection by magnetic resonance imaging of demyelinating lesions and the neuropathology of the brain biopsy. At present amplification of viral DNA is used for the diagnosis of PML; in fact the PCR technique is used to search for JCV DNA in cerebrospinal fluid (CSF) drawn by lumbar puncture.
In our study, the presence of JCV genome and viral particles were searched for by means of a specific and sensitive nested-PCR for the non-coding control region (NCCR) in CSF samples collected from 13 HIV-1 positive and negative subjects (3 with PML and 10 without PML).
The PCR fragments were sequenced and analysed in Genbank, comparing the JCV NCCR structure found in CSF with that of the archetypal structure.
The archetypal NCCR can be divided into six regions or blocks named A, B, C, D, E and F which are consistently retained, duplicated or deleted in JCV genomes found in our patients. In particular, in HIV-positive and negative subjects without PML, the structure of the NCCR presents deletions or duplications by comparison to the archetype structure, whereas the sequences found in CSF of patients with PML show specific and characteristic rearrangements, such as the loss of the F box.
A possible explanation of our results is that rearrangements are generated in the host from a basic archetypal sequence within which there are preferred targets.
During replication of viral genome, specific variants could be selected and transported to the brain and might lead to the development of PML.

Accordo di collaborazione N° 50C.24

Presentazione come poster.

ROLE OF NERVE GROWTH FACTOR (NGF) ON CELL SURVIVAL AND VIRUS MATURATION IN HUMAN HERPESVIRUS 8 (HHV-8)-INFECTED PRIMARY EFFUSION LYMPHOMA CELLS.

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We have previously reported that BC-1 and BCBL-1 (two primary effusion lymphoma cell lines infected by HHV-8 and EBV or by HHV-8 alone, respectively) express NGF receptors and produce NGF, whereas RAMOS cells (a B-cell line that is negative for HHV-8 and EBV) express NGF receptors but do not produce detectable NGF. We also reported that neutralization of endogenous NGF resulted in cell growth inhibition and apoptosis in BCBL-1 cells and, to a minor extent, in BC-1 cells.

We have then studied the effect of NGF on BCBL-1 cell survival during the chemically-activated HHV-8 lytic cycle. We found that when the HHV-8 lytic cycle is induced in BCBL-1 cells by tetradecanoyl phorbol acetate (TPA), an initial reduction of endogenous NGF production is observed, and many cells undergo apoptosis. However, at 48 hours, TPA-treated cells produce significantly more NGF than untreated controls, and a subsequent recovery of cell viability is observed. Consistent with this finding, the addition of exogenous NGF or anti-NGF antibodies to TPA-treated cells reduces or increases, respectively, the rate of apoptosis in response to TPA. To determine whether NGF was able to affect viral maturation in this virus-host cell system, we performed electron microscopy studies on BCBL-1 cells treated with TPA, in the presence or absence of human recombinant NGF or anti-NGF antibodies. The results of this study showed that the addition of exogenous NGF increases the number of BCBL-1 cells producing and releasing complete virions as compared with the controls (25% versus 5%). On the contrary, NGF neutralization leads to the production of defective viral progeny in about 2% of cells. We therefore conclude that NGF is essential for both cell survival and virus maturation in HHV-8-infected cell lines.

These data streghten our previous observations concerning the existence of a close relationship among NGF, HHV-8 infection and HHV-8-associated diseases. Moreover, they provide new insights for the understanding and treatment of HHV-8-related diseases. To fully elucidate the mechanism(s) involved in the observed phenomena, further *in vivo* and *in vitro* studies are currently ongoing.

Contributo n.50 C.25

IN VITRO BACTERICIDAL ACTIVITY OF MONOCLONAL AND RECOMBINANT KILLER ANTIBODIES AGAINST ANTIBIOTIC RESISTANT GRAM-POSITIVE COCCI

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Monoclonal (mAbKT) and recombinant single chain (scFvKT) antiidiotypic antibodies were produced to represent the internal image of a yeast killer toxin (KT) characterized by a wide spectrum of antimicrobial activity. MAbKT and scFvKT had proven to kill in vitro pathogenic eukaryotic and prokaryotic microorganisms such as Candida albicans, C. glabrata, C. krusei, Cryptococcus neoformans, Pneumocystis carinii, Streptococcus mutans, and multidrug resistant strains of Mycobacterium tuberculosis presenting specific cell wall receptors.
The swelling tide of concern over increasing bacterial resistance to conventional antibiotic drugs gives the impetus to develop new therapeutic compounds against microbial threat. Thus, the in vitro bactericidal activity of mAbKT and scFvKT against gram-positive cocci, 5 isolates of Staphylococcus aureus, 4 S. haemolyticus, 1 Enterococcus faecalis, 1 E. facium, and 3 Streptococcus pneumoniae characterized by different patterns of resistance to antibiotics, including methicillin,vancomycin and penicillin, was investigated.
According to the experimental conditions adopted no bacterial isolate proved to be resistant to the activity of mAbKT and scFvKT. ScFvKT exerting a microbicidal activity against multidrug resistant gram-positive cocci may represent, in particular, the basis for the drug modelling of new antibiotics with broad antibacterial spectra to tackle the emergence of microbial resistance.

Accordo di Collaborazione N^{o} 50C.26

THERAPY OF VAGINAL CANDIDIASIS BY KILLER ANTIIDIOTYPES EXPRESSED BY HUMAN COMMENSAL BACTERIA OR MIMOTOPES

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The human commensal Streptococcus gordonii has been engineered to express a candidacidal single chain fragment variable (scFv) antiidiotypic antibody (H6) mimicking a yeast killer toxin (KT) for use as a therapeutic tool in vaginal infections. Two recombinant strains of S. gordonii, both relying on gene fusion with the streptococcal M6 protein, were constructed as to either display the scFv H6 on cell surface or secrete it. The recombinant strains exerted a strong candidacidal activity in vitro clearly dependent on the relative numerical ratio between engineered bacteria and yeast cells and concentration of culture supernatants, respectively. In a rat vaginitis model, both the transformed S. gordonii strains, inoculated intravaginally after a local challenge with Candida albicans cells, were able to colonize the mucosa and accelerated the yeast clearance from the rat organ. In particular, the scFv H6-secreting strain caused a dramatic reduction of fungal intravaginal burden comparing well with the therapeutic efficacy of fluconazole and that of killer mimotopes obtained as synthetic decapeptides from the sequence of scFv H6.

Accordo di Collaborazione N^{o} 50C.26

DENDRITIC CELLS FOR VACCINATION IN FUNGAL INFECTIONS

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The fungus Candida albicans behaves as a commensal as well as a true pathogen of areas highly enriched of dendritic cells, such as skin and mucosal surfaces. The fungus can switch from a unicellular yeast form into various filamentous forms, all of which can be found in infected tissues. The ability to reversibly switch between these forms is thought to be important for Candida's virulence. Although recent studies have clearly shown that the ability to switch from yeast to filamentous form is required for virulence, whether it is the yeast or the hyphal form that is responsible for pathogenicity is still an open question. One interesting observation is that disseminated infection of mice with a low virulence stain of C. albicans, which does not produce hyphae, is associated with the development of protective T helper (Th) 1 immunity and renders mice resistant to an otherwise lethal infection with the high virulent strain CA-6, capable of forming hyphae in vivo. One possibility is that the filamentous growth form is required to evade the cells of the immune system, whereas the yeast form may be the mode of proliferation in infected tissues. For this to be possible, a cell must exist that finely discriminates between the two forms of the fungus in terms of class of immune response elicited. Recent evidence in mice indicates that neutrophils discriminate between the two forms of the fungus, being able to produce IL-12 in response to C. albicans yeasts, and IL-10 in response to C. albicans hyphae. However, the induction of T-dependent immunity against the fungus necessitates prior pathogen-nonspecific triggering of antigen presenting cells (APC) capable to activate antigen-specific Th cells. Dendritic cells (DC) fulfill this requirement, being uniquely able to initiate responses in naive T cells and to participate in Th cell education. Here we show the interaction, and consequences, of different forms of C. albicans with dendritic cells. Immature myeloid dendritic cells rapidly and efficiently phagocytosed both yeasts and hyphae of the fungus. Phagocytosis occurred through different phagocytic morphologies and receptors, resulting in phagosome formation. However, hyphae escaped the phagosome and were found lying free in the cytoplasm of the cells. In vitro, ingestion of yeast activated dendritic cells for IL-12 production and priming of Th1 cells, while ingestion of hyphae inhibited IL-12 and Th1 priming, and induced IL-4 production. In vivo, generation of antifungal protective immunity was induced upon injection of dendritic cells ex vivo pulsed with Candida yeasts but not hyphae. The immunization capacity of yeast-pulsed dendritic cells was lost in the absence of IL-12, whereas that of hypha-pulsed dendritic cells was gained in the absence of IL-4. These results indicate that dendritic cells fulfill the requirement of a cell uniquely capable of sensing the two forms of C. albicans in terms of type of immune responses elicited. As dendritic cells similarly discriminate between nonvirulent (conidia) and virulent (hyphae) forms of Aspergillus fumigatus, all together these results indicate that dendritic cells meet the challenge of Th priming and education in fungal infection and saprophytism.

Accordo di Collaborazione n. 50C.27

HIV-1 TAT INHIBITS CELL GROWTH AND INDUCES ION SECRETION IN HUMAN ENTEROCYTES.

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Although the intestine is a target organ in HIV disease, there is little and not conclusive evidence of a direct interaction between HIV and the enterocyte. The HIV-1 transactivating factor protein (Tat) is a key factor in pathogenesis of AIDS. Tat is released outside the infected cells and inhibits several L-type-Ca^{2+} channels-dependent functions in immune cells. Caco-2 cells are human-derived enterocytes that possess L-type-Ca^{2+} channels, which are involved in their growth and in ion transport processes. Aims. To test the hypothesis that Tat is capable to direct interact with human intestinal cells, inducing ion secretion and growth impairment. Methods. For transport studies, electrical parameters were measured in Caco-2 cells monolayers mounted in Ussing chambers and exposed to Tat. To measure cell growth, cells were counted and ^{3}H-thymidine incorporation was determined, in the presence or absence of Tat. Control experiments were performed by preincubating Tat with the anti-Tat specific antibodies (anti-TatAb) or with Bay K8644 (a specific L-type Ca^{2+} channels agonist). Results. Intestinal transport: the serosal, but not the mucosal, addition of Tat induced a significant Cl^{-}-dependent increase in short-circuit current (Isc) without modification of tissue conductance, consistent with ion secretory effect (maximal Isc increase=2.5$\pm$.2 uA/cm^2). The effect was dose-dependent and saturable. Preincubation with anti-TatAb or with Bay K8644 inhibited the electrical response. Cell growth: 48 h incubation with Tat resulted in up to 40% decrease in cell count and 35% decrease in ^{3}H-thymidine incorporation vs. controls. Tat-induced inhibition of cell proliferation was prevented by anti-TatAb or Bay K8644. Both secretory and cell growth-inhibition effects by Tat were dose-dependent and the maximal effective concentration was identical for each effect (0.1 nM, similar to Tat serum concentration of HIV-infected patients). Conclusions. These results provide compelling evidence of direct interaction of the enterocyte with a HIV product. The ability of Tat of inducing ion secretion and inhibiting cell proliferation may reflect two common intestinal problems of AIDS patients: diarrhea and HIV-associated enteropathy. This in vitro model offers a unique opportunity for further investigating the direct HIV-enterocyte interaction.

N^{o} dell'Accordo di Collaborazione: 50C.28

INTERACTIONS BETWEEN FLUCONAZOLE AND AMPHOTERICIN B AGAINST CRYPTOCOCCUS NEOFORMANS

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The interaction of amphotericin B (AMB) and azole antifungals in the treatment of fungal infections is still a controversial issue. This study was designated to evaluate the in vitro and in vivo effects of combined or sequential therapy with fluconazole (FLC) and AMB against Cryptococcus neoformans. A checkerboard titration broth microdilution-based method that adhered to the recommendations of the National Committee for Clinical Laboratory Standards was first used to analyze drug interactions against 15 isolates of C. neoformans. Seven percent (1/15) of the interactions were synergic (fractional inhibitory concentration [FIC] index ≤0.50), 67% (10/15) were additive (FIC >0.50 to 1.0), 26% (4/15) were indifferent (FIC >1.0 to ≤2.0), while antagonism (FIC >2.0) was not observed. To investigate the effects of combination therapy in vivo, we established an experimental model of systemic cryptococcosis in BALB/c mice by intravenous injection of cells of C. neoformans 2337, a clinical isolates for which in vitro combination yielded an additive interaction. Both survival and tissue burden studies showed that combination therapy was more effective than FLC alone and it was at least as effective as AMB given as single drug. On the other hand, when cells of C. neoformans 2337 were grown in FLC-containing medium a pronounced increase in resistance to subsequent exposures to AMB was observed. In particular, killing experiments conducted in non-replicating cells showed that preexposure to FLC abolished the fungicidal activity of the polyene. However, this apparent antagonism was not observed in vivo. Rather, when the two drugs were used sequentially in the treatment of systemic murine cryptococcosis, a reciprocal potentiation was often observed. Our study shows that: (i) a complete correlation between in vitro and in vivo results of antifungal combination against C. neoformans is lacking, and (iii) the concomitant or sequential use of FLC and AMB in systemic murine cryptococcosis results in a positive interaction.

N° dell'Accordo di Collaborazione 50C.29

INTERACTIONS OF POSACONAZOLE AND FLUCYTOSINE AGAINST CRYPTOCOCCUS NEOFORMANS

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Cryptococcus neoformans is an important cause of morbidity and mortality in immunocompromised patients. Cryptococcal meningoencephalitis in AIDS patients is generally incurable despite aggressive antifungal therapy. Although amphotericin B, alone or associated with flucytosine (FC), remains the standard therapy for cryptococcosis, this therapeutic approach has important clinical limitations including toxic side effects and inconvenience of intravenous dosing. Recently, several antifungal combination therapies have been evaluated, both in vitro and in vivo using models of cryptococcal infections. One of the most promising combination strategies may be FC in association with an antifungal triazole. Posaconazole (SCH 56592) is a new broad spectrum antifungal triazole currently under development. SCH has been shown to have potent in vitro and in vivo activities against Candida spp., C. neoformans, Aspergillus spp., Blastomyces dermatitidis, and Coccidioides immitis.
A checkerboard titration broth microdilution-based method that adhered to the recommendations of the National Committee for Clinical Laboratory Standards was applied to study the in vitro interactions of FC and SCH against 15 isolates of Cryptococcus neoformans. Synergy, defined as a fractional inhibitory concentration (FIC) index of <0.50, was observed for 33% of the isolates. Where synergy was not achieved, there was still a decrease in the MIC of one or both drugs when used in combination. Antagonism, defined as a FIC >2.0, was not observed. The in vitro efficacy of combined therapy was confirmed by quantitative CFU of C. neoformans 486, an isolate for which FC/SCH association yielded a synergistic interaction. To investigate the potential beneficial effects of this combination therapy in vivo, we established two experimental murine models of cryptococcosis by intracranial or intravenous injection of cells of C. neoformans 486. One day postinfection, the mice were randomized into different treatment groups including single drugs alone and in combination. Both survival than tissue burden studies confirmed a potentiation of antifungal activity upon the combination.
Our study demonstrates that combined SCH and FC are significantly more active than either drug alone against C. neoformans in vitro as well in vivo. These findings suggest that this therapeutic approach could be useful in the treatment of cryptococcal infections.

N° dell'Accordo di Collaborazione 50C.29

IMMUNOGENICITY AND PATHOGENICITY OF PENICILLIUM MARNEFFEI, AN EMERGING OPPORTUNISTIC FUNGUS IN AIDS.

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P. marneffei (P.m.) is a dimorphic, intracellular opportunistic fungus which causes disseminated infections in immunocompromised subjects. Virtually unknown until fifteen years ago, P.m is now the third cause of death for AIDS patients in SE Asia. The aim of our study was to investigate the pathogenicity, the immune response and the drug sensitivity of P.m. using both in vitro and in vivo systems.
Using an in vitro macrophage model that allows intracellular P.m growth, we demonstrated that J774 or THP1 cells phagocytize 30% or 45% conidia, respectively. Duplication time in untreated macrophages is about 48h. When macrophages are activated by LPS+IFNγ, intracellular fungus death occurs (Taramelli D. et al. Infect. Immun. 2000). In addition, we demonstrated that aminoquinoline drugs, such as chloroquine (CQ) and amodiaquine at 10 μM are cytotoxic against phagocytized P.m. The fungicidal activity of CQ is lost when the molecule is chemically modified at the amino side chain, which allows drug accumulation in the macrophage phagolysosomes, where P.m grows (Taramelli et al. Antim. Agents. Chem, in the press). CQ and other aminoquinolines are also toxic to P.m in axenic medium with MIC ranging between 16-128 μg/ml. These results suggest that CQ, a safe and cheap drug that possesses anti-HIV activity, could be used in the prophylaxis of P.m infections in AIDS patients.
An in vivo Balb/c mouse model was used to evaluate the immune response to the fungus by examining by RT-PCR and biological assays the cytokines and chemokines produced in the different organs. Doses ε 10^7 conidia I.V./mouse were lethal. At 10^5 conidia/mouse a self-limiting infection developed with disseminated granulomas in the spleen and livers of infected animals. The results indicate that mRNA for IL12, IFNγ, TNFα and iNOS, but not IL10 peaked at day +7 post infection in the spleens and decreased thereafter concomitantly with the decrease in CFUs. Conversely, the infection in the livers resolved more slowly and peak cytokine levels was at day 21-post infection. Again IL12, iNOS and IFNγ predominate over IL10. Chemokine mRNA levels (MCP1, MIP1α and RANTES) and the receptors CCR1, CCR2, CCR5, but not CCR3 and CCR4, were also upregulated in both spleen and liver of infected animals. We conclude that the resolution of sublethal P.m infection is mediated by the activation of Th1 type of immune response first in the spleens and subsequently in the livers of infected animals, with high levels of chemokines and their receptors expressed in both organs. The implications of these results in the development of HIV co-infections are discussed.

Accordo di Collaborazione 50.C.030

INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY BY A CRYPTOCOCCAL DEACETYLASE

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Cell-mediated immunity plays a crucial role in host defense against cryptococcosis. Therefore cryptococcal antigens capable of inducing a delayed type hypersensitivity reaction (DTH) may be useful in the development of alternative strategies in the control of cryptococcosis.The aim of this study is to characterize cryptococcal antigens capable to induce a DTH reaction in mice.
In order to characterize DTH-inducing antigens, culture supernatants from an acapsular strain of Cryptococcus neoformans (CAP67) were separated by anion exchange chromatography. Three different fractions were obtained and then tested for their ability to induce footpad swelling in mice immunized with concentrated culture supernatants or in unimmunized mice. Two fractions only (peak 2 and 3) were capable of inducing significant DTH. After further fractionation of peak 3 material by preparative SDS-PAGE, a purified protein with an apparent molecular weight of 25 kDa was found to produce DTH. This antigen was not a mannoprotein, since it did not react with Con-A in Western blots. Based on the 20 amino acid N-terminal sequence of the 25 kDa protein an upstream primer was synthesized. The sequence of the upstream primer was derived from homology searches of the GenBank database and the Cryptococcus neoformans Genome Project available at the web site of the Stanford Genome Technology Center (http://wwwsequence.stanford.edu/group/C.neoformans/index.html).
A combination of the upstream primer and the downstream T7 primer was used to amplify a specific PCR product d25 from a cDNA λ phage library created from C. neoformans strain B3501. PCR product sequencing was carried out in both orientations after cloning the insert into the pCR2.1 vector and transformation into Escherichia Coli. The size of d25 DNA was confirmed by Northern blot analysis of RNA from C. neoformans. The 25 gene is about 680 base pairs long and encodes 226 amino residues. The gene shows significant homology with Aspergillus nidulans and Mucor rouxii deacetylase The 25p gene was amplified by PCR using a gene-specific upstream primer containing a EcoR1 site and a downstream primer containing a BamH1 site. Both the vector and PCR amplified 25p were then digested, purified, ligated, and transformed into expression vector. Three trasformants were selected and induced with 1mM isopropyl-D-thiogalactopyranoside (IPTG) for recombinant gene expression. The 25p protein was expressed as a fusion protein with glutathione-S-transferase (GST) and purified from lysed cells under nondenaturing conditions by absorption with 1 ml of a 50% slurry of glutathione-agarose beads, followed by elution in the presence of 1 ml of 50mM Tris-HCl (pH 8.0)/5mM reduced glutathione. The yield of fusion protein was estimated using the Bradford protein micro-assay method.
A novel series of DTH experiments was then carried out with the recombinant protein obtained as described above. Preliminary results clearly indicate that the recombinant product DTH-inducing ability matches exactly the one previously observed with the purified natural protein.
In conclusion, we have identified and cloned a 25kDa deacetylase from C. neoformans capable of inducing DTH reactions in mice. Further studies are being performed to: 1) Test the protective activity of the recombinant protein in a mouse cryptococcosis model. 2) Clone and characterize other antigens from C. neoformans using approaches similar to those used for the 25 kDa protein. In this respect we have temptatively identified genes encoding for 45 kDa, 70 kDa and 200 kDa proteins.

Accordo di collaborazione. 50C.31

ANTIBODY TO GLUCURONOXYLOMANNAN OF CRYPTOCOCCUS NEOFORMANS PROMOTES EXPRESSION OF IL-12Rβ2 SUBUNIT ON HUMAN T CELLS IN VITRO[1]

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The ability to mount a Th1 immune response in humans and murine cells may be enhanced via expression of IL-12Rβ2 subunit. In contrast, the Th2 immune response requires IL-1R expression on T cells .In addition to a requirement for Th1 responses in control of C. neoformans infection, the administration of Ab to the capsule has also been shown to prolong survival and reduce tissue fungal burden. The mechanism for Ab efficacy against a pathogen for which Th1 responses are essential is not well understood. However, there is evidence that enhancement of cell mediated immunity contributes to the protective efficacy of Ab Considering that Ab therapy is currently in clinical development, there is a need to better understand the mechanisms by which Ab to the polysaccharide capsule can influence the interactions of this fungus with host immune cells. In this study we evaluated the hypothesis that GXM could hinder the Th1 response and that mAb to GXM may reverse this effect. To this end we carried out qualitative and quantitative analysis of IL-12Rβ2 subunit and IL-1R expression in T cells responding to C. neoformans in the presence and absence of Ab to the polysaccharide capsule.
Glucuronoxylomannan (GXM), the principal constituent of the Cryptococcus neoformans polysaccharide capsule, inhibited expression of IL-12Rβ2 subunit on T cells responding to cryptococcal antigens. Addition of GXM-binding mAb overcame this effect by promoting IL-12Rβ2 expression and by decreasing IL-1R expression on T cells. mAb to GXM enhanced the effect of anti-CTLA-4 $(Fab)_2$ by inhibiting IL-1R. The observed effects on T cells were a consequence of increased antigen presenting cell function due to enhanced phagocytosis. These findings suggest another mechanism by which specific antibody could contribute to an enhanced cellular immune response against C. neoformans.

N°. dell'Accordo di Collaborazione. 50C.32

PROGNOSIS AND OUTCOME MARKERS OF PNEUMONIA IN HIV-INFECTED PATIENTS. OBSERVATIONAL STUDY ON CLINICAL AND EPIDEMIOLOGICAL CHARACTERISTICS OF COMMUNITY ACQUIRED PNEUMONIA (CAP) IN HIV INFECTED SUBJECTS. IDENTIFICATION OF PROGNOSTIC MARKERS AND PARAMETERS OF CLINICAL STABILITY.

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Objective Our aim was to carefully describe the epidemiological, clinical and microbiological features of hospitalized CAP in HIV infected people. Our primary objectives were: 1. to define and analyze the main risk factors which predict unfavorable prognosis (30-day mortality) 2. to develop and validate a prediction rule for prognosis, able to assign patient with CAP to different classes. 3. to describe the time to resolution of abnormalities in vital signs, mental status, and selected biochemistry parameters and assess clinical outcome after achieving stability. Methods Prospective, multicenter observational cohort study involving 60 Infectious Diseases Centers in Italy, conducted in HIV+ patients with radiological evidence of pneumonia on hospital admission. We defined a baseline prognostic score to group the patients in 3 severity classes. This was based on 20 easy valuable variables (resulted independently related to mortality in previous studies in HIV and non-HIV populations) recorded on hospital admission, regarding epidemiological, physical-examination, immunological, biochemistry and instrumental data. Microbiological specimens were also recorded according to single center diagnostic alghoritms. During the course of pneumonia, in order to describe the time to resolution, were recorded: 1. daily measurement of vital signs, 2. determination of oxygen saturation and neutrophil count every 3 days, 3. weekly determination of selected laboratory data. Data about antibiotic regimen and its possible variations, anti-retroviral therapy and development of possible complications were also requested. All the above data were acquired by a structured case record form. To validate the prediction rule we used as outcome measures the mortality rate and the time to overall clinical stabilization. The time to overall stability was defined as the first day that all 4 pre-defined vital signs (heart rate, respiratory rate, body temperature, systolic blood pressure), ability to eat and mental status were normalized. For an evaluation better tailored to common clinical practice we used 3 different definitions of stability, the first less (HR < 100/min, RR < 24/min, BT < 38°C, Systolic Blood Pressure $\geq$ 90 mmHg), the third more conservative (HR < 90/min, RR < 20/min, BT $\leq$ 37°C, Systolic Blood Pressure $\geq$ 90 mmHg). To characterize the time of normalization of vital signs remarkable for clinical stabilization we used the descriptive statistics and the Kaplan-Maier estimate (with Score test). Furthermore we verified the efficiency of the clinical use of the definition of stability, analyzing it as the dependent variable with the main outcome measures by Logistic Regression and Cox model (univariate and multivariate). Results During the first six months of the study, data about 147 CAP were collected. The mean patient age was 38 $\pm$ 8 years (range 25-68); 123 (84%) were men , 24 females. Twenty-five patients (17%) had 1 or more co-morbid illnesses, 45 (30,6%) had a previous AIDS diagnosis. The median length of stay was 10 days (mean 14 $\pm$ 10) and the crude mortality rate was 4,8%. In 101 CAP no etiologic agent was identified, 15

were due to P. carinii, 5 to M. tubercolosis and 26 to other bacteria; among these the most frequent was S. pneumoniae (18/26), cause of bacteremia in 6 cases (33,3%). According to the prognostic rule, 78 subjects were classified as class I, 55 as class II, and 14 as class III. The mortality rate in the latter was 44,9%, as compared with 1,8% in class 2 ($p < .00001$). No deaths occurred in class I. Regarding the second outcome measure – time to stability – 40 patients were not evaluated because of lack of daily data (8 patients), absence of stabilization (10), patients already stable on admission (22). Noteworthy the absence of stabilization was significantly more frequent in class 3 (50% of cases) than in class I and II. On the contrary the 22 cases stable on admission occurred among class I patients. The average vital signs on admission were Body Temperature (BT) 38,2 $\pm$ 1 °C, Heart Rate (HR) 96 $\pm$ 17, Respiratory Rate (RR) 25 $\pm$ 7, O_2 Saturation (O_2S) 90 $\pm$ 7%. For the HR the median time to stabilization ($\leq$ 90/min) was 3 days in class I , 6 in class II ($p < .00001$) and 7 in class III; for the BT ($\leq$ 37°C) was 4 days in class I and 7 in class II and III ($p < .0001$); for the RR ($\leq$ 20/min) was 5,7 and 9 days respectively ($p < .001$). The median times of overall clinical stability were also considered. For the least conservative definition the median time to stability among all patients was 4 days, while for the most conservative definition it was 7 and for intermediate was 6. In the table median time to stability stratified per prediction rule class is reported (§ $p < .00001$).

	Class I	Class II	Class III
Time to stability def. 1 (days)	2	6 §	7
Time to stability def. 3 (days)	4	9 §	10

The percentage of patients with relapse in overall stability (significant clinical deterioration serious enough to merit escalation in antibiotic therapy and/ or prosecution of hospital stay) was globally around 5%. Comment These preliminary data support the possibility to identify at baseline HIV infected patients with CAP who may require hospitalization and/or more aggressive diagnostic and therapeutic approach. Moreover the definition of criteria of stability can provide evidence-based estimate of optimal length of stay and improve the efficiency of in-patients management.

Accordo di Collaborazione Scientifica N. 50C.33.

PHARMACOKINETIC EVALUATION OF ORAL LEVOFLOXACIN IN HIV-INFECTED SUBJECTS WITH COMMUNITY ACQUIRED PNEUMONIA RECEIVING CONCOMITANT ANTIRETROVIRAL THERAPY.

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As a part of an ongoing prospective multicenter observational cohort study related to the evaluation of prognostic features and outcome markers of community acquired pneumonia (CAP) in HIV patients, 24 subjects on steady-state antiretroviral therapy affected with CAP and treated with Levofloxacin (LEV) were recruited for the pharmacokinetic study. LEV is a new quinolone with a broad antibacterial spectrum and pharmacokinetics /dynamics features allowing for effective once-a-daily administration and switch from IV to oral form at the same dosage. Doing to these characteristics LEV appears an attractive choice for the treatment of CAP in HIV-infected patients, in which a larger number of bacteria is involved if compared to the immunocompetent population. The pharmacokinetic (PK) profile of LEV in HIV infected population turned out to be similar to that of healthy subjects and no interaction with Zidovudine concomitant administration was found out. Nevertheless, no data are available on potential interaction with Non Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) and Protease Inhibitors (PI). The purpose of this study was to evaluate the pharmacokinetic profile of oral LEV (500 mg/d) in 12 HIV-positive patients in steady-state treatment with Nelfinavir (NFV-750mgx3/d) and 12 with Efavirenz (EFV-600mg/d) and to determine the effects of LEV on the pharmacokinetic parameters of these two antiretroviral agents. For determination of LEV concentrations, plasma samples were obtained at steady-state during a 24-hour dosing interval and analyzed by a validated and specific HPLC assay with fluorimetric detection. NFV and EFV plasma concentrations were evaluated before and after 4 days of levofloxacin treatment. Analyses were performed by HPLC with UV detection. The main PK parameters of levofloxacin in the two groups of patients were: C_{max}: 6.22±1.98 μg/ml (with NFV) and 5.7±0.8 μg/ml (with EFV); T_{max} : 1.4±0.45 and 3.3 ± 1.1 h , respectively; AUC_{0-24}: 71.8±32.45 and 65.5±20.6 μg.h/ml; C_{trough}: 1.5±1.13 and 0.86±0.5 μg/ml . Comparing Levfx PK parameters of our patients with literature data in healthy subjects or in HIV+ patients not receiving NFV or EFV, we found a substantial overlap of concentrations, though we must stress a delay in levofloxacin absorption rate when combined with EFV. There was no significant difference between NFV and EFV plasma levels obtained with and without LEV. The results of this study suggest that a clinically important PK interaction between LEV and NFV or EFV is not likely to occur.

Accordo di Collaborazione Scientifica N. 50C.33.

TITOLO. INFECTION OF $CD34^+$ HEMATOPOIETIC PROGENITOR CELLS BY HUMAN HERPESVIRUS 7 (HHV-7)

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To investigate the tropism of the T-lymphotropic human herpesvirus 7 (HHV-7) for hematopoietic progenitors, cord blood $CD34^+$ cells were inoculated in vitro with HHV-7 and then induced to differentiate along the granulocytic and erythroid lineages by the addition of appropriate cytokine cocktails. In semisolid assays, HHV-7 modestly affected the growth of committed (CFU-GM and BFU-E) progenitors, while it significantly decreased the number of pluripotent (CFU-GEMM) progenitors. Such inhibitory effect was completely abrogated by incubating HHV-7 inoculum with anti-HHV-7 neutralizing serum. In liquid cultures, HHV-7 hastened the maturation along the myeloid but not the erythroid lineage, as demonstrated by the up-regulation of CD33 early myeloid antigen at day 7 of culture, and of CD15 and CD14 antigens at day 15. Moreover, HHV-7 mRNA was detected by RT-PCR in cells maturating along both the myeloid and the erythroid lineages. To evaluate the relevance of these in vitro findings, the presence of HHV-7 was investigated in bone marrow (BM) unfractionated mononuclear cells (MC) as well as in purified $CD34^+$ and $CD34^-$ cell subsets, obtained from 14 normal adult donors. HHV-7 DNA was detected by DNA-PCR in 4 out of 7 BMMC samples, and it was found to be associated to both the $CD34^-$ (2 out of 7) and the $CD34^+$ (1 out of 7) fractions. These data indicate that HHV-7 infects BM cells in vivo, and shows the ability to affect the survival/differentiation of $CD34^+$ hematopoietic progenitors in vitro by inhibiting more ancestral progenitors and perturbing the maturation of myeloid cells.

N°. dell'Accordo di Collaborazione 50C.34

The National research program on AIDS

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Project

EPIDEMIOLOGY AND HEALTH CARE

Scientific Coordinator: **Giovanni REZZA**

Projects financed N° 4

THE ROLE OF HPV GENOTYPES AND P53 CODON-72 POLYMORPHISM IN THE EVOLUTION OF HPV-RELATED SQUAMOUS INTRAEPITHELIAL LESIONS (SIL): SELECTED RESULTS FROM THE LONGITUDINAL PHASE OF THE DIANAIDS STUDY

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Introduction: According to many studies, cervical infection with human Papillomavirus (HPV) is the most important risk factor for carcinoma of the cervix, the second most common type of cancer among women worldwide. HPV-DNA sequences have been found in over 90% of invasive cervical tumours. Nonetheless, the mechanism involved in the development of cervical carcinoma from HPV-related squamous intraepithelial lesions (SIL) remains unclear.
Objectives: To measure the incidence of cervical HPV infection and to study factors potentially associated with the evolution of HPV-related SIL (i.e., HPV genotype and p53 codon-72 polymorphism).
Methods: The study population consisted of women with, or at-risk for, HIV-1 infection enrolled in clinical centres throughout Italy, with follow-up visits planned every 6-8 months (DIANAIDS cohort). Genotyping was performed with RFLP analysis. To analyse p53 codon-72 polymorphism, techniques based on PCR were adopted.
Results: Of the 239 women in the cohort, 145 (60.6%) returned for at least one follow-up visit and were included in the present longitudinal analysis. Of the 72 women negative for HPV-DNA at enrolment, 12 were positive at the successive follow-up visit (crude incidence: 16.7%). Four of these 12 women (33.3%) had a high-risk genotype **(**61, 55, and 56); 2 (16.6%) had a low-risk genotype (11 and 52); and 3 (25.5%) had rare genotypes with an undetermined level of oncogenicity (pap291, pap 155, and 66) (for 3 women, genotyping could not be performed). Of the 34 women positive for both HPV and HIV-1, 26 (76.5%) had SIL at the first follow-up visit, whereas of the 13 women who were HPV-positive yet HIV-1-negative, 5 (39.0%) had SIL (OR=5.20; 95%CI: 1.10-26.07). Twenty-four women were HPV-DNA-positive at both enrolment and follow-up ("persistent infection"); genotyping was performed for only 16 of the 24 strains: 75.0% (n=12) showed a high-risk genotypes (16, 18, 31, 35, 53, 58, and 59). Among the 15 women who were HPV-DNA-positive at enrolment yet negative at the first follow-up visit ("transitory infection"), 53.3% (n=8) had high-risk genotypes (16, 18, 31, and 58) (OR=2.63; 95%CI: 0.46-16.19). The analysis of p53 codon-72 polymorphism showed that 53.3% of the women were homozygous for the arginine allele (Arg/Arg), 40% were heterozygous (Arg/Pro), and 6.7% were homozygous for the proline allele (Pro/Pro), with no significant differences in this distribution between women with persistent infection and those with transitory infection. Among women with persistent infection, only 26.6% were homozygous for the arginine allele (Arg/Arg).
Conclusions: Among HPV-infected women, those co-infected with HIV-1 seem to be at a much higher risk of developing SIL, compared to HIV-1-negative women. Moreover, the high percentage of high-risk HPV genetoypes in both transitory and persistent infections stresses the need for more in-depth studies on the biomolecular characteristics of high-risk genotypes. Finally, women who are homozygous for the arginine allele do not seem to have a higher risk of developing HPV-related SIL.

DIFFERENTIAL IMPACT OF COMBINED ANTIRETROVIRAL THERAPY ON THE SURVIVAL OF ITALIAN PATIENTS WITH SPECIFIC AIDS defining illnesses.

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Progetto: Epidemiologia e Modelli Assistenziali
Linea di Ricerca: Studio della Mortalità per AIDS in Italia (n. 20 C/B)
Responsabile: Dott. Susanna Conti

Background: A decrease in HIV-related mortality and morbidity has been observed since 1996 in most developed countries, as consequence of the extensive use of combined antiretroviral therapies.

Purpose: To investigate whether combined antiretroviral therapies had a differential impact on the survival of patients with different AIDS Defining Illnesses (ADI).
Methods: 35,318 persons representing all the adult AIDS cases (PWA) diagnosed in Italy from 1.1.90 to 31.8.98 were studied. Actuarial life tables and Kaplan-Meier method were used to estimate cumulative probability of survival; multivariate Cox proportional hazards models were used to estimate adjusted relative hazards of death (RH).

Results: Among PWA diagnosed after 1995, the proportion of survivors at 24 months after diagnosis was more than doubled (66%) compared to that of PWA diagnosed before the end of 1995 (31%). Significantly decreased RH for some ADI were already observed in 1996 (i.e., oesophageal candidiasis, PCP, brain toxoplasmosis, HIV-wasting syndrome and pulmonary tubercolosis). In the last period (1997-1998) the decrease was marked and significant for almost all of the ADI, from 55% to 80% compared to the RH of the reference-year (1995). Conversely, primary lymphoma of the brain and Burkitt's lymphoma showed a low and not statistically significant decrease, thus, resulting the ADI with the worst outcome.

Conclusions: After 1995 there was a rather uniform increase in the survival of PWA diagnosed with most specific ADI, but not for patients affected by primary brain lymphoma and Burkitt's lymphoma. The determinants of this differential effect need to be investigated.

CHANGES IN THE RISK OF OPPORTUNISTIC INFECTIONS AND CANCERS AS FIRST AIDS-DEFINING DISEASES AMONG HIV-INFECTED ADULTS BEFORE AND AFTER THE INTRODUCTION OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

Maria Dorrucci, Patrizio Pezzotti, Catia Valdarchi, Manuela Zazzara, Giovanni Rezza for the HIV-Italian Seroconversion Study (ISS)

The rate of progression to AIDS-defining diseases has been reduced considerably since the introduction of highly active antiretroviral therapy (HAART). It is unclear, however, whether the reduction has been the same for all opportunistic infections and cancers. To this end, a study has been conducted to estimate changes in the risk of developing specific AIDS-defining diseases as first events in different calendar periods.
Data belongs to a cohort of individuals with known dates of HIV-seroconversion (HIV-Italian Seroconversion Study). Using separate Cox proportional hazards models, we evaluated the time from seroconversion to first AIDS-defining disease. Estimates of relative hazards (RH) adjusted for age at HIV-seroconversion were performed for each specific AIDS-defining disease for two calendar period: 1980-1995 and 1996-1999, i.e., before and after the introduction of HAART.
Of 1,671 (1,163 males and 508 females) HIV-seroconverters included in the study, 535 (32.2%) progressed to AIDS up to the end of 1999. Of the AIDS defining diseases, 468 (87.5%) were opportunistic infections and 67 (12.5%) were AIDS-related cancers. Overall, we found a substantial reduction of opportunistic events after the introduction of HAART: compared to the period 1980-1995, the RH of any AIDS defining opportunistic infection was 0.50 (95% CI: 0.39-0.63) for 1996-1999. Little heterogeneity between opportunistic infections was observed. A substantial reduction was also found for Kaposi's Sarcoma (RH = 0.15; 95% CI: 0.06-0.41). No significant trend was observed for lymphomas (non-Hodgkin's lymphoma and lymphoma of the brain) [RH=0.81 (95% CI: 0.25-2.65)]. Restricting the analysis to females participants, we observed an increase of invasive cervical cancer after the introduction of HAART [compared to 1980-1995, RH = 2.54 (95% CI: 0.39-16.46) for 1996-1999].
Our data indicate that after 1996 (i.e., the introduction of HAART), the risk of all opportunistic infections, as well as the risk of Kaposi's sarcoma, was substantially reduced. No evidence of a significant decrease for lymphomas was observed. An increasing trend was observed only for invasive cervical cancer among women.

Accordo di Collaborazione N. 20C/C

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Project

PATHOLOGY, CLINIC AND THERAPY OF AIDS

Scientific Coordinator: **Stefano VELLA**

Projects financed N° 16

VAGINAL TRANSMISSION OF HIV-1 IN A HU-SCID MICE: MODEL FOR THE EVALUATION OF VAGINAL MICROBICIDES.

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Background: A mice model for vaginal transmission of HIV-1 may represent a useful model for the evaluation of vaginal microbicides. Because of it simplicity and because may give faster results compared to other animal model. SCID mice transplanted with human peripheral blood lymphocytes (hu-PBL-SCID) are a useful model in AIDS research.

Methods: CB.17 SCID female mice were divided in 4 different groups (A, B, C, D) three of which (A, C, D) were transplanted intraperitoneally with $4x10^7$ human PBMC. Vaginal infection was performed on group A and B whereas group C and D were used as negative and positive controls, respectively. Progestin-treated mice (A, and B) received at day 12 a non invasive vaginal administration of $2x10^6$ PBMC previously infected in vitro with 10^5 TCID50 of cell free NSI strain of HIV-1. Systemic infection was investigated after two weeks post-inoculation collecting different samples. Plasma viral load and p24 antigen detection were evaluated by using the amplicor HIV-1 monitor kit and cocultivation, respectively. Polymerase chain reaction (PCR) analysis was performed on DNA extracted from tissues (spleen, lymph-nodes, peritoneal cells).

Results: In our experiments HIV transmission was established using NSI virus infected cells inoculated vaginally as shown by FACS, HIV-viral load, p24, and PCR results. We also answered the question whether the human lymphocytes, delivered at vaginal site, could crosses the intact epithelia by examining *in vivo* lymphocytes migration using fluorescently labelled lymphocytes. The mice were killed after 4, 24 and 48 hours, vagina and local lymph-nodes were removed, snap frozen with OCT, sectioned, and examined by fluorescent microscopy. Labeled cells were easily located within the vaginal tissues after 4 h and after 24 h still same cells could be detected. However, no cells could be identified after 48 h at the vaginal level whereas the labeled cells could be seen at the level of regional lymph-nodes.

Conclusions: This results strongly suggest that in the vaginal infection studies the HIV-1 infected cells delivered intravaginally reach the systemic sites via toracic duct. This hu-SCID mouse model may prove useful to test the activity of compounds against HIV and others sexually transmitted diseases.

PRENATAL EXPOSURE TO ANTI-HIV DRUGS: SHORT, -MEDIUM- AND LONG-TERM BEHAVIOURAL EFFECTS OF AZT + 3TC COMBINATION IN MICE

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Treatment of pregnant seropositive women and their neonates with the nucleoside analogues (reverse transcriptase inhibitors) zidovudine (AZT), lamivudine (3TC) and their combination has become a standard of care in industrialised countries to prevent vertical transmission of the HIV-1 virus. Recent evidence from studies on uninfected children has raised concerns about the long-term effects of developmental exposure to AZT and 3TC, especially when used in combination. Animal studies from our and other laboratories have indicated short-, medium- and long-term behavioural changes in both AZT- and 3TC- perinatally exposed offspring, including delayed sensorimotor development, impaired habituation and learning, alteration in social/aggressive behaviour at adulthood as well as alterations in the response to selective pharmacological challenges such as scopolamine or amphetamine. During the past year we investigated the neurobehavioural effects of prenatal exposure to AZT in combination with 3TC in CD-1 mice. Pregnant mice were given *per os* AZT+3TC (160 and 500 mg/kg respectively) or vehicle solution (NaCl 0.9%) twice daily from gestational day 10 to delivery. Data on reproductive performance, such as pregnancy length, litter size, sex ratio and offspring viability were negative. Pups were scored for different somatic and behavioural endpoints including sensorimotor development, homing performances on postnatal day (PND) 10, avoidance learning (PDN 22-23), locomotor activity (PDN 23), social interactions (PDN 35) and open field behaviour (PDN 70). While no effects were observed on maternal reproductive endpoints, treated pups showed long-lasting reduction of body weight and delayed maturation of placing, grasping and pole grasping reflexes. In addiction, ATZ+3TC treated mice showed selective alterations in the social interaction test, and displayed higher frequency of rearing, and lower frequency and duration of self-grooming behaviour in the open-field test. On the whole, we found that the combination of AZT and 3TC induced small but more marked effects on somatic and sensorimotor development than either drug when given separately, affecting social behaviour at the juvenile stage, and altering specific behavioural items in the open-field test at adulthood.

In light of the potential developmental toxicity and side-effects associated with prolonged exposure to combination of anti-HIV-therapies during pregnancy, less frequent effective single therapies are becoming the focus for preventing vertical transmission.

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor that in the last few years appears to be an excellent candidate drug for single-dose intervention during labour, having a potent antiviral activity and being rapidly absorbed when given orally. Over the next period, our aim is to evaluate the short-, medium- and long -term neurobehavioural effects of this drug in the offspring of CD-1 mice with NVP orally in the prenatal phase.

N° dell'accordo di collaborazione 30C/A

NANOPARTICLES AS DELIVERY SYSTEMS FOR VACCINES AND DRUGS AGAINST HIV INFECTION

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Nanoparticles are polymeric colloidal spheres of different dimensions (10-1000 nm) and chemical composition. Many different molecules can be adsorbed on the nanoparticle surface or included inside the nanoparticle. In this way the molecule conjugated to the nanoparticle is delivered to the cell and gradually released. This property permits to utilize lower doses of the molecule to deliver for any kind of purposes. Therefore, nanoparticles are an efficient delivery systems for both molecules whose action is at the intra-cellular level and molecules whose target is a specific cell.
Nanoparticles able to internalize plasmid DNA have been synthesized. Experiments of "DNAse protection" have been performed and indicated that these nanoparticles protect plasmid DNA from DNAse degradation (*Laus et al., J. Biomater Sci, in press*). In vitro toxicity of the nanoparticles, both alone and with associated DNA, has been assayed on H3T1 cells. Results have shown that nanoparticles (alone or with DNA) are not toxic for cells for concentration ranges from 10 μg/ml up to 5 mg/ml. To evaluate the toxicity of the compound DNA/nanoparticle in vivo, Balb/C mice have been inoculated with growing doses of compounds (from 1 to 500 μg) 3 times in 45 days. No signs of toxicity, neither local nor systemic, have been observed. Absence of toxicity was evident also at the histological analysis where no signs of cellular activation were observed (*in preparation*).
Similar results of absence of toxicity were observed when plasmid DNA carried the tat gene (pCV-tat) from HIV-1. Moreover, PBMCs from some of the mice injected with the polymer pCV-tat/nanoparticle proliferated after stimulus with Tat in vitro.
In order to verify whether DNA associated to nanoparticles is taken up by the cell and released, a plasmid carrying both the tat gene and the green fluorescent protein gene (GFP), conjugated to the nanoparticles, was used with H3T1 cell line. The uptake and release assays have indicated that DNA is taken up and released within 48 hours.
Nanoparticles able to bind proteins have been also synthesized. The Tat protein was conjugated to nanoparticles and the toxicity of the compound assayed in both H3T1 and CEM cells. Results from these experiments have indicated that in both cell lines concentrations of compound up to 100 μg/ml were not toxic (*Tondelli et al., in preparation*). The compound (polymer/Tat) has also been assayed for its ability to be taken up by cells and to carry the Tat protein in a biologically active form. To this aim rescue assays on HLM1 cells (cells carrying a tat-defective HIV-1 provirus and therefore not able to replicate) have been performed.
Results showed that the compound, when added to cells, is able to rescue HIV replication, indicating that the Tat protein conjugated to the nanoparticle is able to maintain its biological activity (*Caputo et al., submitted*). These results are important in view of the development of new methods of delivering a Tat-based vaccine.

Numero di collaborazione: 30C/B

HIV-1 PROTEASE INHIBITORS ARE SUBSTRATES FOR THE MDR1-P-GLYCOPROTEIN EXPRESSED ON HUMAN T-LYMPHOCYTES: PHARMACOLOGICAL AND PHYSIOLOGICAL IMPLICATIONS.

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Despite the evidence that the drug resistance is a very complex phenomenon that might involve different aspects of the drug host-cell interaction, the emergency of HIV variants no longer susceptible to the PIs treatment have been mainly attributed to *gag-pol* gene mutations. In this regard, by using cell lines-based approaches and analyses of fluorescent substrates we have demonstrated that the PIs Ritonavir, Saquinavir and Indinavir bind with the P-glycoprotein inhibiting its drug transport mechanism expressed on MDR variants of CD4 positive lymphoblastoid cells. Recently, the ability of P-glycoprotein to mediate the efflux of the PI Indinavir against its concentration gradients has been shown by Liquid Chromatographic determination in biological matrices (*MR Frizzano, L. Valvo, ML Dupuis, V. Mennella, M. Cianfriglia; J. Pharm. Biomed. Anal. 22, 307-314: 2000*). However, in order to identify the relationship between drug resistant problems observed during PIs administration and cellular transporter mechanisms, several aspects remain to be elucidate and in particular the P-glycoprotein expression and function in HIV-1 target cells.
The interaction of PIs with MDR1-P-glycoprotein in CD4 T cells was investigated by monitoring the intracellular accumulation exerted by the absorption of these drugs on the release of fluorescent P-glycoprotein substrates. PBMC isolated from blood of healthy donors were loaded with bodipy-FL- (fluorescent)-Vinblastine, bodipy-FL-(fluorescent)-Colchicine and Daunomicin (an intrinsically dyed compound) and their efflux evaluated after co-incubation with each single PIs. The labelling of PBMC with the CD4 antibody allow to verify if in these T cell sub-set act drug transporter mechanisms inhibited or blocked by the contemporaneous intracellular presence of the PIs. Ritonavir, Saquinavir, and Indinavir at concentrations corresponding to that observed in the plasma of treated patients (10 uM, sufficient to effectively inhibit HIV-1 replication) block the efflux of the fluorescent P-glycoprotein substrates Vinblastine and Daunomicin. This modulation of the P-glycoprotein function exerted by PIs is similar to that induced both *in vivo* and *in vitro* by the MDR reversing agents Verapamil and SDZ PSC 833 used in clinical trials for the treatment of tumors (in combination with cytotoxic compunds). Saquinavir and Ritonavir, but not Indinavir (and Verapamil) block the efflux of the Colchicine in CD4 positive T-lymphocyte sub-set. These results confirmed that also in HIV-1 target T-lymphocytes the transport of PIs is efficiently mediated by the P-glycoprotein and that its activity might interfere with the biodisponibility and efficacy of these drugs. However, the evidence that efflux mechanisms in CD4 T lymphocytes possess distinct PIs binding domains as the inhability of Indinavir to compete with the transport of Colchicine has demonstrated suggest the clinical importance to verify the functional and structural properties of the P-glycoprotein present in other cell types having fundamental role in PIs absorption, metabolism and distribution.

N°. dell'Accordo di Collaborazione. 30/cc

LC DETERMINATION OF INDINAVIR IN BIOLOGICAL MATRICES WITH ELECTROCHEMICAL DETECTION. DIRECT EVIDENCE OF THE PIS TRANSPORT BY THE MDR1-P-GLYCOPROTEIN.

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Significant advances in the treatment of HIV-1 infection have been achieved with the development of HIV-1 protease inhibitors (PIs). These inhibitors when used in combination with other antiretroviral drugs, are higly efficient at reducing viral replication and correcting CD4+ T-cell defects. However, in addition to development of HIV-1 protease mutants, problems with absorption, metabolism, and distribution of these drugs in the body may limit their effectiveness. In this regard, by using cell based approaches and fluorescent substrates we have demostrated that the PIs bind with the MDR1-P-glycoprotein and are actively pump-out the MDR cells. In these studies, the PIs efflux P-glycoprotein-mediated has been indirectly evaluated by measuring the transport inhibition of dyed substrates or the FITC-labelled antitumoral drugs.

By developing a novel high performance liquid chromatographic (HPLC) method capable of directly determining very low Indinavir amounts in the cell culture supernatant we intended to furnish a new and very effective tool for studying the cellular mechanisms involved in PIs transport and biodisponibility. Several HPLC methods to determine the Indinavir concentration in biological fluids with the use of UV detection at 210 nm have been reported; however these methods have shown their limits in both sensitivity and specificity, bearing the risk of interferences from other compounds potentially present in the biological fluids. The Indinavir chemical structure with some oxidation sites suggested that a very effective assay could be developed by coupling HPLC electrochemical detection. Herein, a new and original HPLC system is described and its suitability for the determination of very low amounts (3-5 ng/ml) of Indinavir in cell culture is assessed. The sensitivity and specificity of this technique allow the study of Indinavir metabolims and transport also in those HIV target cells , such as CD4 positive human T lymphocytes, where the reduced level of P-glycoprotein expression and function may seriously limit the effectiveness of an indirect evaluation of the drug transport or metabolism.

N°. dell'Accordo di Collaborazione. 30/cc

ACTIVATION OF MMP-2 AND MT1-MMP IN ENDOTHELIAL CELLS AND INDUCTION OF VASCULAR PERMEABILITY IN VIVO BY THE HIV-1 TAT PROTEIN AND bFGF

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Previous studies indicated that the Tat protein of human immunodeficiency virus type-1 (HIV-1) is a progression factor for Kaposi's sarcoma (KS), an angioproliferative disease very frequent and aggressive in HIV-1 infected individuals (AIDS-KS). Specifically, extracellular Tat released by HIV-1 infected cells cooperates with basic fibroblast growth factor (bFGF), an angiogenic factor highly expressed in KS, in promoting KS and endothelial cell growth and locomotion and in inducing angioproliferative KS-like lesions in mice by mimicking the effects of extracellular matrix proteins (*Ensoli et al., Nature 1990, J. Virol. 1993, Nature 1994; Barillari et al., J. Immunol. 1992, Proc. Natl. Acad. Sci. 1993; Albini et al., Proc. Natl. Acad. Sci. 1995; Fiorelli et al., J. Clin. Invest. 1995, J. Immunol. 1999*). Extracellular Tat can also induce endothelial cell RNA expression of matrix-metalloproteinase-2 (MMP-2), a powerful mediator of tumor invasion, angiogenesis and vascular permeability (*Ensoli et al., Nature 1994; Barillari et al., Blood 1999*). This suggested to us that Tat could also play a role in the pathogenesis of the edema, one major morbidity factor of KS.

Here we show that Tat and bFGF combined, but not Tat and vascular endothelial growth factor (VEGF), increase MMP-2 production, secretion and activation by macro- and micro-vascular endothelial cells in an additive or synergistic fashion. These effects are due to the activation of the membrane type 1 (MT1)-MMP and to the increase of the cell membrane-associated tissue inhibitor of metalloproteinase-2 (TIMP-2) induced by Tat and bFGF combined, and to Tat-mediated inhibition of both basal or bFGF-induced TIMP-1 and -2 secretion. Consistent with the activation of MMPs, Tat and bFGF promote vascular permeability and edema in small animal models. This in vivo effects require both factors and are blocked by a synthetic MMP inhibitor. Finally, high MMP-2 expression levels are detected in AIDS-KS lesions by in situ hybridization as compared to control skin, indicating that these mechanisms are operative in AIDS-KS lesions where Tat and bFGF are present and may represent the key modulators of MMP-2 function. This suggests a novel pathway by which Tat can increase KS aggressiveness in the setting of HIV-1 infection.
(*submitted for publication*)

N. dell'accordo di collaborazione: 30 C/D

ANGIOGENIC EFFECTS OF EXTRACELLULAR HIV-1 TAT PROTEIN IN THE DEVELOPMENT AND PROGRESSION OF AIDS-ASSOCIATED KAPOSI'S SARCOMA.

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The Tat protein of HIV-1 is released by acutely infected cells and, in this form, induces vascular cell invasion, migration, adhesion and growth, thus promoting new blood vessel formation (angiogenesis). The angiogenic properties of extracellular Tat have linked this viral protein to the pathogenesis of Kaposi's sarcoma (KS), a vascular tumor frequent and aggressive in HIV-1 infected individuals (AIDS-KS) (*reviewed in Barillari et al., Clin. Microbiol. Rev, in press*).

However, in order to exert its angiogenic effects, Tat requires the cooperation of inflammatory cytokines including IL-1β, TNFα and IFNγ, whose levels are increased in AIDS-KS lesions (*Fiorelli et al., J. Immunol. 1999*). These cytokines augment tissue levels of bFGF, an angiogenic molecole expressed in primary KS lesions (*Samaniego et al., Am. J. Pathol. 1998*). Indeed, Tat enhances bFGF capability of promoting angiogenesis both in vitro and in vivo (*Ensoli et al., Nature 1994; Barillari et al., Blood 1999; Barillari et al., J. Immunol. 1999*). This is because the highly basic residues of Tat, by competing for heparin-binding sites, retrieve extracellular-bound bFGF into a soluble form which both promotes vascular cell growth and upregulates the expression of the αvβ3 integrin receptor (*Barillari et al., Blood 1999, J. Immunol. 1999*). The αvβ3 integrin, in turn, binds the arginine-glycine-aspartic acid (RGD) region of Tat, mediating Tat-promoted migration of vascular cells. The binding of Tat RGD region to αvβ3 also activates the expression of collagenase IV, a metalloprotease which mediates cellular invasion (*Ensoli et al., Nature 1994; Barillari et al., Blood 1999*). In addition, Tat-αvβ3 interaction provides vascular cells with the adhesion signal they require to grow. These effects of Tat RGD region are consistent with the role of the RGD region of extracellular matrix molecules in angiogenesis (*Hammes et al., Nature Med. 1996*).

The same inflammatory cytokines cooperating with Tat in promoting angiogenesis and KS progression also increase KSC production of VEGF-A, another angiogenic factors expressed in KS lesions (*Samaniego et al., Am. J. Pathol. 1998; Barillari et al., J. Immunol. 1999*). It is noteworthy that Tat binds and phosphorylates VEGFR-2, the receptor mediating most of VEGF-A angiogenic effects (*Albini et al., Nature Med. 1996; Mitola et al., J. Virol. 2000*). The fact that Tat and VEGF-A share the same receptor may explain why, differently from what occurrs with Tat and bFGF, Tat does not enhance VEGF angiogenic effects either in vitro or in vivo (*Barillari et al., J. Immunol. 1999*). Thus, extracellular HIV-1 Tat protein increases the biological effects of true angiogenic factors and mimicks the angiogenic activities of the extracellular matrix, enhancing all the steps of angiogenesis. The finding that elevated levels of bFGF, VEGF and inflammatory cytokines are detectable in AIDS-KS lesions where extracellular Tat co-stains with αvβ3 in vascular cells (*Ensoli et al., Nature 1994*) suggests that the mechanisms of Tat action described here are operating in vivo.

Numero di collaborazione: 30C/D

KSHV (HHV8) SEROLOGY IN EUROPE AND UGANDA: A MULTICENTRIC STUDY WITH MULTIPLE AND NOVEL ASSAYS

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The diagnosis of Kaposi sarcoma-associated herpesvirus/Human herpesvirus-8 (KSHV/HHV8) infection (E*nsoli et al., Sem. Cancer Biol. 2000, Adv. Cancer Res, in press; Stürzl et al., Adv. Cancer Res., in press*) is difficult due to the low concordance of the available HHV8 serologic assays. We undertook a multicentric study to define combinations of novel or second-generation assays with increased inter-assay concordance, sensitivity, specificity, and predictive value for the serological diagnosis of HHV8 infection.

Five serum panels for a total of 562 sera including European and African (Ugandan) HIV-infected or uninfected individuals with or without Kaposi's sarcoma (KS), at risk or not for KS, and regular blood donors were analyzed in a coded fashion with 18 different assays in seven different European laboratories. Nine of these assays were used to screen all the 5 serum panels. Concordance was evaluated by *observed agreement* and *k for pairways agreement*. The "true positive sera" were identified according to two assumptions: (i) all sera from patients with KS were considered positive; (ii) non-KS sera were considered positive only if they scored positive with at least 6 (70%), 7 (80%), or 8 (90%) out of the 9 assays used to screen all the 5 serum panels. The *validity* of the assays was then evaluated by univariate logistic regression analysis.

Two immunofluorescence assays (IFA) for detection of antibodies against HHV8 lytic (Rlyt) (*Rezza et al., J. Natl. Cancer Inst., 1999; Andreoni et al., J. Natl. Cancer Inst., 1999*) or latent (Llana) antigens and two enzyme-linked-immunosorbent assays (ELISA) (M2, EK8.1) for detection of antibodies against HHV8 structural proteins encoded by HHV8 ORF 65 and K8.1 were found to be highly concordant, specific and sensitive, with odd ratios (OR) indicative of a high predictive value. When used together, the two IFA (Rlyt-Llana) showed the best combination of sensitivity (89.1%) and specificity (94.9%).The performance of Rlyt, Llana, M2, Ek8.1, or Rlyt-Llana combined indicate that these assays may be used for the clinical management of risk individuals including allograft recipients.

Although none of the present assays is standardized, this study identified serologic tests suitable for the diagnosis of HHV8 infection and useful for the surveillance and prevention of HHV8-associated diseases in risk individuals (*Schatz et al., J. Med. Virol., in press*).

Numero di collaborazione: 30C/D

MECHANISM OF BCL-2 ACTIVATION IN AIDS-KS

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Kaposi's sarcoma (KS) is a vascular disease particularly frequent and aggressive in HIV-1 infected individuals and characterized by angiogenesis and by the growth of spindle cells of endothelial cell origin. Although early KS has a polyclonal nature, it can progress into a true sarcoma, likely in association with the deregulated expression of anti-apoptotic genes (*Stürzl M et al., Adv. Cancer Res, in press*). We have previously observed that Bcl-2 is upregulated in spindle cells and endothelial cells of KS lesions (*Bohan-Morris et al., Am. J. Pathol. 1995; Stürzl et al., J. Natl. Cancer Inst. 1999*) and that its expression increases with lesion progression. In fact, the highest levels of Bcl-2 expression are observed in the nodular late-stage lesions, in which low or no apoptosis is detected as compared to early stage lesions. The importance of Bcl-2 in KS pathogenesis is also supported by the observation that paclitaxel, a drug highly effective in patients with advanced AIDS-KS, promotes the down-regulation of Bcl-2 protein expression that is associated with apoptosis of KS cells and regression of experimental KS-like lesions induced by the inoculation of primary KS cells in nude mice (*Sgadari et al., J. Immunol. 2000*).

bFGF, a potent angiogenic factor, acts as a survival factor for endothelial cells, protecting them from apoptotic stimuli through induction of Bcl-2 expression. bFGF is highly expressed in human primary KS lesions or KS-like lesions of mice and plays a key role in KS cell growth and angiogenesis (*Ensoli et al., Science 1989, Nature 1994, J. Clin. Invest. 1994; Samaniego et al., Am. J. Pathol. 1998*). In addition, bFGF synergizes with the HIV-1 Tat protein to induce both KS growth and angiogenesis (*Ensoli et al., Nature 1990, J. Virol. 1993, Nature 1994; Barillari et al., J. Immunol. 1992, Proc. Natl. Acad. Sci. 1993; Fiorelli et al., J. Clin. Invest. 1995; Albini et al., Proc. Natl. Acad. Sci. 1995*), explaining the higher frequency and aggressiveness of KS in HIV-1 infected individuals.

We investigated the mechanism(s) of Bcl-2 up-regulation in AIDS-KS by utilizing the two key pathogenetic factors of KS: bFGF and Tat. We found that bFGF promotes vascular lesions in mice closely resembling KS lesions that are characterized by a high Bcl-2 expression. Tat further increases both the formation of angiogenic KS-like lesions and the expression of the Bcl-2 protein. Moreover, combined Tat and bFGF synergize to increase Bcl-2 mRNA and protein expression in endothelial cells, and to protect them from apoptosis induced by deprivation of extra-cellular matrix interactions. This effect is associated with a rescue of Bcl-2 expression, which is downregulated in apoptotic non-adherent cells (*Sgadari et al., submitted*).

Thus, bFGF and Tat represent two key mediators of Bcl-2 activation in AIDS-KS suggesting that the activation of Bcl-2 expression is a common pathway of different stimuli leading to angiogenesis and cell growth. Finally, these data provide a rationale for paclitaxel activity in KS patients and suggest that Bcl-2 downregulation may represent a pathogenetic approach to late-stage KS, usually refractory to conventional therapies.

N. dell'accordo di collaborazione: 30 C/D

REGRESSION OF LEUKOPENIA-ASSOCIATED AGGRESSIVE CLASSIC KAPOSI'S SARCOMA ACCOMPANIED BY CLEARANCE OF HUMAN HERPESVIRUS 8 FROM BLOOD BY α-INTERFERON.

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One of the most used therapeutic approaches for KS (*Ensoli et al., Sem. Cancer Biol. 2000, Adv. Cancer Res, in press; Stürzl et al., Adv. Cancer Res., in press*) is the administration of recombinant α-interferon (αIFN), however, the mechanism(s) of αIFN efficacy as a monotherapy for KS is still poorly understood. In particular, αIFN is known to block the production of angiogenic factors that are key to KS development, whereas our recent work has shown that αIFN inhibits HHV8 replication in infected cell lines and PBMC from patients with KS or at risk for KS (*Monini et al., J. Virol., 1999; Ensoli et al., Adv. Cancer Res, in press*).

To gain insights in the effects of αIFN on KS, we studied the case of a human immunodeficiency virus (HIV)-negative 31-year-old female patient affected by severe and advanced classic KS who showed complete clinical remission in response to long-term αIFN treatment. At base-line the patient presented with idiopathic leukopenia, reduced NK cell activity and HHV8 infection of all circulating cell types including $CD4^+$ and $CD8^+$ T lymphocytes, B cells and monocytes. Antibodies against HIV, HIV p24 antigenemia, and plasma HIV RNA were undetectable. The patient was also negative for infections known to be associated with leukopenia, including B19 parvovirus and active cytomegalovirus infection. The patient was treated with recombinant αIFN-2b at the starting dose of 3 millions international unit subcutaneously 3 times a week. The clinical conditions started to improve after 8-months therapy, with oedema and lesion regression. After 11 months of therapy the patient leukocytes, T cells, NK cell counts, and NK cytotoxic activity were significantly increased, HHV8 anti-lytic antibody titers (*Rezza et al., J. Natl. Cancer Inst., 1999; Andreoni et al., J. Natl. Cancer Inst., 1999*) were decreased, and PBMC had turned PCR-negative. However, PBMC were still PCR-positive after culture with inflammatory cytokines (IC) known to rescue HHV8 infection (*Monini et al., Blood, 1999*). After 24 months of therapy, $CD3^+$ T cells and NK cells counts and cytotoxic activity were restored to normal levels, and PBMC had turned PCR-negative even after culture with IC. At this time, nodular lesions were disappeared although slight oedema and regressing plaque-like lesions were still visible. The patient is still under follow-up and is in good clinical conditions, without notable side-effects.

These data show that therapy with αIFN resulted in stable remission of KS that was accompanied by resolution of the leukopenia, restoration of NK cell activity, reduction of the antibody titers against HHV-8 lytic antigens and complete clearance of HHV-8 from the circulation. Thus, in addition to its known effects in blocking production of angiogenic factors, αIFN therapy appears to induce KS regression by both a direct inhibition of HHV8 infection and by increasing the antiviral NK cytotoxic activity (*Submitted for publication*).

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THE HIV-1 PROTEASE INHIBITORS INDINAVIR AND SAQUINAVIR INDUCE KAPOSI'S SARCOMA (KS) REGRESSION BY INHIBITING KS CELL INVASION, ANGIOGENESIS AND VASCULAR PERMEABILITY

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HIV-1 protease inhibitors (PI) have potent antiviral activity that reduces HIV-1 viral load and increase CD4 T cells restoring the immune response toward HIV-1 and other infectious agents. Treatment with PI is frequently associated with the regression or resolution of both initial or advanced Kaposi's sarcoma (KS), an angioproliferative tumor arising in HIV-1 and human herpesvirus-8 (HHV-8) co-infected individuals (*Stürzl M et al., Adv. Cancer Res, in press*). At least in part, these effects may be mediated by the block of HIV replication and Tat production and, consequently, by a better immune response against HHV-8. However, PI have also shown effects on several methabolic pathways, cellular proteasome 20S and fungal proteases, suggesting that they may have a direct effect on KS cell locomotion, angiogenesis or vascular permeability, all key features of KS that require proteolytic activity.
Here we show that indinavir or saquinavir induce the regression of angiogenic KS-like lesions and the vascular permeability promoted by inoculation of KS cells in nude mice (*Sgadari et al., in preparation*). In vitro, indinavir and saquinavir inhibited the invasion of KS cells but not their adhesive, proliferative or migrating responses. This suggested that PI inhibit the activity of proteases degrading the extracellular matrix and that are required for cell invasion and vascular permeability, two key events for angiogenesis and tumor growth. In fact, PI inhibited the secretion and the proteolytic activation of matrix-metalloproteinase (MMP) -2 produced by KS cells both in vitro and in primary human KS lesions. Moreover, PI inhibited different steps of the angiogenic process in vitro and in vivo and the secretion of activated MMP-2 by EC in response to bFGF (*Sgadari et al., in preparation*). Thus, HIV-1 PI have direct inhibitory effects on KS and may be effective in the treatment of angiogenic diseases and tumors.

Numero di collaborazione: 30 C/D

TRANSCRIPTION PATTERN OF THE K3 OPEN READING FRAME OF HHV8 IN PRIMARY EFFUSION LYMPHOMA AND KAPOSI'S SARCOMA.

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Human herpesvirus 8 (HHV8) is found in immunoblastic B cells from multicentric Castelman's disease (MCD) and, as a predominant latent form, in primary effusion lymphoma (PEL) and Kaposi's sarcoma (KS) spindle cells (*Ensoli et al., Sem. Cancer Biol. 2000, Adv. Cancer Res, in press; Stürzl et al., Adv. Cancer Res., in press*). Recent studies have shown that upon reactivation (*Monini et al., Blood, 1999*) HHV8 expresses factors downregulating MHC class I proteins and co-activation molecules which may enable productively infected cells to escape cytotoxic T lymphocytes and NK cell responses. One of these viral factors is encoded by the open reading frame (ORF) K3. However, the molecular pattern and kinetic of K3 transcription have not yet been elucidated and no data are available on K3 expression in KS tissue. To analyze the expression pattern of the K3 gene product, PEL-derived cells (BCBL-1) were cultured for various periods of time with or without TPA (*Monini et al., J. Virol., 1999*) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) and total RNA was analyzed by Northern blot hybridization with a DNA probe spanning the HHV8 ORF K3. The results show that in PEL cells ORF K3 is expressed through viral transcripts that are induced very early upon virus reactivation. Specifically, a K3 transcript was found to be expressed in the absence of de novo protein synthesis, thereby identifying a novel HHV8 immediate-early (IE) product, whereas the other K3 transcripts were expressed with a kinetic typical of delayed-early (DE) genes. To identify and characterize the HHV8 IE and DE K3 transcripts, total RNA from BCBL-1 induced with TPA in the presence or absence of CHX was subjected to suppression subtractive hybridization (SSH), a PCR-based technique that allows to isolate cDNA sequences from genes that are differentially expressed upon specific stimuli, and to rapid amplification of cDNA ends (RACE), to determine the 5' and 3' ends of the HHV-8 K3 RNA molecules. The results showed that K3 is expressed through multiple mono-cistronic and bi-cistronic RNA molecules containing coding sequences from the viral ORF K3 and ORF 70. Several features of the RNA molecules encoding the K3 product, including multiple transcriptional start sites, multiple donor splicing sites and potential alternative ATG usage, suggest a finely tuned modulation of K3 expression. By contrast, ORF K3 transcripts were not detected in the majority of cells present in KS lesions that are latently infected by the virus suggesting other as yet unknown mechanisms of immune evasion for infected KS spindle cells. Nevertheless, as HHV8 viremia in risk individuals precedes the development of KS (*Monini et al., Blood, 1999; Monini et al., J. Virol., 1999*) and is associated with the recrudescence of MCD symptoms, the prompt expression of ORF K3 in productively infected circulating cells may be key for virus pathogenesis. Thus, molecules targeting host or viral inducers of ORF K3 expression or inactivating the biological functions of the K3 product should be exploited for the prevention or therapy of HHV\8-associated diseases in risk individuals (*submitted for publication*).

Numero di collaborazione: 30C/D

HUMORAL IMMUNE RESPONSE AGAINST THE HIV-1 TAT PROTEIN IS PREVALENT IN ASYMPTOMATIC INDIVIDUALS AND CORRELATES WITH NON PROGRESSION TO AIDS

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The Tat protein of HIV-1 plays a key role in the viral life cycle, in the pathogenesis of HIV infection and in AIDS-associated tumors. Because of these properties and since Tat is highly conserved among the different HIV-1 subtypes, the Tat protein has been used as a vaccine against HIV/AIDS for both preventive and therapeutic approaches (*Cafaro et al., Nat. Med. 1999; Ensoli and Cafaro, AIDS Clin. Rev. 2000/2001; Cafaro et al., J. Med. Primatol. 2000; Cafaro et al., Vaccine in press*). In order to evaluate the anti-Tat immune response during natural HIV infection and to verify whether it correlates with a reduced progression to AIDS, a cross-sectional analysis of 302 HIV-infected Italian individuals at different stages of disease and 132 HIV-negative individuals was performed by testing sera for the presence of anti-Tat IgG and IgM antibodies by two different ELISA assays, using a highly purified and biologically active Tat protein. The results indicated that the presence of anti-Tat antibodies significantly correlates with the asymptomatic stage of the disease. In fact, anti-Tat IgG positive patients in stage A were 37 out of 220 (16.8%), whereas only 1 patient out of 33 in stage B (3.0%) and 2 out of 49 (4.1%) in stage C, had anti-Tat IgG ($p = 0.01$). Anti-Tat IgM paralleled the anti-Tat IgG. Specifically, 20 out of 220 (9.1%) of patients in stage A had anti-Tat IgM antibodies, whereas 1 out of 33 (3.0%) of those in stage B and 0 out of 49 in stage C (0%) showed Tat-specific IgM antibodies ($p= 0.01$). Among asymptomatic patients, a higher prevalence of anti-Tat antibodies was observed in Long Term Non Progressors [20.0% (6/30) for IgG, 10.0% (3/30) for IgM].The prevalence of anti-Tat antibodies was also higher in patients with a CD4 T cell numbers > 200/μl. In addition, longitudinal analysis of 99 individuals with 2 or more samples more than 6 months apart indicated that asymptomatic patients with undetectable levels of anti-Tat IgG antibodies were more likely to progress to the symptomatic stages of infection. By utilizing synthetic peptides representing the aminoacid sequence of Tat, one major linear B cell epitope was defined within the proline-rich aminoterminal domain (aa 1-20) of Tat (42.5%, 17/40 of the anti-Tat positive patients). Additional reactive epitopes were identified at residues 36-50 (12.5%, 5/40) and at the residues 83-102 (12.5%, 5/40), spanning a region not represented by the BH10 recombinant Tat protein utilized in the ELISA tests. Anti-Tat positive sera had also neutralizing activity against Tat as determined by the inhibition of the rescue of a tat-defective proviruses induced by exogenous Tat in the HLM-1 cell line. The neutralizing activity was observed in sera depleted of epitope-specific antibodies, suggesting that this biological activity is mostly due to conformational anti-Tat antibodies. These data point out to a protective role of anti-Tat antibodies in the progression of AIDS and have strong implications in the use of Tat as a therapeutic vaccine in HIV-1 infected patients (*Buttò et al., submitted*).

Numero di collaborazione: 30C/E

ANALYSIS OF THE TAT NUCLEIC ACID SEQUENCE AND OF THE IMMUNE RESPONSE TO A BIOLOGICALLY ACTIVE TAT PROTEIN DERIVED FROM AN HIV-1 SUBTYPE B VIRUS, IN UGANDAN INDIVIDUALS INFECTED WITH DIFFERENT HIV SUBTYPES

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Our previous studies have demonstrated that a vaccine based on the Tat protein of HIV-1 is able to control the replication of the highly pathogenic SHIV89.6P and to block diseases onset in the monkey model (*Cafaro et al., Nat. Med., 1999; Cafaro at al., J. Med Primatol.,2000; Cafaro et al., Vaccine, in press*). Based on these results, phase I trials for evaluation of safety and immunogenicity of both the preventive and therapeutic approaches, are being started in Italy. In addition, phase II studies are being organized in South Africa and Uganda and will be performed at the completion of phase I studies in Italy. Due to the conservation of the Tat protein, the vaccine should be effective against HIV subtypes different from the HIV subtype B from which the Tat vaccine has been derived. To this aim we studied a population of 109 HIV infected Ugandan patients attending the St. Mary's Lacor Hospital (Gulu, North Uganda) for gag and tat nucleic acid sequence variation and immunological cross-reactivity to the Tat protein. Firstly, we PCR-amplified and sequenced the p6/p7 gag region from 11 individuals. Phylogenetic analyses based on these sequences confirmed, as also reported in the literature, that the A and D clades are the most represented subtypes. However, other subtypes with a lower prevalence have also been identified (*Buttò et al., in preparation*).
Secondly, to verify the conservation of Tat antigenic sequence, the Tat encoding region was PCR amplified from PBMC samples from 11 Tat-reacting patients and directly sequenced. Results have shown that the first exon of Tat, which contains both the principal B-cell epitope and the functional regions is relatively well conserved among the different HIV-1 subtypes, including the B subtype, whereas the second exon is more variable (*Buttò et al., in preparation*).
Finally, we analyzed the ability of sera from the 109 infected individuals to bind the biologically active B-derived Tat protein utilized as vaccine (aa 1-86, derived from the BH-10, subtype B virus), using two different, highly reliable and standardized Elisa assays. A prevalence of 24.8% of anti-Tat IgG antibodies was found, with titers ranging from 100 to1600, which is similar to that obtained in the Italian individuals, infected with the HIV-1 B subtype in other studies from our group. Furthermore, by utilizing synthetic peptides spanning the entire Tat sequence of the BH-10 clone (aa 1-102), a major linear epitope has been identified at residues 1-20, and a minor linear epitope was identified at residues 36-50, as observed also for the Italian population. These data also indicate that conformational epitopes should be present and should play an important role in the humoral response to Tat. Finally, sera from the Tat-reactive Ugandan patients neutralized the ability of the Tat protein to rescue HIV replication in the HLM1 cell line containing a Tat-defective HIV-1 provirus (*Buttò et al., submitted*).
Studies to verify the cell-mediated immune response to Tat in these individuals as well as similar studies in South Africa are in progress.
These data provide important information on the ability of the Tat-based vaccine to be effective in individuals infected with different HIV-1 subtypes.

Numero di collaborazione: 30C/E

MULTICENTER TRIALS FOR THE EVALUATION OF THERAPEUTIC STRATEGIES IN hIV INFECTION

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The Laboratory of Virology is conducting important multicentre controlled trials for the definition of the best therapeutic strategies for the long-term treatment of HIV infection. These trials have been designed and conducted within the framework of the National Institutes of Health ACTG program (trials ACTG 384 and 388) and the multinational collaborative group coordinated by the UK Medical Research Council (INITIO trial). The three trials are ongoing and have cumulatively enrolled about 200 patients as of January 1, 2001. All trials include substudies, which will evaluate virology, immunology, pharmacokinetics, resistance, quality of life and adherence issues.

Trial ACTG 384 is a randomized, partially double-blind trial with an overall sample size of 800 treatment-naive HIV-positive patients with detectable RNA (>500 copies per ml). Objective is to compare as first-line treatments strategies based on 3-drug and 4-drug regimens and on protease inhibitors (PI) versus non-nucleoside reverse transcriptase inhibitors (NNRTI). Regimens will be compared in terms of long-term virological and immunological outcomes and in terms of level of adherence and toxicity.

Trial ACTG 388 is a randomized trial designed to assess virological efficacy of different 3-drug and 4-drug regimens in previously pretreated patients with treatment failure (n: 517), defined by a low CD4 count (below 200/mm^3) or high RNA levels (above 100,000 copies per ml). Study duration is 96 weeks and results are expected in 2001 after study closure.

INITIO is a multinational randomised trial which will evaluate different therapeutic pathways in previously untreated patients (n= 900). First-line treatments include 3-drug and 4-drug regimens, based on PI , NNRTI, or both. In the case of virological failure patients will switch according to predefined criteria to second-line 4-drug or 5-drug regimens composed of drugs with a different class or resistance pattern. Main outcome is immunological status (CD4 count) at three years.

PART (Pulsed AntiRetroviral Therapy) is a wide randomised national trial (100 centres, 600 patients) which will compare in patients with good virological response to HAART (HIV-RNA viral load below 400 copies/ml, for at least 6 months and CD4+ lymphocytes above 300 cells per mm^3) the immunological efficacy of standard (i.e. continuous) versus intermittent antiretroviral treatment. Main objective of the trial is to define the role of Structured Treatment Interructions (STI) in the treatment of HIV infection. Immunological, virological, metabolic and quality of life aspects will be investigated in detail in specific substudies. Trial start is expected in early 2001.

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30C/G, 30C/N, 31B/A, 32B/A, 35C/A (ACTG 384-388)
31C/A (PART)

No publications available (ongoing trials)

ALTERED OUTWARD-RECTIFYING K^+ CURRENT REVEALS MICROGLIAL ACTIVATION INDUCED BY HIV-1 TAT PROTEIN

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The importance of the HIV-1 regulatory protein Tat stands not only on its role as a promoter of viral replication, but also on the ability to modulate the functional activity of a number of cells. In this light, we addressed the question whether it could affect the phenotype of microglial cells, which are known to play a key role in the progression of neuro AIDS.
Among the electrophysiological changes accompanying microglial activation, the appearance of outward-rectifying potassium currents (OR) is the most remarkable. Although a clear view of the role played by this current in microglia is still lacking, in cells other than microglia OR have been linked to cell proliferation or the modulation of cytoplasmic calcium transients.
In order to study the possible influence of Tat on the microglial electrical properties we employed the single cell "patch-clamp" recording technique on microglial cell cultures obtained from newborn rats.
In these experiments Tat (ε 100 ng/ml) was able to induce depolarization-activated OR which, according to pharmacological sensitivity, potassium permeability and kinetic behavior, depicted the presence of functional Kv1.3 channels.
The effect of Tat was concentration- and time-dependent and could be abolished by heat-denaturation of the protein or by a specific anti-Tat polyclonal antibody.
In order to elucidate the mechanism by which this effect occurred we used two agents able to block the activation of nuclear factor-κB in different ways. Both were able to prevent the Tat effect on OR current, suggesting the involvement of this transcription factor.
In conclusion, our data confirm the ability of Tat to activate the NF-κB pathway in microglia and illustrate a novel potentially relevant change in microglial phenotype induced by the HIV-1 protein.

Accordo di Collaborazione N. 30 C/H a Giulio Levi

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 TAT PROTEIN DECREASES CYCLIC AMP SYNTHESIS IN RAT MICROGLIA CULTURES

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We have studied the modulation of cyclic AMP accumulation by the HIV-1 protein Tat in microglia and astrocyte cultures obtained from neonatal rat brain. Pre-treatment of microglia with recombinant Tat resulted into a dose- and time-dependent decrease of cyclic AMP accumulation induced by subsequent exposure to isoproterenol (1 μM). The inhibitory action of 100 ng/ml Tat approached 50% after 4 h of preincubation and reached a maximum of 70% after 24 h. The Tat-induced time- and dose-dependent decrease of cyclic AMP accumulation was observed also when microglial cultures were stimulated with the adenylyl cyclase activator forskolin (100 μM). In both cases Tat inhibitory action was 70% reverted by a specific monoclonal anti-Tat antibody, but was not prevented by the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xantine (100 μM), nor by a 16 h pre-treatment of microglial cultures with the Gi protein inhibitor pertussis toxin (10 ng/ml). All these results suggested the viral protein to act at a step of the cyclic AMP transduction pathway other that receptors, G proteins and phosphodiesterases. The target of Tat appeared to be adenylyl cyclase, whose activity was markedly reduced (up to 60%) in membranes prepared from Tat-treated microglial cells, both in basal conditions and after stimulation with isoproterenol and forskolin. The inability of the competitive inhibitor of nitric oxide synthase N^G-monometyl-L-arginine (20 and 200 μM) to revert Tat action on forskolin-induced cyclic AMP accumulation, and of two potent nitric oxide donors, PAPA and DETA (0.1-2 mM), to alter forskolin-induced cyclic AMP accumulation, excluded an involvement of nitric oxide in Tat-induced adenylyl cyclase inhibition. On the contrary two inhibitors of nuclear factor kB activation, N-tosyl-$_L$-phenylalanine chloromethyl ketone (10 μM) and SN50 (25 μM), markedly prevented the reduction of forskolin-evoked cyclic AMP accumulation by Tat, suggesting a possible role of this nuclear trascriptional factor in the regulation of adenylyl cyclase by Tat in microglia. This assumption was strengthened by the ability of lipopolysaccharide (100 ng/ml, 4 h) to mimic the inhibitory effect of the viral protein. Conversely, astrocyte cyclic AMP accumulation was unaffected by the viral protein, as tested at various concentrations and time points. Finally, Tat inhibition of microglial adenylyl cyclase was not due to aspecific cytotoxicity. As cyclic AMP has been reported to exert a neuroprotective role in several vivo and in vitro models of brain pathologies and microglia is believed to mediate Tat-induced neurotoxicity, these results suggest that the ability of Tat to inhibit cyclic AMP synthesis in microglia may contribute to neuronal degeneration and cell death associated to HIV-infection.

No. accordo di collaborazione 30 C/H

HIV PROTEASE INHIBITORS HINDERS APOPTOSIS VIA A TARGET EFFECT ON MITOCHONDRIA

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Treatment with a combination therapy of at least three antiviral drugs has become of widespread use in the management of patients with HIV-1 infection. In most cases, treatment regimens include nucleoside reverse transriptase inhibitors (NRTI) and HIV protease inhibitors (PI). The use of this highly active antiretroviral therapy (HAART) led to reduced morbidity and mortality. This is mainly due (or associated with) immune reconstitution characterized by quantitative and functional recovery of peripheral blood mononuclear cells (PBMC), mainly CD4+ cells. This seems to at least partially depend on a significant reduction of cell loss by apoptosis by CD4+ cells. The role of apoptosis in the pathogenesis of HIV infection and in the progression of the disease was in fact described since many years. Precisely, cell loss by "spontaneous" apoptosis was described as the main cause of CD4+ bystander cell depletion observed HIV+ patients. The effects of both NRTI and PI on apoptotic cell death in the immune system was in fact deeply investigated by several research groups indicating that i) NRTIs are apoptotic inducers while ii) PIs are apoptotic hindering drugs. Namely, the use of NRTIs, although of relevance in the control of the progression of the disease via a direct activity on viral replication, was demonstrated to be *per se* cytotoxic to the immune system cells. On the other hand, some very recent *in vitro* and *ex vivo* studies suggested that PIs were capable to impair PBMC cell death by apoptosis and to restore impaired T-cell proliferative response. Importantly this appeared to be independent from viral infection. Proliferation/apoptosis balance is thus still considered as a key factor in the immune system homeostasis in the management of AIDS patients. Apoptosis is a widespread suicide mechanism that involves a series of subcellular modifications. In particular, a specific activity of mitochondrion has generally been associated to the apoptotic triggering. This orgenelle, via cytochrome c release, might in fact activate the complex caspase cascade leading to apoptotic cell death. In particular, in vitro and ex vivo studies, including ours, clearly indicated that NRTI did induce a mitochondrial dysfunction acting on mitochondrial polymerase gamma, decreasing mitochondrial DNA and increasing mutant mitochondrial DNA. NRTI are also irreversibly incorporated into mitochondrial DNA and inhibit oxidative phosphorylation. On the other hand, PI have been described to inhibit apoptotic cell death in both HIV-infected and uninfected cells by a mechanism that seems still a matter of debate. In the present work we partially address this mechanism by pointing to mitochondrial membrane potential of activated lymphocytes as a key factor in PI activity. These results endeavor for a reappraisal of PI as immune system homeostatic drugs that act independently from HIV infection by a target activity on mitochondrial function..

Contract N. 30C/I

ROLE OF HHV-8, INFLAMMATORY CYTOKINES, AND HIV TAT IN KAPOSI'S SARCOMA AND IN PRIMARY EFFUSION LYMPHOMAS: AN ULTRASTRUCTURAL AND IMMUNOCYTOCHEMICAL STUDY

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Lytic growth of human herpesvirus 8: morphological aspects. HHV-8, a recently identified gamma-Herpesvirus, is associated with several human diseases including primary effusion lymphomas (PEL) and Kaposi's sarcoma (KS). Limitations in systems allowing productive viral replication have hampered the characterization of the HHV-8 life cycle and further definition of its role in the development of KS and PEL. PELs are a rare form of non-Hodgkin's lymphomas in which HHV-8 is present often in association with Epstein-Barr virus (EBV) infection. HHV-8 is also present in a latent state or in a state of low level persistence in different primary effusion lymphoma (PEL)-derived cell lines such BCBL-1 cells that lack EBV infection. This cell line was induced to produce mature virions by treatment with 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the characteristic ultrastructural features of HHV-8 lytic replication were identified and compared to those of the other members of *Herpesviridae* family. Our findings improve the knowledge of the different steps of HHV-8 morphogenesis and the nature of modifications induced by the virus in lytically infected cells that may be helpful to recognize HHV-8 infection and to identify targets for specific antiviral drugs.

Ultrastructural characterization of KS spindle cells. Comparative morphological studies have been carried out on spindle cells of vascular and lymphatic endothelial cell origin isolated from KS lesions and grown in the presence of activated T-cell conditioned media (TCM) and on cultured human macrovascular endothelial cells (HUVEC). All these cell types showed signs of cell activation including dilatation, ipertrophy, and electrondensity of rough endoplasmic reticulum and condensation of mitochondria. However, spindle cells invariably showed characteristic osmiophilic cytoplasmic inclusions that were only occasionally observed in normal endothelial cells. KS cells of vascular endothelial cell origin and normal macrovascular endothelial cells presented wery small sized mitochondria. Neoformations of viral origin were never observed. To further compare KS cells of different origin at the ultrastructural level, lesions have been enzymatically disrupted, cellular types have been isolated with specific antibody-coated magnetic beads, and analyzed by electron microscopy. Immunocytochemical studies on ultrathin sections of endothelial spindle cells of lymphatic origin labeled with anti-VEGF-R3 antibodies indicated that this marker of endothelial cells of lymphatic origin is mainly expressed on the cell apical surface. Studies combining immunological and ultrastructural approaches to associate cell specific antigenic determinants with cellular characteristic morphological markers are ongoing.

HIV Tat effects on lytic growth of HHV-8 in BCBL-1 cells. The effect of different concentrations (0.01-1 μg/ml) of soluble Tat on lytic growth of HHV-8 in BCBL-1 cells has been investigated. Results obtained demonstrated that soluble Tat treatment induces an inhibition of HHV-8 lytic growth in both TPA-treated and untreated cells, being the inhibition greater with the higher Tat concentration utilized (1 μg/ml). Our findings suggest that Tat play an important role in HHV-8 infectious cycle by inhibiting viral reactivation and lysis of BCBL-1 cells. Experiments are in progress to evaluate the effects of Tat, immobilized on tissue culture plates to enhance cell membrane signaling, on lytic growth of HHV-8.

N°. dell'Accordo di Collaborazione. 30C/K

IMMUNORICONSTITUTION IN HIV+ CHILDREN TREATED WITH PI-CONTAINING HAART.

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§ Clinica Pediatrica, Università Degli Studi di Milano

Objectives: To evaluate the immunologic response to a nelfinavir containing regimens in PI naïve HIV+ children.

Patients and methods: Nine HIV+ vertically infected PI naïve children, aged 5 months- 10,5 years (mean 5,7 years), started a triple antiretroviral regimen including nelfinavir (NFV) plus 2 NRTIs; three children had been previously treated with a dual NRTIs combination, one had received AZT only. Follow-up was 12 months for all patients.
The immunologic response to antiretroviral therapy was evaluated by the following assays: T lymphocyte phenotyping; lymphoproliferative response to PHA, *Candida* and HIV antigens; Vβ T cell repertoire (TCR) analysis on CD4+ and CD8+; TREC (Thymic Recent Emigrants) presence in PBMC.

Results: At baseline, mean plasma HIV RNA level was 4.51 log.
plasma HIV RNA values below 50 copies/ml were achieved only in the 5 naïve children and in the AZT-experienced one (virologic responders).
Mean number of CD4+ was 816 cells/mmc at baseline and 1365 after 12 months. The increase in total CD4+ T cell count was comparable in virologic responders and non responders; however, among the virologic responders the recovered cells were mainly of naïve phenotype, whereas in those with virologic failure percentage of naïve and memory CD4+ cells did not change significantly over time.
At baseline, positive lymphoproliferative response to PHA and *Candida* was observed in 9/9 and 5/9 children, respectively, and was conserved after 12 months of therapy. Four children responded to HIV-1 p24 protein and 2 to gp160 at baseline. These proportions increased to 7/9 and 4/9, respectively, after 12 months of therapy
To analyse Vβ T cell receptor repertoire, HIV+ children were compared with 12 age-matched healthy subjects. With respect to controls, three Vβ families on CD4+ cells and 6 on CD8+ cells showed a significant perturbation at baseline; after 12 months of HAART a trend to normalisation was observed in all these Vβ families. TREC analysis are currently under evaluation.

Conclusions: **The results of this study are consistent with those reported by other Authors, confirming that the pattern of immunoricostitution after HAART in paediatric patients differs from adults. The recovery of CD4+ naïve lymphocytes, the lymphoproliferative response to HIV antigens and the partial normalisation of Vβ repertoire indicate a more complete immunologic restoration and provide the rationale for novel therapeutic approaches.**

GRANT: 30C/L

DEVELOPMENT OF AN INTERFERON-GAMMA ELISPOT ASSAY FOR THE QUANTIFICATION OF HIV-1 SPECIFIC CD8+ CYTOTOXIC T LYMPHOCYTES CELLS STIMULATED WITH AUTOLOGOUS BLCL INFECTED WITH HIV-1 RECOMBINANT VACCINIA VIRUS.

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Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy

Background: HIV-1 specific cytotoxic T lymphocytes (CTL) play an important role in the control of primary HIV infection and in the suppression of viral replication during the course of the disease.
Several assays are available to measure anti HIV-1 CTL activity in PBMC.
The classical limiting dilution assays (LDA) and in vitro stimulation followed by measurement of cytoxicity are time consuming, technically difficult and poorly sensitive. In addition, they require remarkable amounts of patient PBMC.
A more sensitive assay to detect CD8+ CTL frequencies is based on staining with HLA class I peptide tetrameric complexes.
A further method is represented by the ELISPOT assay, where antigen specific memory T cell are detected by IFN-γ secretion; this technique is more sensitive than LDA.
Since both ELISPOT and tetramers assays are dependent on the use of HLA restricted peptides, patients haplotype and peptides presented by each MHC class I haplotype should be known in advance.

Methods: These observations have driven us to develop a variant of the Elispot assay where infected autologous BLCL infected with HIV-1 (gag-pol and gp160) recombinant Vaccinia Virus were used to stimulate PBMC from a patient naïve for antiretroviral therapy. CD8+ T cells were positively selected with MACS Micro Beads and tested in an ELISPOT assay for the production of IFN-γ.
As positive control HTLVIIIB infected BLCL and PHA mitogen stimulus were used. Unpulsed BLCL and BLCL infected with Vaccinia control vector were used as negative controls. PBMC from the same patient were tested in a LDA ^{51}Cr release assay.

Results and conclusions: The ELISPOT assay results were coherent with the LDA analysis: both assays demonstrated a CD8+ cells positive response to gp160 and HTLVIIIB stimuli.
Moreover a CD8+-induced production of IFN-γ was shown by the ELISPOT assay when Gag-Pol Vaccinia Virus infected BLCL were utilized as stimulus, indicating its sustained sensitivity.

The ELISPOT/Vaccinia Virus assay is presently under validation in a population of HIV+ subjects at different stages of disease. If preliminary results are confirmed, it will provide a simple and accurate tool for the detection of CTL activity in HIV infection.

GRANT: 30C/M

THE EUROSIDA STUDY: AIDS ACROSS EUROPE, 1994-98: THE EUROSIDA STUDY.
Published on: Lancet. 2000 Jul 22;356(9226):291-6.

The EUROSIDA Study Team: Italian Group, Coordinating Centre: S. Vella, A. Chiesi, Di Nallo, Istituto Superiore di Sanita, Rome. **Italian Centres:** C. Arici, Ospedale Riuniti, Bergamo; R. Pristerá, Ospedale Generale Regionale, Bolzano; F. Mazzotta, F.Vichi, Ospedale S. Maria Annunziata, Florence; R. Esposito, A. Bedini, Università di Modena, Modena; A. Chirianni, E. Montesarchio, Presidio Ospedaliero A.D. Cotugno, Naples; V. Vullo, P. Santopadre, Università di Roma La Sapienza, Rome; Antonucci, Franci, Narciso, Antinori, Zaccarelli, Ospedale Spallanzani, Rome.

BACKGROUND: The clinical presentation of HIV-1 related diseases could have changed after the introduction of highly active antiretroviral treatment (HAART). We aimed to assess changes over time in the incidence of ADIs overall and within CD4 lymphocyte count strata, the relationship with treatment and degree of immunodeficiency at diagnosis of ADIs.

METHODS: We did a prospective observational multicentre study of over 7300 patients in 52 European HIV-1 outpatient clinics. Incidence rates per 100 patient-years of observation were calculated.

FINDINGS: In total, we recorded 1667 new ADIs; the incidence of ADIs declined from 30.7 per 100 patient-years of observation during 1994 (95% CI 28.0-33.4) to 2.5 per 100 patient-years of observation during 1998 (95% CI 2.0-3.0, p<0.0001, test for trend). Median CD4 lymphocyte count at diagnosis of a new ADI increased from 28 cells/microL to 125 cells/microL between 1994 and 1998 (p<0.0001), yet a steep decline in the rate of ADIs was seen after stratification by latest CD4 lymphocyte count within each year (< or = 50, 51-200, and > 200 cells/microL). Patients on HAART had a lower rate of ADIs than patients not on this treatment within each CD4 lymphocyte count strata. The proportion of ADIs attributable to cytomegalovirus retinitis and Mycobacterium avium complex declined over time (p=0.0058 and 0.0022, respectively), whereas the proportion of diagnoses attributable to non-Hodgkin lymphoma has increased (p<0.0001). In 1994, less than 4% of ADIs were non-Hodgkin lymphoma, in 1998 the proportion was almost 16%. This condition has become one of the most common ADIs in patients on HAART.

INTERPRETATION: Our findings lend support to the idea that treatment regimens can lower the incidence of ADIs. The immediate risk of an ADI for a given CD4 lymphocyte count has declined over time and is lower among patients on HAART. Long-term follow-up of patients on combination treatment is essential to monitor the incidence of new and emerging diagnoses.

Main articles of the past two years:

1. Relations among CD4 lymphocyte count nadir, antiretroviral therapy and HIV-1 disease progression: results from the EuroSIDA study. Ann Intern Med. 1999 Apr 6;130(7):570-7
2. Anaemia is an independent predictive marker for the clinical prognosis in HIV-infected patients from across Europe. AIDS 1999 May 28;13(8):943-50
3. Discontinuation of Pneumocystis carinii pneumonia prophylaxis after the initiation of highly active antiretroviral therapy in HIV infection. Lancet 1999 Apr 17;353(9161):1293-8
4. Regional survival differences across Europe in HIV positive People: The EuroSIDA Study. AIDS 1999; 13:2281-2288
5. Use of observational databases to evaluate the effectiveness of antiretroviral therapy for HIV infection: comparison of cohort studies with randomized trials. AIDS 1999; 13:2075-2082

6. Predictors of Virological Success and Ensuing Failure in HIV-Positive Patients Starting Highly Active Antiretroviral Therapy in Europe. Results From the EuroSIDA Study. Arch Intern Med. 2000 Apr 24;160(8):1123-32.
7. Does European or non-European origin influence health care and prognosis for HIV-patients in Europe?
   HIV Medicine 1999; 1:2-9
8. A comparison of exposure groups in the EuroSIDA study: starting highly active antiretroviral therapy (HAART), response to HAART, and survival. J Acquir Immune Defic Syndr 1999 Dec 1;22(4):369-78
9. Infections with Mycobacterium tuberculosis and Mycobacterium avium among HIV-infected Patients after the Introduction of Highly Active Antiretroviral Therapy. Am J Respir Crit Care Med 2000 Sep;162(3 Pt 1):865-72
10. AIDS across Europe, 1994-98: the EuroSIDA study. Lancet. 2000 Jul 22;356(9226):291-6.
11. Virological failure among patients on HAART from across Europe: results from the EuroSIDA study.
    Antivir Ther 2000 Jun;5(2):107-12
12. Interruption of secondary prophylaxis against Pneumocystis pneumonia in AIDS-patients responding to antiretroviral therapy. N Engl J Med (in press, 2000).

GRANT: 30C/O

THE EUROSIDA STUDY: INFECTIONS WITH MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM AVIUM AMONG HIV-INFECTED PATIENTS AFTER THE INTRODUCTION OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY.

Published on: Am J Respir Crit Care Med 2000 Sep;162(3 Pt 1):865-72

The EUROSIDA Study Team:
Italian Group, Coordinating Centre: S. Vella, A. Chiesi, Di Nallo, Istituto Superiore di Sanità, Rome.
Italian Centres: C. Arici, Ospedale Riuniti, Bergamo; R. Pristerá, Ospedale Generale Regionale, Bolzano; F. Mazzotta, F.Vichi, Ospedale S. Maria Annunziata, Florence; R. Esposito, A. Bedini, Università di Modena, Modena; A. Chirianni, E. Montesarchio, Presidio Ospedaliero A.D. Cotugno, Naples; V. Vullo, P. Santopadre, Università di Roma La Sapienza, Rome; Antonucci, Franci, Narciso, Antinori, Zaccarelli, Ospedale Spallanzani, Rome.

The impact of highly active antiretroviral therapy (HAART) among human immunodeficiency virus (HIV)-infected patients on the incidences of mycobacterial infections has not been studied in detail. We assessed incidences of mycobacterial diseases among HIV-infected patients following the introduction of HAART, using data from the EuroSIDA study, a European, multicenter observational cohort of more than 7,000 patients. Overall incidences of Mycobacterium tuberculosis (TB) and Mycobacterium avium complex (MAC) were 0.8 and 1.4 cases/100 person-years of follow-up (PYF), decreasing from 1.8 (TB) and 3.5 cases/100 PYF (MAC) before September 1995 to 0.3 and 0.2 cases/100 PYF after March 1997. After adjustment for changes in CD4 cell count and use of antiretroviral treatment in Cox proportional hazards models, the risk of MAC decreased with increasing calendar time (hazard ratio per calendar year; HR = 0.58 [95% confidence intervals: 0.45-0.74], whereas this was not the case for TB; 0.95 [0.74-1.22]).
In conclusion, we documented marked decreases in the incidence of TB and to an even larger extent of MAC among HIV-infected patients from 1994 to 1999. The decrease in TB was associated with the introduction of HAART and changes in CD4 cell count. These factors could also explain some of the decrease in MAC over time, though there remained a significantly lower risk of MAC than expected.

Main articles of the past two years:

1. Relations among CD4 lymphocyte count nadir, antiretroviral therapy and HIV-1 disease progression: results from the EuroSIDA study. Ann Intern Med. 1999 Apr 6;130(7):570-7
2. Anaemia is an independent predictive marker for the clinical prognosis in HIV-infected patients from across Europe. AIDS 1999 May 28;13(8):943-50
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Antivir Ther 2000 Jun;5(2):107-12
12. Interruption of secondary prophylaxis against Pneumocystis pneumonia in AIDS-patients responding to antiretroviral therapy. N Engl J Med (in press, 2000).

GRANT: 30C/O

THE EUROSIDA STUDY: PREDICTORS OF VIROLOGICAL SUCCESS AND ENSUING FAILURE IN HIV-POSITIVE PATIENTS STARTING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY IN EUROPE: RESULTS FROM THE EUROSIDA STUDY.

Published on: Arch Intern Med. 2000 Apr 24;160(8):1123-32.

The EUROSIDA Study Team: Italian Group, Coordinating Centre: S. Vella, A. Chiesi, Di Nallo, Istituto Superiore di Sanita, Rome. **Italian Centres:** C. Arici, Ospedale Riuniti, Bergamo; R. Pristerá, Ospedale Generale Regionale, Bolzano; F. Mazzotta, F.Vichi, Ospedale S. Maria Annunziata, Florence; R. Esposito, A. Bedini, Università di Modena, Modena; A. Chirianni, E. Montesarchio, Presidio Ospedaliero A.D. Cotugno, Naples; V. Vullo, P. Santopadre, Università di Roma La Sapienza, Rome; Antonucci, Franci, Narciso, Antinori, Zaccarelli, Ospedale Spallanzani, Rome.

BACKGROUND: Predictors of virological response to highly active antiretroviral therapy (HAART) have never been systematically evaluated in a large continental multicenter cohort of unselected human immunodeficiency virus (HIV)-infected people. **OBJECTIVE**: To determine the factors related to achieving and maintaining undetectable plasma HIV-1 RNA levels among HIV-1-infected patients first starting protease inhibitor- or nonnucleoside retrotranscriptase inhibitor-containing HAART in Europe. **DESIGN**: Prospective multicenter cohort study. **SETTING**: Fifty-two clinical centers in 17 European countries included in the EuroSIDA Study Group, from August 1996 to April 1999. **PATIENTS**: A total of 1469 HIV-positive patients first starting HAART recruited from an unselected cohort of more than 7300 HIV-positive patients. **MAIN OUTCOME MEASURE**: Detection of factors related to virological success after first starting HAART (baseline) and ensuing failure by standard survival techniques, including Kaplan-Meier techniques and Cox proportional hazards models. All analyses were intention to treat. **RESULTS**: Most patients (80%) achieved plasma HIV-1 RNA levels of less than 500 copies/mL during follow-up (60.4% at 6 months from the onset of HAART). Patients with higher baseline HIV-1 RNA levels (relative hazard [RH], 0.76 per log higher; 95% confidence interval [CI], 0.69-0.84; P<.001) and those taking saquinavir mesylate hard gel as a single protease inhibitor (RH, 0.62; 95% CI, 0.47-0.82; P<.001) were less likely to reach undetectable HIV-1 RNA levels. Conversely, higher CD4+ lymphocyte counts (RH per 50% higher, 1.09; 95% CI, 1.02-1.16; P = .008) and the initiation of 3 or more new antiretroviral drugs (RH, 1.29; 95% CI, 1.03-1.61; P = .02) were independent predictors of higher success. Once success was achieved, HIV-1 RNA levels rebounded in more than one third of all patients during follow-up (24% at 6 months). Antiretroviral-naive patients (RH, 0.50; 95% CI, 0.29-0.87; P = .01), older patients (RH, 0.86 per year older; 95% CI, 0.75-0.99; P = .04), and those starting a protease inhibitor other than saquinavir hard gel (RH, 0.66; 95% CI, 0.44-0.98; P = .04) were at decreased hazard for virological failure. Higher baseline HIV-1 RNA level (RH, 1.18 per log higher; 95% CI, 0.99-1.40; P = .06) and a longer time to achieve virological success (RH per 12 months, 1.53; 95% CI, 0.99-2.38; P = .06) were marginally significant predictors of a decreased hazard of ensuing virological failure. **CONCLUSIONS**: HAART is associated with a favorable virological response if started when the baseline HIV-1 RNA level is low, if at least 2 new nucleoside retrotranscriptase inhibitors are added, and if standard doses of saquinavir hard gel capsule are avoided as a single protease inhibitor. Older patients are more likely to achieve virological success. Thereafter, the higher durability of virological response is predicted by an antiretroviral-naive status and by the use of specific regimens. Lower baseline HIV-1 RNA levels and rapid maximal viral suppression seem to be other important factors in the durability of virological response.

Main articles of the past two years:

1. Relations among CD4 lymphocyte count nadir, antiretroviral therapy and HIV-1 disease progression: results from the EuroSIDA study. Ann Intern Med. 1999 Apr 6;130(7):570-7
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12. Interruption of secondary prophylaxis against Pneumocystis pneumonia in AIDS-patients responding to antiretroviral therapy. N Engl J Med (in press, 2000).

GRANT: 30C/O

THE EUROSIDA STUDY: VIROLOGICAL FAILURE AMONG PATIENTS ON HAART FROM ACROSS EUROPE: RESULTS FROM THE EUROSIDA STUDY.
Published on: Antivir Ther 2000 Jun;5(2):107-12

The EUROSIDA Study Team. Italian Group, Coordinating Centre: S. Vella, A. Chiesi, Di Nallo, Istituto Superiore di Sanita, Rome **Italian Centres:** C. Arici, Ospedale Riuniti, Bergamo; R. Pristerá, Ospedale Generale Regionale, Bolzano; F. Mazzotta, F.Vichi, Ospedale S. Maria Annunziata, Florence; R. Esposito, A. Bedini, Università di Modena, Modena; A. Chirianni, E. Montesarchio, Presidio Ospedaliero A.D. Cotugno, Naples; V. Vullo, P. Santopadre, Università di Roma La Sapienza, Rome; Antonucci, Franci, Narciso, Antinori, Zaccarelli, Ospedale Spallanzani, Rome.

OBJECTIVES: To monitor the response to highly active antiretroviral therapy (HAART) over time and the proportions of patients with poor virological control in order to help provide some insight into drug resistance. DESIGN: Analysis of data from the EuroSIDA study; an observational study initiated in 1994 of almost 8500 patients with HIV from across Europe.
METHODS: Patients who initiated HAART, and had both a CD4 lymphocyte count and viral load measured in the 3 months prior to starting HAART, were included in analyses. The proportion of patients with a poor virological response (defined as a viral load of > 10,000 copies/ml, using either a single measure or two consecutive measures) at 16 and 48 weeks was determined. Multivariate logistical regression was used to determine the factors associated with a poor virological response at both time points.
RESULTS: Median CD4 cell count at starting HAART was 218 cells/mm3 [interquartile range (IQR), 113-327 cells/mm3] and median viral load was 4.36 log10 copies/ml (IQR, 3.57-5.04 log10 copies/ml). At 16 weeks, 16% had a viral load of > 10,000 copies/ml based on a single viral load measure and 10% if the more stringent definition of two consecutive viral loads above this level was used. At 48 weeks these proportions were 19% and 13%, respectively. Compared with patients from Southern Europe, patients from both Central and Northern Europe had approximately half the chance of a poor virological response at 16 weeks (odds ratios 0.53 and 0.47, $P = 0.0015$ and $P < 0.0001$, respectively), while at 48 weeks both regions still had approximately a 25% reduced chance of a poor virological response, but this was no longer statistically significant (odds ratio 0.77 and 0.75, $P = 0.17$ and $P = 0.13$, respectively).
CONCLUSIONS: There were marked difference in virological response to HAART across regions of Europe, which may be partly explained by regional differences in access to HAART and utilisation. If drug resistance is closely related to virological failure, these results may help to provide an early insight into the potential problem of drug resistance across Europe. Continued follow-up is essential to monitor patients with poor virological control.

Main articles of the past two years:

1. Relations among CD4 lymphocyte count nadir, antiretroviral therapy and HIV-1 disease progression: results from the EuroSIDA study. Ann Intern Med. 1999 Apr 6;130(7):570-7
2. Anaemia is an independent predictive marker for the clinical prognosis in HIV-infected patients from across Europe. AIDS 1999 May 28;13(8):943-50
3. Discontinuation of Pneumocystis carinii pneumonia prophylaxis after the initiation of highly active antiretroviral therapy in HIV infection. Lancet 1999 Apr 17;353(9161):1293-8
4. Regional survival differences across Europe in HIV positive People: The EuroSIDA Study. AIDS 1999; 13:2281-2288
5. Use of observational databases to evaluate the effectiveness of antiretroviral therapy for HIV infection: comparison of cohort studies with randomized trials. AIDS 1999; 13:2075-2082

6. Predictors of Virological Success and Ensuing Failure in HIV-Positive Patients Starting Highly Active Antiretroviral Therapy in Europe. Results From the EuroSIDA Study. Arch Intern Med. 2000 Apr 24;160(8):1123-32.
7. Does European or non-European origin influence health care and prognosis for HIV-patients in Europe?
   HIV Medicine 1999; 1:2-9
8. A comparison of exposure groups in the EuroSIDA study: starting highly active antiretroviral therapy (HAART), response to HAART, and survival. J Acquir Immune Defic Syndr 1999 Dec 1;22(4):369-78
9. Infections with Mycobacterium tuberculosis and Mycobacterium avium among HIV-infected Patients after the Introduction of Highly Active Antiretroviral Therapy. Am J Respir Crit Care Med 2000 Sep;162(3 Pt 1):865-72
10. AIDS across Europe, 1994-98: the EuroSIDA study. Lancet. 2000 Jul 22;356(9226):291-6.
11. Virological failure among patients on HAART from across Europe: results from the EuroSIDA study.
    Antivir Ther 2000 Jun;5(2):107-12
12. Interruption of secondary prophylaxis against Pneumocystis pneumonia in AIDS-patients responding to antiretroviral therapy. N Engl J Med (in press, 2000).

GRANT: 30C/O

IP REGISTRY: NATIONAL REGISTRY OF HIV INFECTED PERSONS TREATED WITH PROTEASE INHIBITORS.

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Progress Report:

Activity Program and methods: The National Registry of HIV Infected Persons Treated with Protease Inhibitors (IP Registry) was established at the ISS, Laboratory of Virology, Sec. of Retrovirology, with the 23 Dec 1996 n. 18 Disposition of the Ministry of Health. Its primary scope is to be a surveillance Registry monitoring the short term and long term toxicity/efficacy aspects related with the expanded access and the long term use of the new combination treatments based on Protease Inhibitors. The secondary scopes are to investigate efficacy and toxicity of different combinations of drugs in terms of clinical outcome, compliance to treatments and immunological and virological response.
The IP Registry is organised as a prospectic cohort representing a significant sample of the overall patients treated with a combination therapy including IP. The data collection form collects detailed information on prescribed drugs, adverse events and long term toxicity, clinical, immunological and virological outcome, compliance to treatments, frequency of hospitalisation, prophylactic treatments, deaths. Data are collected in an anonymous coded way.

Progress report and expected results: 8203 patients have been consecutively enrolled in 123 clinical centres trough out Italy during the entire 1997. All patients were IP naive at enrolment. Data are collected trough a dedicated software and, more recently via internet. The principal characteristics of the cohort, based on enrolment data are the following: Mean age: 36 years (±7.9); median CD4 count: 157 cells/mm^3; gender: 73% maschi; infection stage: 29.4% AIDS, 34.7% symptomatic non AIDS and 35.9% asymptomatic. The male population is in majority represented by IV drug users (57%), homosexuals (20%) and heterosexuals (17%). The female population mainly consists of heterosexuals (49%) and IV drug users (44%). The 21% of all patients has a CD4 count < 50 CD4/mm^3, 38% between 50 and 199 CD4/mm^3, and 34% between 200 and 499 CD4/mm^3. 48% of AIDS patients had less than 50 CD4/mm^3, 46% of symptomatic non AIDS patients had between 50 and 199 CD4/mm^3 and 52% of asymptomatic patients had between 200 and 499 CD4/mm^3. The median value of HIV-RNA, among those patient who have a measure at enrolment (about 66% of patients), is 69.700 copies/ml. The 85% of them has more than 5.000 copies/ml, 41% of which with at least 100,000 copies/ml. 55% of AIDS patients has at least 100.000 copies/ml. Several protocols of data analysis are under evaluation within a collaborative project with the statistical group of researcher of the Royal Free Hospital in London headed by Prof. Andrew Phillips, specialised in the analysis of HIV cohort studies. Such analyses will provide important information on several parameters of toxicity and efficacy on used drugs, but also on the variation of mortality and morbidity rates in the past three years, on the progression rate to a new/first AIDS defining event, on the toxicity-free time, on the mean efficacy duration of a specific HAART regimen (with respect to immunological and virological surrogate markers), according to the person-years method of analysis.

External Collaborations: 123 Italian Clinical Centres; Department of Biostatistic of the Royal Free Hospital in London headed by Prof. Andrew Phillips.

Grants: 30C/O, 36C/A

DETERMINATION OF PLASMA LEVEL OF ANTIRETROVIRAL DRUGS BY LIQUID CHROMATOGRAPHY

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All the compounds that are used for the treatment of HIV infections belong to one of the following classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). The measurement of these drug plasma levels may have a clinical role, provided that antiretroviral efficacy and drug toxicity can be managed with this information.
Two methods, one for the determination of indinavir, saquinavir and ritonavir and one for the determination of zidovudine and nevirapine were developed.
The first one used a liquid chromatography with ultraviolet (UV) and electrochemical detection (ED) for the simultaneous quantitation of indinavir, saquinavir and ritonavir. Sample pre-treatment consisted of solid-phase extraction prior to ion-pair reversed phase high performance liquid chromatography with ultraviolet detection at 240 nm (Saquinavir and Ritonavir) and electrochemical detection at + 750 mV (Indinavir and Saquinavir).
The second one system was a reversed phase high performance liquid chromatography method for the simultaneous determination of zidovudine and nevirapine in human plasma, using high performance liquid chromatography (HPLC) with dual-wavelength UV detection at 265 and 280 nm. After solid-phase extraction using Waters Oasis HLB cartridges chromatography was performed using a Zorbax SB-C18 columns with a mobile phase composed of 10 mM potassium dihydrogen phosphate pH 6.5- acetonitrile (83:17, v/v).
For each method peak-areas are linear and correlation coefficients are better than 0.998 for all compounds. Extraction recoveries are higher than 89% for PIs, 94% for zidovudine and 92% for nevirapine. The limits of detection were 13.05 pg and 110.76 pg injected onto the column at 265 and 280nm respectively for zidovudine and 35.37 pg and 541.22 pg injected onto the column at 265 and 280nm respectively for nevirapine, 200 pg injected onto the column for Indinavir, 500 pg injected onto the column for Saquinavir ED and 240 pg injected onto the column for Saquinavir UV and 240 pg injected onto the column for Ritonavir. The limits of quantification were 40 pg and 335.64 pg injected onto the column at 265 and 280nm respectively for AZT, 251.18 pg and 1.64 ng injected onto the column at 265 and 280nm respectively for NVP, 1 ng for Indinavir and Saquinavir and 10 ng for Ritonavir. Each method was also validated respect to precision and accuracy. For all compounds both precision and accuracy were < 15%.
The proposed methods were employed for the analysis of HIV patient's plasma and additional studies are under way.

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Emilia Marchei, Roberta Pacifici, Gianna Tossini, Rita Di Fava, Luisa Valvo, Piergiorgio Zuccaro. "Simultaneous liquid chromatographic determination of indinavir, saquinavir and ritronavir in human plasma with combined ultraviolet absorbance and electrochemical detecion". Journal of Liquid Chromatography, (2001) in press.

Emilia Marchei, Roberta Pacifici, Gianna Tossini, Rita Di Fava, Luisa Valvo, Piergiorgio Zuccaro. "Determination of plasma level of protease inhibitors by liquid chromatography". British Journal of Clinical Pharmacology, Abs n. 1017; pag. 261 (2000)

Programma Nazionale di ricerca sull'AIDS (1999) contratto 30C/P.

The National research program on AIDS

(ISS research projects)

Project

PATHOGENESIS AND IMMUNITY

Scientific Coordinator: **Paola VERANI**

Projects financed N° 12

A DROSOPHILA MODEL OF HIV-TAT RELATED PATHOGENICITY

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To analyze the mechanism of Tat-mediated HIV pathogenicity, we produced Drosophila melanogaster strain transgenic for HIV-tat gene and induced the expression of the protein during Drosophila development. By in vitro and in vivo experiments, we demonstrated that (i) Tat specifically binds to tubulin via the MAP-binding domain of tubulin; (ii) this interaction delays the polymerization of tubulin and (iii) induces a premature stop to microtubule-dependent cytoplasmic streaming. The delay in the polymerization of microtubules, the tracks for the transport of the axes determinants, alters the positioning of the dorso-ventral axis as shown by the mislocalization of Gurken and Kinesin in oocyte of Drosophila after Tat induction. These results validate the use of Drosophila as a tool to study the molecular mechanism of viral gene products and strongly suggest that Tat-tubulin interaction is responsible for neurodegenerative diseases and oncogenesis associateted with AIDS.

No. dell'Accordo di Collaborazione 40C/A

INTERFERON REGULATORY FACTORS REGULATE ACTIVATION OF HIV-1 VIA PHYSICAL AND FUNCTIONAL INTERACTIONS WITH TAT

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Transcription of the human immunodeficiency virus type 1 (HIV-1) is controlled by the cooperation of virally-encoded and host regulatory proteins. The HIV-1 Tat protein is the transcriptional activator of HIV-1 gene expression and is essential for viral replication. However, expression of Tat after virus entry requires the activation of the HIV-1. A sequence containing a binding site for transcription factors of the Interferon Regulatory Factors (IRF) family has been recently identified downstream of the 5' HIV-1 LTR, and it appears to play a critical role in HIV-1 transcription and replication. Here we show that IRF-1 is produced early upon viral infection, binds this sequence and activates transcription from the HIV-1 LTR in a dose-dependent fashion. In addition IRF-1 cooperates with suboptimal doses of Tat in increasing HIV-1 transcription. The cooperation of IRF-1 and Tat occurs in virally infected cells and depends on a direct physical interaction between the two proteins. This interaction is blocked by overexpression of IRF-8 which acts as the natural repressor of IRF-1 activity on the HIV-1 LTR by displacing Tat-IRF-1 interaction and thus inhibiting virus replication. These data suggest a key role of the IRF-1 in early phases of viral replication and/or during viral reactivation from latency, when viral transactivators are absent or present at very low levels.

N. dell'Accordo di Collaborazione: 40C/B

DUAL ROLE OF THE HIV-1 VPR PROTEIN IN THE MODULATION OF THE APOPTOTIC RESPONSE OF T CELLS

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We investigated the effect of vpr, physiologically expressed during the course of an acute HIV-1 infection, on the response of infected cells to apoptotic stimuli as well as on the HIV-induced apoptosis. At 48 h after infection, Jurkat cells exhibited a lower susceptibility to undergo apoptosis with respect to uninfected cells. This effect was not observed following infection with either a vpr-negative virus or a wild-type strain in the presence of antisense oligodeoxynucleotides targeted at vpr mRNA. Single-cell analysis, aimed at simultaneously identifying apoptotic and infected cells, revealed that resistance to apoptosis correlated with productive infection. Notably, vpr-dependent protection from induced apoptosis was also observed in HIV-1 infected PBMC. In contrast, at later stages of infection, a marked increase in the number of cells spontaneously undergoing apoptosis was detected in infected cultures. This virus-induced apoptosis involved vpr expression and predominantly occurred in productively infected cells. These results indicate that HIV-1 vpr can exert opposite roles in the regulation of apoptosis, which may depend on the level of its intracellular expression at different stages of HIV-1 infection. The dual function of vpr represents a novel mechanism in the complex strategy evolved by HIV to influence the turnover of T lymphocytes leading to either viral persistence or virus release and spreading.

Contract number: 40C/C

IMPAIRMENT OF HIV-1 ENTRY IN T JURKAT CELLS BY CONSTITUTIVE EXPRESSION OF THE HIV-1 VPR PROTEIN

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In this study, we investigated the susceptibility to HIV infection of Jurkat T cells constitutively expressing the HIV-1 vpr protein. Vpr expression in Jurkat clones resulted in a strong inhibition of the replication of two different T-tropic HIV-1 strains (i.e., NL432 and IIIB). A dramatic reduction of proviral DNA as well as of viral proteins was detected in vpr-expressing cells with respect to mock-transfected cells. The vpr-mediated restriction of HIV-1 replication was not due to the acquisition of a general antiviral state, as these cells were permissive to infection with an unrelated virus. FACS analysis of HIV-1 receptor and co-receptors revealed a consistent down-modulation of surface CD4 receptors, but not of HIV-1 co-receptors, in vpr-expressing clones. Notably, vpr-expressing clones were fully competent to infection with a VSV-G pseudotyped HIV-1 virus, revealing that a block at the level of HIV-1 entry occurred in these cells. The protective effect exerted by the constitutive expression of vpr on HIV replication suggests that this protein can negatively regulate the establishment of a productive HIV-1 infection when expressed before virus entry. This would suggest a potential use of this viral product as a therapeutic strategy for limiting viral spreading.

Contract number: 40C/C

INFECTION OF SIMIAN B CELL LINE BY SIV AND HIV STRAINS.

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Infection of CD4-negative cells by variants of tissue culture-adapted human immunodeficiency virus HIV-1 or HIV-2 strains has been shown to be mediated by the CXCR4 co-receptor. Here we show that two in vitro established $CD4^-/CCR5^-/CXCR4^+$ human pre-T cell lines (A3, A5) can be productively infected by wild type-laboratory-adapted T-cell line-tropic (T-tropic) HIV-1 and HIV-2 strains in a CD4-independent, CXCR4-dependent fashion. Despite the absence of CCR5 expression, A3 and A5 cells were susceptible to the simian immunodeficiency viruses SIVmac239 and SIVmac316 infections. Thus, at least in A3 and A5 cells, one or more of the chemokine receptors can efficiently support the entry of HIV/SIV isolates in the absence of CD4. These findings suggest that to infect cells of different compartments HIV/SIV could have evolved in vivo to bypass CD4 and to interact directly with an alternative receptor (Borsetti et al., J. Virol. 74:6689-6694, 2000).
We extended our studies on a simian lymphoblastoid B cell line (SL691) infectable by SIV in a CD4 independent fashion (Titti et al., submitted). Previous studies reported that HIV-1 is restricted for replication in macaque cells. Here we show that SL691 are infectable by HIV-1. Although expression of CD4 mRNA was detected by RT PCR in SL691 cells, no CD4 surface expression was ever detected on these cells using a CD4 panel of monoclonal antibodies that recognize different epitopes of the CD4 molecule. Using an envelope complementation assay to produce recombinant HIV-1 virions containing T cell line adapted (HXBc2, MN), dualtropic (89.6), M tropic (ADA, YU2), primary T tropic (ELI) or SIVmac239, SIVmac316 envelope glicoproteins, we obtained an efficient entry in SL691 cells compared control cells. Our preliminary data indicate also that differently from monkey PBMC, SL691 cells are productively infectable by SIVmac239, SIVmac251 and HIV-1 as determine by measuring p24 level in the supernatans. It is known that HIV-1 replication is blocked at different stages of infection in monkey PBMC in culture. One possibility is that the differences in the tropism between HIV and SIV are due to cell surface restrictions, which are mediated by the ability of the viral envelope protein to interact with certain species-specific cellular entry cofactors. To determine where the block could be overcome in SL691 cells at the early phase of viral infection we first indagated the expression of viral coreceptors in SL691 cells by flow cytometry. CXCR4 was expressed at high level whereas no expression of CCR5 was detected. RT PCR analysis revealed that SL691 cells expressed high level of CXCR4, low level of CCR5, CCR2, CCR3, CCR8 and no expression of gpr1, gpr15, strl33 mRNAs. We are currently performing inhibition of infection experiment using specific ligands of chemokine receptors to determine which chemokine receptors support the entry of SIV and HIV-1 into SL691 cells. Furthermore we are analyzing the levels of viral DNA by a semiquantitative PCR assay at 1 and 2 days after infection with HIV-1 to figure out the step(s) at which HIV-1 replication is blocked in monkey PBMC but it is successful in SL691 cells .

Numero di collaborazione: 40C/D

INFLUENZA VIRUS AS VECTOR OF HIV-1 ANTIGENS

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The induction of long-term mucosal and systemic immune response, particularly to broadly cross-reactive determinants, is considered of major importance for the development of an effective vaccine against HIV-1 infection. In this project, we evaluate the potential use of influenza virus as vector of HIV-1 antigens. Previous studies on recombinant influenza virus carrying foreign epitopes demonstrate the ability of this vector to induce a specific and protective immune response against other infectious agents, suggesting that a similar immunization approach might be efficient in eliciting a significant immune response at mucosal surfaces against HIV-1. As a preliminary goal of the project, recombinant influenza viruses bearing HIV-1 CTL epitopes have been generated by reverse genetics. In particular, P18IIIB of gp160 (aa 315-329) (H-2^d) and p24 Gag epitope (aa 193-212) (H-2^d) have been inserted in the neuraminidase (NA) stalk of influenza virus. To determine whether Flu/P18IIIB and Flu/$Gag_{193-212}$ recombinant viruses could induce a specific immune response against the HIV-1 epitopes, Balb/c mice were immunized with a single dose of either wild-type WSN or the mutant viruses by i.n. route. HIV-1 specific CTLs recovered by BAL from infected mice were able to lyse both P18IIIB and p24 peptides pulsed target cells in primary cytotoxic assay. Similarly, the lytic activity of spleen cells restimulated in vitro by the addition of specific peptides demonstrate that CTL epitopes expressed in influenza virus can be processed and presented in the context of class I MHC and induce specific cytotoxic T cells in vivo, comparably to the immunodominant CTL epitope $NP_{147-155}$ of influenza virus.

Furthermore, we are comparing the effectiveness of infectious recombinant influenza viruses to either inactivated virus or recombinant influenza virus derived virosomes in eliciting a specific immune response against HIV antigens. We also generated Flu/Sen recombinant influenza virus expressing the CTL $NP_{324-332}$ epitope (H-$2D^b$) of Sendai virus. By this way, we will be able to compare the ability of the different immunogens in eliciting a protective immune response in mice against Sendai virus challenge.

AIDS Project N. 40C/E

ROLE OF ERM FAMILY PROTEINS IN P-GLYCOPROTEIN1-MEDIATED MULTIDRUG RESISTANCE AND NK FUNCTION.

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Riassunto
This project involves investigation on the role of membrane/cytoskeleton interactions in both: (1) multidrug resistance and (2) NK function.

(1) We have recently shown that P-glycoprotein 1 (Pgp-1) may be polarized, co-localizing with ezrin and actin in human primary monocytes (Puddu et al., 1999). Thus, we have preliminary investigated the possible association of Pgp-1 with the actin cytoskeleton through the ERM family proteins (Bretscher, 1999). and the possible role of this association in the development of the multidrug resistance. To this purpose we used as a cellular model CEM1.3 cells and its multidrug-resistant variant, CEM-VBL100 cells, obtained by selection of CEM1.3 in medium supplemented with 100 ng/ml of vinblastine. These cells express high levels of membrane Pgp-1 and resistance to a wide spectrum of drug, including protease inhibitors. The preliminary results of this study showed that: (i) Pgp-1 was highly polarized on CEM-VBL100 uropods; (ii) on uropods Pgp-1 co-localized with actin and ERM proteins; (iii) Pgp-1 co-immunoprecipitated with actin and ezrin, moesin, radixin; (iv) treatment with ERM antisense oligonucleotides induced on CEM-VBL100 a clear redistribution of Pgp-1 on the cell membrane, the susceptibility to vinblastine and doxorubicine treatment, together with a marked reduction of the Pgp-1-mediated efflux activity. These results strongly suggested that the Pgp-1-mediated multidrug resistance highly depends on the Pgp-1 association to the actin cytoskeleton through ERM proteins. Experiments are in progress in evaluating the specific role of Pgp-1/actin association in the cellular resistance to antiviral drugs.

(2) Evidences have been provided that an impairment of NK function is involved in HIV-1 pathogenesis. Particularly, a marked derangement in the formation of NK/HIV-1 infected cell have been shown conjugates (Sirianni et al., 1990). A crucial role of actin cytoskeleton in the NK/target cell conjugate formation has been strongly suggested (Trichieri, 1989). Thus, in this part of the project we have evaluated the importance of ERM proteins in human NK function. The results showed that: (i) in NK cells both ezrin, radixin and moesin were expressed, while human lymphocytes exclusively expressed ezrin and moesin, as assessed by both RT-PCR and Western Blot analysis ; (ii) the distribution of ERM proteins markedly varied in NK cells either resting or following IL-2 activation; (iii) ezrin and radixin polarized in the NK-to-target cell contact sites, while moesin did not. These results suggested that ezrin and radixin are full involved in the conjugates formation during the development of NK function and showed that radixin is a specific NK marker. Notably, the fact that NK cells and neurons shared radixin and CD56 (N-CAM) expression further suggests a common origin between these two cell types. Experiments are in progress in evaluating the possible derangement in ERM proteins expression and distribution in NK cells following HIV-1 infection

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N°. dell'Accordo di Collaborazione: 40C/F

STUDIES ON CD95 (APO-1/FAS)/ACTIN CYTOSKELETON ASSOCIATION IN GP120-INDUCED APOPTOSIS IN HUMAN T-LYMPHOCYTES.

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Riassunto.
CD95(APO-1/Fas)-mediated apoptosis is one of the major pathogenic mechanism involved in the HIV-1-mediated CD4+ T-cell depletion. CD95 is a member of the tumor necrosis factor receptor family, which can trigger apoptosis in a variety of cell types. However, little is known on the mechanisms underlying cell susceptibility to the CD95-mediated apoptosis. In the present project we have preliminary investigated the possible involvement of CD95/actin association in the development of human T-lymphocyte susceptibility to the CD95-mediated apoptosis. The results showed that human T cells that are susceptible to CD95-mediated apoptosis: (i) exhibit a constitutive polarized morphology; (ii) CD95 co-localizes with ezrin at the site of cellular polarization; (iii) CD95 co-immunoprecipitates with ezrin exclusively in lymphoblastoid CD4+ T cells and primary long-term activated T lymphocytes, that are prone to CD95-mediated apoptosis , but not in short-term activated T lymphocytes, that are refractory to the same stimuli, even expressing equal levels of CD95 on the cell membrane; (iv) pre-treatment with ezrin antisense oligonucleotides specifically protected from the CD95-mediated apoptosis and (v) the actin cytoskeleton integrity is essential for this function (Parlato et al., 2000). These findings strongly suggest that the CD95 cell membrane polarization, through an ezrin-mediated association with the actin cytoskeleton, is a key intracellular mechanism in rendering human T lymphocytes susceptible to the CD95-mediated apoptosis.
It has been shown that ERM proteins need phosphorylation to become active (Fais et al., 2000). To this purpose evidence has been provided that the CD4 crosslinking on human T-lymphocytes results in ezrin phosphorylation (Thuillier et al., 1994). Thus we investigated the possible effect of gp120 stimuli on ERM protein phosphorylation in human T-lymphocytes. The preliminary results showed that gp120+ rIL-2 stimuli induced in human T-lymphocytes: (i) cellular polarization with pseudopods formation; (ii) polarization of CD95 on uropods; (iii) stable ezrin phosphorylation and (iv) increased susceptibility to CD95-mediated apoptosis. These results suggest that the gp120-mediated ezrin phosphorylation may have a role in the pathogenesis of CD4 depletion. Experiments are in progress in evaluating the CD95/ezrin/actin association in gp120-stimulated human T-lymphocytes.

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N°. dell'Accordo di Collaborazione: 40C/F

THE EXPRESSION OF THE NATURAL OCCURRING F12-HIV NEF ALLELE BLOCKS HIV REPLICATION: ANALYSIS OF THE MECHANISM OF ACTION AND NEW ANTI-HIV GENE THERAPY APPROACHES.

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We have already demonstrated that the expression of the F12-HIV *nef* allele blocks the HIV release and that this strictly depends on the presence of CD4 intracytoplasmic tail. Through a genetic approach, we gained further insights on the mechanism of the antiviral effect induced by F12-HIV Nef. We established that the F12-HIVNef induced antiviral action depends on the concomitant presence of three unique amino acidic substitutions (i.e. G140E, V153L and E177G). We also tested the F12-HIV Nef and its back mutants alleles in terms of known effects of Nef on host cells. Our data indicate that only F12-HIVNef was defective in both accelerated CD4 internalization rate and p62NAK activation, whereas in all back mutants at least one Nef function was restored. Infection of cells expressing Nef resistant CD4 molecules with HIV strains expressing F12-HIVNef back-mutants demonstrated that both the lack of accelerated CD4 endocytosis and a still unrevealed positive function are required for the F12-HIVNef inhibitory phenotype.

A new anti-HIV gene therapy model was approached by exploiting the evidence that F12-HIVNef retains its antiviral phenotype even when it is part of a fusion protein (i.e. CD8-Nef). Thus, we constructed a lentiviral vector with the following features: a) it expresses a fusion protein consisting in the Low Affinity Nerve Growth Factor receptor (NGFr) truncated in its intracytoplasmic domain (NH_2 terminus) fused with F12-HIVNef; b) it is a Tat-inducible vector, being the fusion product under the HIV LTR control; c) the fusion product acts at the same time as a selection marker (extracellular NGFr moiety) and as an effective antiviral product (intracytoplasmic F12-HIVNef moiety). Of note, the presence of a typical membrane protein as NGFr at the NH_2 part of the fusion product allows F12-HIV Nef to concentrate at the inner part of the cell membrane, the site where the antiviral action effectively occurs. At the present, we are testing the effectiveness of the NGFr/F12-HIVNef expression in HIV susceptible *ex vivo* cell cultures (i.e., PBLs, monocyte/macrophages, megakaryocytes, and CD34+ human progenitor cells).

N. Accordo di collaborazione: 40 C/G

T-TROPIC HIV-1 NEF PROTEIN ENTERS HUMAN MONOCYTE/MACROPHAGES AND INDUCES RESISTANCE TO HIV REPLICATION: A POSSIBLE MECHANISM OF HIV T-TROPIC EMERGENCE IN AIDS

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Increasing interest has been devoted to the role that monocyte-macrophages play in the pathogenesis of acquired immunodeficiency syndrome (AIDS). The hypothesis of an involvement in AIDS pathogenesis of human/simian immunodeficiency virus (HIV/SIV) Nef also is currently under evaluation by many investigators. The original basis of this hypothesis came from the evidence that monkeys infected with a *nef*-deleted SIV strain failed to develop the disease. We show that treatment of human monocyte-derived macrophages (MDM) with recombinant HIV-1 Nef protein (rNef) induces a strong inhibition of the replication of either macrophage (M-) or dual-tropic HIV-1 strains. Through cytofluorimetric analyses, we detected internalization of fluorescein isothiocynate-conjugated rNef in MDM as early as six h after treatment. Confocal microscope observations demonstrated that the intracellular distribution of internalized rNef strictly resembled that of endogenously produced Nef. Down-regulation of the CD4 HIV receptor detected upon rNef treatment of MDM suggested that the rNef induced HIV inhibition occurred at level of viral entry. This hypothesis was strengthened by the observation that CD4 independent infection was totally insensitive to the rNef treatment. The specificity for all observed effects was demonstrated by rNef immunodepletions. Finally, we showed that the resistance to HIV replication induced by rNef treatment in MDM favors the spread of T cell (T-) over M-tropic HIV strain in doubly infected CD4+ lymphocyte/MDM co-cultures. We propose that extracellular Nef contributes to the AIDS pathogenesis by inducing resistance to M-tropic HIV replication in MDM, thereby facilitating the switching from M- to T-tropic HIV prevalence frequently correlating with AIDS progression.

N. Accordo Di Collaborazione: 40 C/G

INHIBITION OF HIV-1 REPLICATION IN HUMAN PERIPHERAL BLOOD MACROPHAGES BY SELECTIVE NEUTRALIZATION OF ENDOGENOUS MCP-1

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We have previously shown that MCP-1 is constitutively expressed at high levels in human peripheral blood monocytes and its expression is further up-modulated during monocytes differentiation into macrophages (*Fantuzzi et al. 1999, Blood 94:875*) as well as in the course of HIV infection (*Mengozzi et al. 1999, Blood 93:1851*). To investigate whether this spontaneous secretion had some physiological role in the control of HIV replication by macrophages, MCP-1 production was abrogated by using specific polyclonal and monoclonal antibodies. Infection of 7 day-cultured monocytes with the monocytotropic strain BaL of HIV-1 in the absence or in the continuous presence of antibodies to MCP-1 resulted in a markedly lower level of p24 release in cultures where the expression of MCP-1 was neutralized with respect to control cultures. Time-course experiments in which p24 release was monitored at 21, 28 and 35 days post-infection revealed that maximal inhibition of viral replication occurred at the peak of viral replication, temporally correlated with the virus-induced MCP-1 up-modulation and remained quite stable at later time points. Similar results were obtained with macrophages maintained in the presence of antibody to MCP-1 and infected with the HIV-1 strain ADA and a primary isolate (R5X4). In contrast, no relevant effects on HIV replication were detected in the presence of antibody to MCP-3. No correlation was found between the extent of viral replication inhibition observed following MCP-1 neutralization and the level of secretion of some cytokines, such as TNF-α and IL-6, involved in the modulation of HIV-1 replication. Experiments are in progress to characterize the step(s) of the viral life cycle which are blocked in the absence of MCP-1 and to determine the role of other soluble factors whose expression might be modulated following MCP-1 neutralization. Altogether these results suggest that MCP-1, either endogenously expressed or induced by viral infection, may play a role in the control of HIV replication in macrophages.

Contract number 40C/H

HIV-1 GP120 STIMULATES THE PRODUCTION OF β-CHEMOKINES IN HUMAN PERIPHERAL BLOOD MONOCYTES THROUGH A CD4-INDEPENDENT MECHANISM

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The present study was designed to evaluate the effect of the HIV-1 gp120 envelope glycoprotein on the expression of β-chemokines in cultured monocytes/macrophages. Treatment of either freshly isolated 1-day monocytes or 7-day monocyte-derived macrophages (MDM) with recombinant HIV-1 gp120 protein resulted in a specific and dose-dependent enhancement of secretion of MCP-1, MIP-1β and RANTES as well as in a clear-cut increase in the accumulation of the corresponding transcripts. The expression of these mRNA was increased, but not superinduced, in the presence of cycloheximide. β-chemokine secretion was also induced after exposure of monocyte cultures to AT-2 inactivated R5 and X4 HIV-1 strains, retaining conformational and functional integrity of envelope proteins. The gp120-mediated effect was independent of its interaction with CD4, as preincubation with soluble CD4 did not abrogate β-chemokine induction. Moreover, triggering of CD4 receptor by a specific antibody did not result in any β-chemokine secretion. Interestingly, engagement of CCR5 and CXCR4 receptors by specific antibodies as well as treatment with CCR5 and CXCR4 ligands induced β-chemokine secretion. On the whole, these results indicate that HIV-1 stimulates monocytes/macrophages to produce β-chemokines by a specific interaction of gp120 with HIV-1 co-receptors on the cell membrane. The expression of these related polypeptides may represent an important cellular response for regulating both the extent of viral infection and the recruitment of immune cells.

Contract number: 40C/H

IDENTIFICATION OF A NOVEL POSTTRANSCRIPTIONAL REGULATORY ELEMENT USING A MUTATED HIV-1 DNA PROVIRAL CLONE AS A MOLECULAR TRAP

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Retroviruses use posttranscriptional control to ensure export and expression of their unspliced mRNA in the cytoplasm. Lentiviruses export their RNA using the CRM1 export pathway, whereas type D retroviruses use CTE, an RNA element binding to the cellular mRNA export factor TAP/NXF1.
HIV and the other lentiviruses utilize the essential viral protein Rev, which binds to RRE RNA, to export their unspliced and partially spliced mRNAs from the nucleus. In order to establish a method for the rapid isolation of posttranscriptional regulatory elements by selecting for rescue of virus replication, we have used a *rev* and RRE defective HIV-1 molecular clone in complementation experiments to identify new posttranscriptional control elements from the mammalian genome. Viruses rescued by this method contained a novel element from the mouse genome, with homology to mouse and Syrian hamster intracisternal A particle retroelements (IAP). A functional element was contained within a 247-nucleotide fragment named RNA Transport Element (RTE), which was able to promote replication of the Rev/RRE defective HIV-1 in both human lymphoid cell lines and primary lymphocytes, demonstrating its potent posttranscriptional function. RTE is predicted to form extensive secondary structure and shows no homology to other identified posttranscriptional control elements. RTE was functional in many cell types, indicating that the cellular factors that recognize RTE are widely expressed and evolutionarily conserved. Leptomycin B treatment did not affect RTE-mediated expression, indicating that RTE-mediated RNA transport was CRM1-independent. In addition, RTE did not bind specifically to the mRNA export factor TAP/NXF1. Since CRM1 and TAP/NXF1 are critical export receptors associated with the two recognized mRNA export pathways, these results suggest that RTE functions via a distinct export mechanism. Taken together, our results identify a novel posttranscriptional control element that uses a conserved cellular export mechanism and provide a method for the rapid isolation of additional functional posttranscriptional regulatory elements from the mammalian genome.

Nappi F, and Pavlakis GN. Generation of an HIV-1 based vector for the posttranscriptional control elements from the mammalian genome. In "The Fourth European Conference on Experimental AIDS Research". Bologna, Monduzzi Editore, 123-128, 1999.

“Posttranscriptional Control Elements from the Mouse Genome Useful for Increased Gene Expression” Pavlakis G.N and F. Nappi. Submitted Patent Application.

Nappi F, Schneider R, Zolotukhin A, Bear J, Michalowski D, Felber BK, and Pavlakis GN. Identification of a novel posttranscriptional regulatory element using a *rev* and RRE mutated HIV-1 DNA proviral clone as a molecular trap. Submitted.

Numero di collaborazione: 40C/I

EFFECT OF HIV-1 ON MATURATION AND FUNCTION OF DENDRITIC CELLS DERIVED FROM CD34+ HEMATOPOIETIC PROGENITORS OR MONOCYTIC PRECURSORS.

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Dendritic cells (DC) are effective antigen presenting cells involved in the pathogenesis of human immunodeficiency virus (HIV) infection. It is now largely accepted that DC can be entered by macrophage tropic (R5) and lymphotropic (X4) HIV, although it is still unclear if and at which stage of maturation these cells support viral replication. The aim of this research project is to investigate the effect of HIV on cells able to generate DC progeny: CD34+ hematopoietic progenitor cells (HPCs) and monocytic precursors. Thus, after purification CD34+ cells were challenged with either R5 (BaL) or X4 (NL4-3) HIV strains and then induced to unilineage DC differentiation; alternatively, monocytic precursors obtained from CD34+ were infected and then switched to DC.
At day 10-14 of culture cells viability of DC from infected CD34+ was more than 50% reduced although very low levels of p24 HIV antigen were measured in supernatants from either R5 or X4 treated cells. This is in accordance with the in situ hybridization results demonstrating that only about 1% of cells were positive to HIV mRNA. Phenotypical analysis of surface markers of infected counterpart revealed downmodulation of CD1a expression thus suggesting an impaired maturation. In addition both the reduced expression of CD40 costimulatory molecule and a reduced stimulatory activity in Mixed Lymphocyte Reaction (MLR) indicated a strong impairement in the antigen presenting capacity. The observation that a minority of DC derived from CD34+ was infected with HIV in spite of a reduced T-cell stimulation activity and a consistent cell death, suggests an indirect role of the virus possibly mediated by a deregulated production of cytokines.
On the other hand, DC derived from R5 and X4 infected monocytic precursors efficiently replicated HIV as demonstrated by p24 analysis although cell viability was only slightly reduced. Even in this type of DC culture a significative reduction of CD40 molecule was coupled with an impaired antigen presenting capacity observed in MLR experiments.
Taken together our results demonstrate that DC derived from HIV treated cells, either HPCs or monocytic precursors, show a significative reduction in their antigen presenting capacity.

N° Accordo Collaborazione 40C/J

SIV AND LYMPHOMAGENESIS IN THE MACACA ANIMAL MODEL: CHRONIC NON-CYTOLITIC SIV INFECTION OF SIMIAN B CELL LINE UPREGULATES THE EXPRESSION OF CD23 AND CD40 CELL SURFACE MARKERS.

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SIV as well as HIV retroviruses induce in vivo polyclonal B cell activation and have been associated with the appearance of lymphomas, but their pathogenetic role in the development of the lymphoproliferative disease is not yet understood. We have already described the presence of SIV in tumor cells of oligoclonal B- and T-cell lymphomas in SIV-infected cynomolgus monkeys (*Maggiorella et al., Blood, 1998*). To elucidate the possible role of SIV in the development of lymphoproliferative disorders, two simian lymphoblastoid B cell lines (SL-691 and SL-P1) were established in vitro to investigate: 1) the genotypic and phenotypic profile of these cells, 2) their susceptibility to SIV infection, and 3) the phenotipic modifications possibly associated with SIV infection. Both cell lines had a detectable level of CD4 mRNA (RT-PCR); expressed typical B cell lineage markers, such as CD20; could be discriminated by the differential expression of CD23, CD40, CD28, κ- and λ-chains; were coinfected with the *Macaca fascicularis* Herpes virus (HVMF-1) and with a simian type D retrovirus (SRV-2). Of importance, SL-691 but not SL-P1 cells were susceptible to chronic non-cytolitic, highly productive SIV infection. The differential susceptibility to SIV infection might be not attributed to different expression of CXCR4 (present on both cell lines as determined by cytofluorimetric analysis) or of CCR5 (absent on both cell lines). Of note, similarly to EBV infection/transformation in human cells, SIV infection upregulated the expression of CD23 and CD40 cell surface markers whereas CD20 expression, that disappeared in SL-691 cells, was maintained in the SIV-infected counterpart (*Titti et al., submitted*). Our results, even if they do not prove the transforming properties of SIV because of the simultaneous presence of other coinfecting viruses with known transforming activity, nevertheless shows that SIV or its products (such as tat, nef or env) induce relevant phenotypic changes on a simian B cells, add new data on the role of SIV in the development of lymphoproliferative disease.

Numero di collaborazione : 40C/K

WHOLE SIV VIRUS AS A VACCINE APPROACH AGAINST AIDS

a) Vaccination with live attenuated virus..

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The aim of this study, was to investigate whether a SIVmac251/C8 virus carrying an in frame 12 bp deletion in the nef gene (Δ-nef), is able to establish and to maintain a protective status following challenges with pathogenic SIVs, and heterologous chimeric SIV/HIV virus (SHIV). The infection of cynomolgus monkeys with the C8 attenuated variant of the SIVmac251/32H virus established a persistent but attenuated infection associated with an extremely low viral burden in PBMCs. At 40 weeks after the primary infection, the monkeys were challenged with SIVmac251/BK28 grown on macaque cells (50 MID_{50}) (*Titti et al., J. Gen. Vir., 1997*) followed by a second challenge (at 103 weeks) with a more pathogenic monkey-grown SIVmac251/32Hspl (50 MID_{50}). All animals resulted protected as judged by the absence of isolation of the challenging virus, anamnestic response and diagnostic PCR. Of importance, stimulation with tetanus toxoid, although capable of inducing specific humoral and T cell proliferative responses, failed to induce a reactivation either of the C8 virus or the challenging virus (*Sernicola et al., Virology, 1999*). At approximately 4 years after infection with the C8 virus, the same monkeys were challenged with the highly pathogenic SHIV89.6P. Since there was no evidence of an active replication of the challenging virus, C8-vaccinated monkeys did result again protected (*Titti et al., submitted*). The persistent but attenuated infection established by the C8 virus a) did not abrogate the capability of the protected monkeys to respond to recall antigens and to SIV antigens, b) did confer a long lasting protection which seems to be independent from the pathogenic potential of the challenging and from a viral interference phenomenon. This protection, in addition to other cell-mediated responses, seems to be mediated by the ability of the C8 virus to mount and to maintain a TH1-like immune responses and an antiviral activity mediated by $CD8^+$ cells (*Goletti et al., submitted*).

b) Pentavalent MVA-SIV vaccine in cynomolgus monkeys: suppression of primary infection and effects of the vaccination on the dynamic of viral replication in cynomolgus monkeys.

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To overcome ethical issues possibly associated to the use of a live attenuated virus, an alternative vaccine candidate is necessary that should mimic the protective efficacy of attenuated virus without inducing potentially dangerous chronic infection in humans. Within the framework of an European Collaborative study, the protective efficacy of a multi-component vaccination with Modified Vaccine Ankara (rMVA) constructs expressing structural (*gag/pol*, *env*) and regulatory (*tat, rev*, *nef*) genes of SIVmac251/J5 was investigated in cynomolgus monkeys. rMVA-J5 vaccination induced antibodies against Gag, Tat, Rev and Env but not

aganist Nef. The antigen-specific *in vitro* proliferative responses against whole SIV, were moderate and more pronounced after the third dose of antigens. Two months after the last boost, these monkeys along with four naive monkeys were challenged i.v. with 50 MID_{50} of SIVmac251 grown on macaque cells. All control monkeys were infected and seroconverted by week 4-8 and their proliferative responses to SIV were moderate. In contrast, challenge induced a strong anamnestic response in rMVA-J5 vaccinated monkeys, with a sharp increase of both humoral (Ab to Gag, Rev, Tat and Env but not to Nef) and proliferative responses at 2 wpc as compared with control monkeys. Although all vaccinated monkeys were infected, nevertheless vaccination with rMVA-J5 did result in a statistically significant control of viral replication during the acute and late phase of infection. These results indicate that rMVA-J5 vaccination was able to control viral replication even if a moderate activation of the immune system was achieved following this vaccination schedule (*Negri et al., J. Med. Prim., in press; Baroncelli et al., submitted*). In order to obtain a more effective and stable delay or control of viral replication further studies based on 2nd generation MVA vectors or on a combined approach with different vectors are necessary.

Numero di Collaborazione: 40C/K

HIV-1 RECOMBINANT NEF PROMOTES PHENOTYPIC AND FUNCTIONAL MATURATION OF HUMAN DENDRITIC CELLS

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HIV-1 Nef protein is required for efficient virus replication *in vivo* and displays different biological activity *in vitro*. Therefore, Nef plays an important role in HIV-1 pathogenesis and is involved in the impairment of the immune response. We have previously demonstrated that recombinant Nef (rNef) activates normal human T cells and up-regulates IL-15 production entering monocytes.
In vitro data and *in vivo* studies support the concept that dendritic cells (DCs) play a central role in the HIV-1 primary infection as well as in its evolution. DCs are among the primary cells infected and function as an important reservoir for the virus and source of viral replication because of their capacity to accumulate virions intracellularly, their relative resistance to the cytophatic effect of HIV-1, and because of their function as antigen-presenting cells.
Because phenotypic and functional maturation may play an important role in the handling of HIV-1, we examined the changes in the monocyte derived DCs induced by rNef in order to define its role in the immunophagenesis of AIDS.
We have found that rNef up-regulates the expression of immature DCs (iDCs) surface molecules known to be critical for their APC function. These molecules include CD1a and HLA-DR involved in the presentation of lipidic and antigenic peptides to CD4+ T cells respectively; CD80 (B7-1) and CD86 (B7-2) costimulatory molecules acting as ligands for CD28 and CD154 expressed at the T cell membrane; CD40 which transduces activation signals; CD83 a maturation antigen for DCs; CXCR4 a constitutive chemokine receptor and HIV-1 coreceptor. On the other hand, rNef down-regulates surface expression of HLA-ABC molecules involved in the presentation of antigenic peptides to CD8+ T cells and molecules involved in antigen capture such as MR and CD32 (FcγRII). The functional consequences of rNef treatment of iDCs are a decrease in endocytic and phagocytic activities and an increase in cytokine and chemokine production as well as in their stimulatory capacity in autologous/allogeneic MLR.
These results indicate that rNef induces a coordinate series of phenotypic and functional changes promoting iDCs differentiation performing them more competent APCs. Indeed, Nef-matured DC, enhancing lymphocyte recruitment and activation, could promote virus dissemination.

XI AIDS Project. Grant N. 40 C/L.

SHORT SYNTHETIC PEPTIDES DERIVED FROM VIRAL PROTEINS COMPETE WITH HIV gp120 FOR THE BINDING TO CD4 RECEPTORS

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In the complex mechanism of adhesion, internalization, and infection of cells by HIV-1 viral particles, a determinant role is played by the viral envelope glycoprotein gp120, which binds to CD4 receptors of T cells and monocytes
We tested the ability of a panel of 7-to-12 residue synthetic peptides, selected from the region 414-434 of the HIV-1 gp120, to inhibit the binding of the viral protein to CD4 receptors of cultured human lymphoid cells. The assay was based on the observation that the binding of gp120 to the receptors interferes with the binding of a specific anti-CD4 monoclonal antibody, as a result of the masking of the antibody epitope: thus, we tested whether preincubation of cells with the peptides before gp120 addition might restore the recognition of the CD4 molecule by the antibody. High expression of CD4 receptors was thus assumed as indication that the binding of the viral protein had been inhibited. Maximum activity was displayed by a 9-residue peptide located near the amino terminal end of the 414-434 fragment. In addition, several fragments deduced from other viral proteins, possessing partial amino acid sequence homology with the HIV gp120 fragment, exhibited a similar type of interaction with the CD4 receptor. All active peptides contain the Cys residue (pos. 423 of gp120). This residue is essential, although not sufficient, for inhibiting gp120 binding, as few other amino acid residues within the fragment play a complementary role in increasing or decreasing the inhibitory ability.

XI AIDS Project. Grant N. 40 C/L.

The National research program on AIDS

(ISS research projects)

Project

DEVELOPMENT OF A VACCINE AGAINST AIDS

Scientific Coordinator: **Barbara ENSOLI**

Projects financed N° 3

USE OF THE HU-PBL-SCID MOUSE MODEL FOR THE EVALUATION OF DENDRITIC CELL-BASED VACCINATION STRATEGIES AGAINST HIV-1: ROLE OF TYPE I IFN

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The development of practical animal models for the *in vivo* testing of human vaccines is an important issue in vaccine research. SCID mice reconstituted with human cells would represent a unique model for testing the *in vivo* interactions of human vaccines with human immune cells and the resulting protection from human pathogens. However, the use of hu-PBL-SCID mice for active immunisation studies has been hampered by the limited human immune repertoire, which may be shaped by murine environment and become poorly responsive to immunogens. We postulated that an efficient immune response could be elicited in hu-PBL-SCID mice, provided that appropriate antigen presentation could occur *in vivo* as a result of transfusion of autologous APCs at early times after reconstitution. We found that the best approach to generate a human primary antibody response in this model was when the hu-PBL-SCID-mice were immunised with autologous antigen-pulsed dendritic cells (DC) few days after reconstitution. We compared monocyte-derived DC generated in the presence of IL-4/GM-CSF (IL-4-DC) with those obtained after treatment with type I IFN and GM-CSF (IFN-DC) for their capability to induce a primary immune response after an *in vitro* pulse with HIV inactivated by AT2 (Aldrithiol-2) and subsequent transfer in hu-PBL-SCID mice. IFN-DC proved to be far superior with respect to IL-4-DC in inducing a human primary response against HIV-1, as evaluated by the detection of specific human antibodies against the all spectrum of viral proteins. At 7 days after primary immunisation, human antibodies were mostly IgM, while HIV-1-specific IgG1 antibodies were detected after boosting (J. Exp. Med. 191:1777-88, 2000). Notably, the antibodies detected in the sera of mice injected with DC generated in the presence of IFN exhibited a remarkable neutralising activity against HIV-1. When these immunised hu-PBL-SCID mice were infected with HIV-1, some reduction in the extent of virus infection was observed. Of interest, IFN-DC showed some characteristics of mature DC, as indicated by up-regulation of CD83 expression, production of IL-15 and MIP-3β, and enhanced expression of certain chemokine receptors (especially CCR7), which was associated with a pronounced capability to migrate in response to chemokine stimulation. We conclude that: i) type I IFN represents a powerful adjuvant in certain vaccination strategies; ii) the hu-PBL-SCID mouse model can be successfully used for testing DC-based immunisation strategies against HIV and, because of the versatility and prospects for implementation, could represent a valuable preclinical model for testing some human vaccines.

Contract number: 45C/A

A BIFUNCTIONAL INTRANASAL VACCINE CONTAINING HIV-1 TAT AND MP65 FROM *C. ALBICANS* ELICITS SYSTEMIC AND MUCOSAL IMMUNE RESPONSES IN MICE

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HIV-1 Tat is a candidate antigen for a human anti-HIV vaccine. We studied the immunogenicity of native HIV-1 Tat when delivered through the mucosal route and we tested the feasibility of a mucosal vaccine containing Tat and the protein MP65 from *C. albicans*. Following intranasal administration of four doses of Tat, C57BL/6 and BALB/c mice showed no Tat-specific serum antibody responses. Administration of Tat plus the heat-labile enterotoxin of *E.coli* (LT) as a mucosal adjuvant, resulted in significantly high Tat-specific serum IgG as well as intestinal and vaginal IgA titers only in Balb/c mice. The lack of immunogenicity in C57BL/6 mice was also observed when Tat was given subcutaneously emulsified in CFA or adsorbed to alum. The recently reported immunosuppression mediated by Tat prompted us to test whether intranasally administered Tat protein would be immunosuppressive toward the mannoprotein MP65 of *C. albicans*. Interestingly, BALB/c mice receiving four doses of an intranasal vaccine containing Tat, MP65 and LT showed high levels of Tat-specific and MP65-specific antibodies in both serum and secretions. Furthermore, the serum IgG titers to MP65 in these mice were similar to those obtained in mice immunized with MP65 in the absence of Tat. The lack of any immunosuppressive effect was also found when a nontoxic derivative of LT, LT-R72, was used as an adjuvant. Our results show that HIV-1 Tat protein is immunogenic when delivered through a mucosal route together with specific mucosal adjuvants. Further, since intranasal delivery of HIV-1 Tat does not interfere with the immune response to MP65, the development of a divalent mucosal vaccine against both HIV and *C. albicans* seems feasible.

Fasc. 45C/B

CONTROL OF VIRAL REPLICATION AND DISEASE ONSET IN CYNOMOLGUS MONKEYS BY HIV-1 TAT PROTEIN OR DNA VACCINE

Cafaro A.,[(a)] Maggiorella M.T.,[(a)] Baroncelli S.,[(a)] Caputo A.,[(b)] Borsetti A.,[(a)] Fanales-Belasio E.,[(a)] Moretti S.,[(a)] Negri D.M.R.,[(a)] Macchia I.,[(a)] Michelini Z.,[(a)] Tripiciano A.,[(a)] Ridolfi B.,[(a)] Sernicola L.,[(a)] Belli R.,[(a)] Comini A.,[(a)] Ten Haft P.,[(c)] Buttò S.,[(a)] Verani P.,[(a)] Titti F.,[(a)] and Ensoli B.[(a)]

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The Tat protein of HIV is produced very early after infection, plays a key role in the virus life cycle and in AIDS pathogenesis, is immunogenic and well conserved among all virus clades. Notably, a Tat-specific immune response correlates with nonprogression to AIDS. We have recently shown that vaccination of cynomolgus monkeys (*Macaca fascicularis*) with biologically active Tat of HIV is safe and induces both humoral and cellular immune responses (including CD8+ CTLs). More importantly, the anti-Tat vaccine blocked primary infection with the simian/human immunodeficiency virus (SHIV) 89.6P and prevented the CD4 T cell decline and disease onset in cynomolgus monkeys (*Cafaro et al., Nat. Med. 1999*). No signs of virus replication were found in 5 out of 7 vaccinated macaques during the 2 years of follow up. Since the inoculated virus (derived from rhesus or from cynomolgus macaques) is shown to be highly pathogenic in cynomolgus macaques (*Cafaro et al, J. Med. Primatol., 2000*), the results indicate efficacy of Tat vaccination in protection against highly pathogenic virus challenge. Protection correlated with the presence of CD8+ CTLs at pre-challenge time and of CD8-mediated antiviral activity at post-challenge, indicating the importance of CD8+ cells in controlling the infection, in the contest of both the natural and adaptive immune responses (*Cafaro et al, Nat. Med., 1999, Ensoli and Cafaro, AIDS Clin. Rev. 2000/2001, 2000, Goletti et al, in preparation*). These results have been recently confirmed in a new protocol in which 4 monkeys were immunized IM with a vector expressing *tat* DNA and rich in unmethylated CpG sequences. All vaccinated monkeys successfully controlled the infection after IV challenge with 10 MID_{50} of SHIV89.6P (*Cafaro et al, Vaccine, in press*). Again, protection correlated with the CD8-mediated responses, since anti-Tat antibodies were not induced by this type of vaccination. To our knowledge this is the first report of an immunogen delivered in a CpG-rich plasmid to exploit its intrinsic adjuvanticity. In order to detect residual virus hidden in resting cells, all the monkeys from both protocols were boosted twice with the anamnestic antigen tetanus toxoid (TT), a stimulus known to activate the immune system and increase viral expression. All monkeys responded to the TT boost, as indicated by increased antibody titers and lymphoproliferative response to TT. However, no virus was detected by QC-RT-PCR in any of the protected monkeys either in the plasma or in lymph node, whereas increased plasma viremia was readily detected in three of the infected monkeys (*Maggiorella et al, in preparation*).

Based on these results 2 new immunization studies were conducted, one with the Tat protein given intradermally (7 monkeys), and one with the *tat* DNA injected intramuscularly (12 monkeys). In this latter protocol the animals were divided into 4 groups and inoculated with either pCV-*tat* (group A) or pCV-tat-cys22 (a transdominant negative mutant lacking of transactivating activity) (group B), or the empty plasmid vector pCV-0 rich in CpG motifs unmethylated (group C) or purposely methylated (group D) to abolish the adjuvanticity of the CpG sequences and control for their impact on the immune response and protection. In both protocols the safety and the induction of Th1 responses and CD8 responses were confirmed and the animals are scheduled for challenge in the next months to evaluate the efficacy. Together, these results indicate that a Tat-based vaccine is a promising candidate for vaccination in humans. Therefore, both preventive and therapeutic phase I clinical trials are currently being organized ad expected to be started in Italy within the year 2001.

Numbero di collaborazione: 45C/C

CORRELATION OF $CD8^+$ T CELL-MEDIATED NONCYTOLYTIC ANTIVIRAL ACTIVITY WITH PROTECTION AGAINST SHIV89.6P CHALLENGE IN TAT PROTEIN OR DNA-VACCINATED MONKEYS AND STRATEGIES FOR ITS IDENTIFICATION.

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Vaccination of cynomolgus monkeys with the HIV-1 Tat protein or Tat expressing plasmid DNA blocked infection with the highly pathogenic simian/human immunodeficiency virus (SHIV)-89.6P and prevented the CD4 T cell decline in 9 of 12 vaccinated animals (*Cafaro et al., Nat. Med. 1999; Cafaro et al., J. Med. Primatol. 2000; Cafaro et al., Vaccine in press*). In addition to Tat-specific CTLs, all the protected monkeys also showed a potent and stable anti-CD3-induced CD8+-mediated noncytolytic antiviral (CD8-NCA) activity that suppressed virus replication in autologous CD-8 depleted peripheral blood mononuclear cells (PBMC) upon SHIV89.6P infection. Conversely, CD8-NCA activity was absent in 4 of 5 infected animals early after challenge but was regained over time in correspondence with the decline of plasma viremia. Similarly, the same level of CD8-NCA activity detected in the protected monkeys was found in 21/27 (77.8%) of uninfected naïve animals. This CD8-NCA activity was mediated by soluble factor(s) distinct from β-chemokines, required cell-to-cell contect for prolonged virus inhibition and did not inhibit T cell proliferation. These results suggest that the CD8-NCA activity belongs to the innate immunity, is present in the majority of naïve animals and is maintained in the Tat vaccinated and protected monkeys, thus contributing to the establishment and maintenance of the protective status conferred by the Tat vaccine (*Goletti et al, in preparation*).
In order to evaluate the possibility that the Tat protein might induce this CD8-NCA activity, experiments aimed to identify the presence of CD8-NCA in CD8+ lymphocytes derived from PBMC of HIV-1-negative healthy subjects after anti-CD3 stimulation in the presence or absence of Tat are being performed. To facilitate the identification of the soluble factor(s) involved in the CD8-NCA activity, mRNA derived from CD8+ T cells will be screened on an array of 3,500 known genes, among which will be identified transcripts of the soluble factors possibly contributing to the CD8-NCA. Candidate genes will be expressed in eucaryotic cells and characterized for their inhibitory activity against HIV-1 replication. In addition, in order to evaluate whether the CD8-NCA activity acts at the transcriptional level on the HIV-1 LTR promoter, the inhibitory activity against HIV-1 replication will be further dissected utilizing several HIV-1 LTR mutants already prepared. Preliminary data indicate that supernatants derived from CD8+ lymphocytes derived from PBMC of HIV-1-negative healthy subjects after anti-CD3 stimulation produce soluble factors able to diminish both the basal and the Tat-induced activity of the HIV-1 LTR promoter.

Numero di collaborazione: 45C/C

PRECLINICAL VACCINE TRIALS IN NONHUMAN PRIMATES WITH THE HIV-1 TAT PROTEIN OR DNA VACCINE ALONE OR COMBINED WITH OTHER HIV/SIV ANTIGENS AND STUDIES OF NOVEL DELIVERY SYSTEMS.

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We have recently shown (*Cafaro et al., Nat. Med., 1999; Cafaro et al., Vaccine, in press*) that vaccination of cynomolgus monkeys (*Macaca fascicularis*) with a biologically active Tat protein or tat DNA of HIV-1 is safe, induces both humoral and cellular immune responses (including CD8+ CTLs) and blocks primary infection after IV challenge with 10 MID_{50} of the simian/human highly pathogenic immunodeficiency virus (SHIV) 89.6P (*Cafaro et al., J. Med. Primatol., 2000*), preventing the CD4 T cell decline and disease onset. In particular, no signs of virus replication were found in 9 out of the 12 vaccinated macaques with either Tat protein or DNA during the 2 years of follow up. Protection correlated with the presence of CD8+ CTLs at pre-challenge and with a CD8-mediated noncytolitic antiviral activity (NCA) at post-challenge, indicating the importance of CD8+ cells in controlling the infection (*Cafaro et al., Nat. Med., 1999; Ensoli and Cafaro, AIDS Clin. Rev. 2000/2001, 2000; Cafaro et al., Vaccine, in press; Goletti et al., in preparation*).
Within the recently established "Italian concerted Action for the development of a vaccine against HIV/AIDS" (ICAV), several projects for new vaccination strategies have started and will be soon ready for monkey studies. These include studies on novel delivery systems of Tat, such as the use of nanoparticles to deliver either Tat protein or tat DNA, and the identification of new approaches for the induction of mucosal immunity, such as the use of viral and bacterial vectors expressing HIV-1 and SIV regulatory and structural genes, and of new mucosal adjuvants.
In addition, a number of collaborative studies are ongoing in Europe, USA and Australia. Specifically, two tat-DNA trials are currently ongoing in The Netherlands in collaboration with Jonathan Heeney (BPRC, Rijswijk, The Netherlands) with the aims of a) reproducing the DNA protection data, b) to compare short (4 immunizations) versus long (9 immunizations) vaccination schedules, and c) to compare vaccination with tat-DNA alone (4 rhesus macaques injected intramuscularly with 0.5 mg of HIV-1 tat-DNA) versus combination with the DNA of HIV-1 Env and SIV Gag/Pol (same route and doses). Again, the safety and immunogenicity data have been reproduced and an intravenous challenge with 20 MID50 of the pathogenic SHIV89.6P is scheduled early in 2001.
As part of the “Italy-USA inter-institutes scientific collaboration for the development of an HIV vaccine” a collaborative study with Marjorie Robert-Guroff (NIH, NCI, Bethesda, USA) has started with the aim of evaluating the safety, immunogenicity and efficacy of a prime-boost approach for mucosal vaccination of rhesus macaques with HIV-1 Tat alone or in association with HIV-1 Env delivered mucosally in an adenovirus vector. This project will also compare several prime-boost regimens in order to optimize the vaccine strategy. The monkeys will be challenged intrarectally with 50 MID50 of the pathogenic SHIVSF162, and the most promising approach successively reproduced with a larger number of monkeys and the SIV-Gag antigen will also be included.
Finally, a collaborative study with Stephen Kent (University of Melbourne, Australia) has recently started with the aim of evaluating a prime-boost approach for mucosal vaccination in pigtailed macaques with Tat alone or associated with Rev and the structural antigens Gag and Pol.

Numero di collaborazione: 45C/C

UPTAKE AND FUNCTION OF TAT ON MONOCYTE-DERIVED DENDRITIC CELLS AND HUMAN UMBELICAL VEIN ENDOTHELIAL CELLS

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HIV-1 Tat is a regulatory protein essential for HIV gene expression and virus replication that has been shown to be released in the extracellular fluid and be taken up by neighbour cells (*Ensoli et al., Nature 1990, J. Virol. 1993; Chang et al., AIDS 1997*). Immune response to Tat arises early during the viral infection and has been associated with a slower disease progression. Vaccination trials with Tat protein or DNA have been shown to induce protection of monkeys challenged with a pathogenic virus (*Cafaro et al., Nat. Med. 1999, Vaccine, in press*). Tat has also being shown to have profound effects on endothelial cells (*Barillari et al., Blood 1999, J. Immunol. 1999*). Most of these effects may be due to a direct action on target cells, since Tat recognizes surface structures as integrins and negatively charged proteoglicans, present on EC and other cells with antigen presenting function. Based on this evidence, we investigated the capacity of activated human umbelical vein endothelial cells (HUVEC) and monocyte-derived dendritic cells (MDDC) to specifically take up recombinant Tat protein, as assessed by intracellular staining with polyclonal anti-Tat antibody and flow cytometry analysis. In addition, we studied the effects of Tat on the induction of maturation, cytokines production and antigen presenting function of MDDC. We found that HUVEC, upon treatment with proinflammatory cytokines, were capable to efficiently take up the Tat protein at doses ranging from 0.1 to 10,000 ng/ml and the uptake peaked after 5-10 minutes of culture. A very similar pattern and kinetic of Tat uptake was found for MDDC. Tat was mostly intracellular since the staining was markedly weaker in cells not permeabilized. Oxidation of the Tat protein, by exposure to air and light, or pre-treatment with heparin markedly impaired its uptake by MDDC. Further, treatment with Tat promoted maturation of immature MDDC in a dose-dependent fashion as indicated by the increased expression, in flow cytometry, of the surface molecules HLA-ABC, HLA-DR, CD40, CD80, CD83 and CD86 and by the enhanced secretion of the cytokines IL-12 and TNF-α and the β-chemokines RANTES, MIP-1α and MIP-1β. Accordingly, the antigen presenting function of Tat-treated MDDC was increased in comparison to untreated cells as assessed by their capacity to induce the proliferative response of allogeneic or autologous lymphocytes primed for tetanus toxoid. Finally, MDDC treated with Tat also induced specific priming of naive lymphocytes (*Fanales et al., in preparation*).
Overall, these data suggest that the Tat protein is efficiently and promptly taken up by activated EC and by MDDC, and that immature MDDC, upon exposure to the protein, undergo a maturative process that augments their antigen presenting function.

Numero di collaborazione: 45C/C

The National research program on AIDS

(ISS research projects)

Project

OPPORTUNISTIC INFECTIONS AND TUBERCULOSIS

Scientific Coordinator: **Antonio CASSONE**

Projects financed N° 6

EXPRESSION OF P-GLYCOPROTEIN LIKE MOLECULES IN AZOLE RESISTANT STRAINS OF *CANDIDA ALBICANS* FROM AIDS PATIENTS

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Candida albicans is an opportunistic pathogen causing a variety of human infections, particularly in immunocompromised patients. Recent studies have reported an increasing incidence of resistance to fluconazole (FZ), one of the most effective anti-Candida drug, in clinical isolates of *C. albicans* from AIDS patients. Azole resistance in *C. albicans* may be due to several mechanisms, including augmentation of drug efflux mediated by molecular pumps belonging to two different families: the major facilitators and the ATP-binding cassette (ABC) transporters. It has recently been demonstrated that *C. albicans* possesses sequences with a high degree of homology with human MDR-1 gene coding for P-glycoprotein (P-gp), the well known drug transporter responsible of the multidrug resistance (MDR) in tumor cells.

On this basis, we have investigated the possible expression and intracellular localization of human P-gp in *C. albicans* strains showing a different sensitivity to FZ. In particular, control sensitive 3153 strain, highly resistant AIDS 68 strain (isolated from an AIDS patient) and *C. krusei* ATCC 6458 strain, whose resistance is not mediated by the presence of ABC transporters, were investigated mainly by immunoelectron microscopy, using a panel of monoclonal antibodies recognizing different epitopes of the P-gp molecule. Post-embedding immunolabeling revealed that the MAb MM4.17, which recognizes an external epitope on the apical part of the 4th loop of human P-gp, reacted with both 3153 and AIDS 68 strains of *C. albicans*. However, immunoreactivity of the FZ resistant AIDS 68 strain was significantly higher than that of the drug-sensitive 3153 strain. *C. krusei* cells were not reactive at all. The specificity of the MAb MM4.17 immunolabeling was confirmed by competitive inhibition assay performed by using phage clone particles capable of mimicking the MM4.17 epitope. In addition, other MAbs, directed against different P-gp epitopes, did not show any reactivity with *C. albicans* cells. To evaluate the functional activity of P-gp like molecules revealed by MAb MM4.17, the effect of two MDR inhibitors, cyclosporin A and verapamil, was assessed. Both inhibitors proved to strongly reduce the MICs for FZ and itraconazole of resistant AIDS 68 cells whereas they did not influence the MICs of either the sensitive 3153 strain of *C. albicans* or the ATCC 6458 strain of *C. krusei*.

In conclusion, our observations seem to suggest that drug transport mechanisms, structurally and functionally similar to those present in mammalian cells and mediated by P-glycoprotein, are present in *C. albicans*. This finding might contribute to better understand the mechanisms of azole resistance and to develop innovative therapeutical strategies against opportunistic infections in AIDS patients.

N° dell'Accordo di Collaborazione: 50C/A

DIFFERENTIAL CYTOKINE RESPONSE OF HUMAN MONOCYTES TO YEAST AND HYPHAL FORMS OF *CANDIDA ALBICANS* AND ITS RELATION TO THE BETA-1,6 GLUCAN OF THE FUNGAL CELL WALL.

Torosantucci A., Chiani P., Bromuro C., Cassone A.

Hyphae formation from yeast cells is a virulence trait which enables the human opportunistic pathogen *Candida albicans* to invade host tissues. Hyphal cells were shown to be much less efficient than yeast cells in stimulating production of macrophage inflammatory protein-1 alpha (MIP-1 alpha), MIP-1beta, interleukin-8 (IL-8), and particularly, monocyte chemotactic protein-1 (MCP-1) by human monocytes. This different stimulation did not depend on the monocyte inability to ingest the hyphae nor did it imply hyphal resistance to the extracellular killing by the monocytes. Purified hyphal and yeast cell walls reproduced the differences shown by the cells, and chemical-enzymatic dissection of cell wall components suggested cell wall beta-1,6, rather than beta-1,3 glucan was the main chemokine inducer. Coherently, immunofluorescence studies with an anti-beta-1,6 glucan serum that the surface expression of this polysaccharide was much lower on hyphae than on yeast cells. In addition, hyphal cells induced much less production of IL-12 (even in the presence of IFN-γ) as compared to yeast cells and LPS. For this cytokine, however, phagocytosis was an essential step in the production process by monocytes. After intraperitoneal inoculation of identical number of yeast and hyphal cells in mice, the latter induced in the peritoneal exudates an early chemokine response significantly lower than that generated by the former cells. This was coupled with the influx of a lower number of inflammatory cells in the peritoneal exudate. Overall, the data strongly suggest that the formation of hyphal filaments by *C. albicans* invading host tissues effectively minimizes cytokine induction, and, as such, it may facilitate fungal escape from host immunity.

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SPECIFIC CELLULAR RESPONSES RECOVERY TO MICROBIAL ANTIGENS IN HAART TREATED HIV+ PATIENTS BY PROTEASE INHIBITORS OR HIV REVERSE TRANSCRIPTASE NON NUCLEOSIDE INHIBITORS

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Recently, a (PI)- sparing regimen of HIV protease inhibitors has been adopted, matching a non-nucleoside (NN) inhibitor with virus reverse transcriptase nucleoside inhibitors. Several results show the efficacy of this alternative therapy, although, it is, in general, administered to subjects with less severe immunological deficit at baseline. However, few studies on functional recovery of the cellular response to antigens of opportunistic agents have been comparatively carried out in both therapeutic regimens. In particular, the proliferative T cell response to *Candida albicans*, a main opportunistic infectious agent susceptible to HAART and marker of the immunological recovery in the HIV+ subject, has not been comparatively investigated in the two regimens.

Hence, we compared the proliferative response to three major antigens of *C. albicans* (mannoprotein, enolase and protease) to that of tetanus toxoid and GP160, beside a policlonal stimulant, such as PHA, in two groups of 20 subjects under chronic treatment (>12 months) with IP-HAART or with NN-HAART, which were not different in age or sex. Furthermore, such a response has been assessed in other two groups of naive subjects 6 months after initiation of the two therapeutic regimens in comparison with the baseline responses.

In the chronic therapy treated groups the two regimens resulted in i) a not significant difference neither in the number of CD4+ cells (368+230 and 386+225, average + SD in PI and NN-HAART, respectively) nor in the viremia reduction (1369+3024 versus 634+2177, respectively); ii) a comparable positive cellular responses to at least one Candida antigen (11/20 in PI-HAART and 7/20 in NN-HAART) with a stimulation rate and a positive response range not significantly different (38 ; 5-136 versus 18 ; 8-107, average and range of stimulation index). Besides, both groups were similarly responsive (or non responsive) to the tetanus toxoid (2/20 and 1/20) or to the GP160 antigen (2/20 and 2/30), while almost all subjects were responsive to the polyclonal stimulus (52 ; 15-182 versus 34 ; 5-140, average and range, respectively).

In the naive subject groups, assessed at 6 months from the beginning of treatment, proliferative responses to Candida antigens were significantly more numerous (9/15 versus 4/15) in NN-HAART subjects, and these patients were proven to functionally recover to a higher degree with respect to both baseline values also again tetanus antigen.

No subject was found to be responsive to GP160 in any of the two groups. However, even almost all of them were responsive to PHA, at 6 months.

In conclusion, the two therapeutic regimens showed a substantially equivalent capability of inducing or non-inducing recovery and maintenance of cellular responses at a systemic level in response to microbial antigens. Altogether, our data support the efficacy of the PI sparing regimens as far as some of the functional immune responses are concerned.

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DEVELOPMENT OF NEW PROPHYLACTIC AND THERAPEUTIC VACCINES AGAINST CERVICAL CANCER USING PLANT-DERIVED ANTIGENS OF THE HUMAN PAPILLOMAVIRUS 16.

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Background: Human papillomaviruses(HPV) infect mucocutaneous surfaces where they induce a variety of pathologies ranging from benign warts to malignant cancers (cervical and anal cancer). A correlation has been established between invasive cervical cancer and HPV types 16 and 18, thus called "high risk" types.
High risk HPVs and HIV are sexually transmitted viruses and they have infection risk factors in common. In HIV seropositive women the probability to get infected by HPV is five times higher than in HIV seronegative ones. Due to the high incidence of genital intraepithelial neoplasia in HIV+ women, the invasive cervical cancer is one of the pathologies which define the AIDS. At the present time, prevention of high-risk HPV-associated cancer is performed by cervical cytology (Pap test), whereas surgery is the choice for therapy.
The close relationship between viral infection and cancer has stimulated efforts to develop prophylactic and therapeutic vaccines. The most promising candidate for the development of a prophylactic vaccine is represented by the "virus-like particles" (VLPs"), while for the therapeutic vaccine a good candidate is the E7 protein, the viral oncogene, expressed at high level in HPV-associated cancer and considered as "tumor-associated antigen".
Plants are currently being used as a cost effective and safe heterologous system for the expression of functional biomolecules. Plant-derived recombinant antigens of animal viruses or bacteria have already been successfully used as experimental vaccines, administrated either by parental inoculation or by oral administration.

Objective: To express L1, the coat protein that self assemble in VLPs when expressed in eukaryotic cells, and E7 viral proteins in plants and to evaluate their immunogenicity in mice to possibly develop HPV vaccines.

Methods: The HPV16 genes encoding L1 coat protein and E7 oncoprotein were cloned into the *Potato virus X* (PVX)-derived epichromosomal expression vector under the duplicated coat protein promoter of PVX. Infective RNA transcripts or DNA plasmids were used to infect *Nicotiana benthamiana* plant leaves. Expression of the proteins in inoculated or systemic leaves was assayed by RT-PCR, ELISA and immunoblotting using mouse polyclonal or monoclonal antibodies.
Mice (C5black/6) were inoculated with crude plants extracts, containing E7, with or without adjuvant (Quil-A, Freund's).

Results: In transient expression no recombinant L1 protein was detected in infected plants. Most probably the adjunctive HPV16 coat protein gene interfered with PVX replication. However a high amount of soluble E7 protein was produced in plants. The E7 in the crude leave extracts was inoculated in mice. Preliminary results indicate that this antigen induce circulating antibodies as the purified protein expressed in bacteria.

Accordo di Collaborazione N. 50C/C

EVIDENCE FOR HIV-DEPENDENT IMPAIRED FUNCTION OF APC IN HIV-DISCORDANT MONOZYGOTIC TWINS.

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The development of protective T cell responses is largely dependent on the function of professional antigen presenting cells (APC). Impairment of APC function would result in T cell unresponsiveness or apoptosis. HIV infection of APC progressively increases during the course of the disease. Because antigens from opportunistic or commensal organisms are more frequently encountered by the immune system in both normal and chronically HIV infected individuals, if HIV infection of APC is associated to an impaired function, it can be hypothesized that T cells specific for antigens from opportunistic or commensal organisms contribute more than other to the T cell depletion observed in AIDS patients. To verify this hypothesis we compared A) the APC function in a couple of monozygotic twins discordant for HIV infection and B) the frequency of T cell precursor for antigen from *C. albicans* and M. avium in comparison to a recall antigen (tetanus toxoid: TT) in a one-year follow-up. Antigen specific T cell clones (TCC) specific for *C. albicans*, M. avium, and TT were isolated from the healthy twin while lymphoblastoid B cell lines (LCL), monocytes (Mø) and dendritic cells (DC) were isolated from both the healthy and the HIV infected individual. The APC function of PBMC, Mø, LCL and DC from both individuals was evaluated in antigen specific T cell proliferation assays using PBMC and TCC from the healthy twin. The analysis of antigen specific T cell precursors was evaluated in ELISPOT assays at six-month intervals. We observed an impairment of the APC function of PBMC, Mø and DC in the HIV positive individual when using the antigen specific proliferation of both PBMC and TCC from the healthy donor as a read-out. On the other hand, the APC function of LCL was not impaired in the HIV positive subject. The reduced responsiveness could not be ascribed to HIV transmission from APC to lymphocytes. The precursor frequency of M. avium specific T cells was observed to decrease in the HIV infected as compared to the healthy twin in a one-year follow-up. These data confirm an APC impairment in HIV infected individuals and suggest an antigen specificity in the $CD4^+$ T cell depletion that characterizes late stages of HIV infections.

N°. dell'Accordo di Collaborazione: 50C/D

ETIOPATOGENIC, IMMUNOLOGIC AND THERAPEUTIC ASPECTS OF *MYCOBACTERIUM AVIUM* INFECTIONS IN AIDS PATIENTS

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Mycobacterium avium complex is an environmental group of mycobacteria that can cause disseminated infections in AIDS patients. Part of our study consisted in the identification and purification of *M.avium* antigens relevant for the study of the natural infection with the organism, or to be used as possible vaccines. Among the immunodominant antigens of *M.avium,* the gene codifying for the chaperonin 10 (Cpn10) has been cloned, sequenced and expressed in *E.coli* and the recombinant Cpn10 protein purified. By immunoelectronmicroscopy it was shown that this cytoplasmic antigen is also associated to the external surface of *M.avium* and may be released in the extracellular environment to stimulate the immune system. Intranasal immunization of BALB/c mice with Cpn10 and CpG oligodeoxynucleotides significantly inhibited *M.avium* multiplication in the spleens and lungs of mice challenged with 10^3 *M.avium* CFU by the same route. On the same line, we compared by metabolic labeling techniques and 2D-electrophoresis the pattern of proteins synthesized intracellularly by *M.avium* after phagocytosis by the macrophage-like cell line THP-1 with that produced during growth in media. The proteins overexpressed inside THP-1 cells were identified by MALDI-TOF (matrix assisted laser desorption/ionization time of flight) mass spectrometry with database search for known *M.tuberculosis* and *M.avium* proteins. Three major proteins were found to be overexpressed (>5 times) inside THP-1 cells: 1) beta-ketoacyl-ACP synthase (kasA), involved in elongation of meromycolate acid intermediates in the biosynthetic pathway of the mycolic acids; 2) acyl-CoA dehydrogenase (fadE2) and 3) electron transfer protein (fixA); both fadE2 and fixA proteins are involved in fatty acid oxidation. This indicates that the response of *M.avium* to Mø results in overexpression of proteins related to the biosynthesis and catabolism of fatty acids. These results can be useful to identify new molecular vaccinal targets.
The natural events of *M.avium* infections in AIDS patients have been studied in BALB/c and nude mice following exposure to low *M.avium* doses (20 CFU) inoculated through the intranasal, intradermal or intraperitoneal routes. Bacterial growth in organs was associated with a modulated increase of IL-12, IFN-γ, IL-4, $CD4^+$ T cells, B cells, $CD3/\gamma\delta^+$ T cells, IgG1 and IgG2a antibodies. All low-dose exposed BALB/c mice were significantly protected against a 10^5 CFU challenge dose but, in general, low bacterial burdens in organs and/or lymph nodes (as observed after immunization by the intranasal route) favored CD4 upregulated Th1 responses, while high bacterial burdens (after intradermal and intraperitoneal immunization) favored CD4 upregulated mixed Th1/Th2 responses. These observations indicate that exposure to low *M.avium* doses in the environement, as occurs in HIV^+ patients can trigger different immune responses depending on the route of infection.
As for the therapy, among eight regimens tested in the beige model of infection with *Mycobacterium celatum*, a new *Mycobacterium* species similar to *M.avium* recently isolated from AIDS patients, we found that, as previously observed by our group for *M.avium*, clarithromycin and azithromycin were the most active drugs in terms of CFU reduction and mutant selection after 56 days of observation.

N°. dell'Accordo di Collaborazione: 50C/E

GENERATION OF *CRYPTOSPORIDIUM* SPECIFIC HUMAN T CELL LINES

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Objectives: Evaluation of the cell mediated immune response to *C. parvum* and identification of the cytokine profile induced by this parasite. Selection of molecules of *C. parvum* that preferentially induce an immune response mediated by CD4+ and/or characterized for the production of IFN-γ.
Methods: *Cryptosporidium* crude extract (CCE), 5 anion exchange chromatography fractions (F1-F5) and two recombinant peptides (SA35 and SA40) have been used to generate *C. parvum* specific T cells lines. Peripheral blood mononuclear cells were separated by Ficoll-Paque gradient centrifugation, suspended in complete medium and dispensed in plates with antigen at the optimal concentration. Recombinant IL-2 has been added weekly to cultures. Fourteen days after, cultures have been re-stimulated with antigen, expanded and cloned.
Results: Twenty CCE specific T-cell lines have been generated from a donor. Furthermore, 18 specific T-cell lines have been generated from another donor using F1, SA35, and SA40 peptides. These T-cell lines consisted of an expanded CD3-CD4 T-helper populations with an high proportion of cells expressing CD45R0 and αβ forms of the TCR.
Conclusions: The generation of T cell lines and their derived clones shows that CCE, F1, F2, F3, SA35 and SA40 are powerful to induce a cellular response. Most of *Cryptosporidium* specific T cells are CD4+CD45RO+ lymphocytes, i.e., the typical phenotype of the memory T cell.

N°. dell'Accordo di Collaborazione fascicolo 50C/F

CHARACTERIZATION OF PROTEINS FROM *CRYPTOSPORIDIUM PARVUM* SPOROZOITES

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Objectives: To characterize *C. parvum* sporozoite antigens, which may play a role in host-parasite interaction; to identify antigens, which may be used in specific and sensitive immunodiagnosis assays; and to identify and characterize genes coding for parasite specific proteins.
Methods: New antigenic peptides of *C. parvum* from a sporozoite-derived expression library have been expressed after screening with rabbit hyperimmune-serum. Peptides have been expressed as histidine-tagged proteins in *E. coli* and purified by affinity chromatography. The peptides SA35 and SA40 (an immunogenic portion of GP 900) have been used to immunize BALB/c mice. MoAbs have been selected by their reactivity with the two peptides in ELISA. A genomic library has been constructed and screened with a radioactive DNA probe. The 5' rapid amplification of cDNA ends (RACE) method and Northern blot (Nb) analysis have been performed using total *C. parvum* RNA.
Results: Specific MoAbs have been obtained against SA35 and SA40. Western blot (Wb) analysis showed that a specific MoAb to SA35 recognized a 135 kDa protein on both sporozoite and oocyst lysates. The same MoAbs evidence selectively the localization of the native protein on one end of the sporozoite by an immunofluorescence assay. As expected, Wb analysis showed that the specific MoAb to SA40 recognizes an high molecular weight protein in oocyst lysates, which corresponds to the membrane associated GP 900 glycoprotein. Two positive clones of the genomic library have been identified using the SA35 probe. Approximately 2900 bp have been sequenced and an uninterrupted ORF of 2100 bp has been found. This gene has been named Cpa135. A 3000 bp mRNA has been identified by Nb hybridization using the Cpa 135 DNA as a probe. The transcription starting point of the Cpa135 gene was determined by the 5'RACE method.
Conclusions: Two sporozoite proteins of 135 kDa and 900 kDa of *C. parvum* have been characterized using chimeric peptides corresponding to the antigenic portion of the entire proteins. The 135 kDa protein is a new one of unknown function and its gene has been identified and characterized. The N-terminus of this protein shows an homology with the tropomyosin family, whereas the GP 900 protein has been previously identified as an immunodominant protein involved in the host-cell invasion. These two antigens could be candidates to develop an immunotherapy and to get ready new diagnostic aids.

N°. dell'Accordo di Collaborazione fascicolo 50C/F

THE PETRA STUDY: A RANDOMIZED, DOUBLE-BLIND, MULTICENTER TRIAL FOR THE EVALUTATION OF THREE SHORT ANTIRETROVIRAL REGIMENS FOR THE PREVENTION OF MOTHER-TO-CHILD TRANSMISSION OF HIV-1 IN SUBSAHARAN AFRICA

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The PETRA (PErinatal TRAnsmission) study is a randomized, four-arm, double-blind trial conducted, under the auspices of UNAIDS, in South Africa, Tanzania and Uganda. The objective of the study was to assess both the short-term and long-term efficacy of three short-course regimens of zidovudine/lamivudine combination therapy for the prevention of mother-to-child HIV-1 transmission in subsaharan Africa. A total of 1797 women were randomized to one of the four regimens : A) zidovudine/lamivudine given at week 36 of pregnancy until the onset of labor + intrapartum + one week following delivery (mother + child); B) zidovudine/lamivudine intrapartum + one week following delivery (mother + child); C) zidovudine/lamivudine intrapartum only; P) placebo.
A total of 1802 children were born, 70% of whom received breastfeeding. Primary outcomes were HIV infection and child mortality at week 6 and month 18 after birth. HIV status was based on HIV- DNA or RNA PCR results at week 6 and ELISA results at month 18.

HIV transmission and death rates of children is summarized in the following table :

	HIV infections + deaths at week 6	HIV infections + deaths at month 18
Arm A	8.2%	21.3%
Arm B	12.3%	24.9%
Arm C	18.8%	27.8%
Arm P	19.1%	26.8%

There was a significant reduction in HIV transmission at 6 weeks of life for arm A when compared to placebo ($p<0.001$), as well as for arm B ($p=0.021$). The intrapartum only arm C was no different from placebo. At month 18 of life, no significant difference was found between the arms. ($p> 0.05$). In a multivariate model caesarean section and high maternal CD4+ cell count were associated with a low risk of HIV-1 transmission at week 6 (odds ratio 0.63 and 0.87, respectively). At month 18 breastfeeding was associated with a high risk of HIV-1 transmission (odds ratio 2.11).
Introduction of short-course regimens to prevent mother-to-child transmission of HIV in developing countries should be accompanied by interventions to minimise the risk of subsequent transmission via breastfeeding.

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AUTHOR INDEX

Abbate I. 181
Abbondandolo A. 83
Abrescia N. 42, 44
Accardi L. 388
Accolla R. 149, 150, 155
Adorni F. 8, 10, 273, 274
Affabris E. 151
Agnoli A. 392
Agosta T. 29
Agostara F. 11
Agostini C. 256
Agrati C. 73
Aiello A. 29
Ailles L. 236
Aiuti A. 153
Aiuti F. 79, 80, 152, 153, 333, 389
Akihiko S. 357
Alba L. 115
Albanesi C. 144
Albano F. 95
Alberici F. 42, 44
Albini A. 114, 123, 154, 155, 208, 246
Albini Riccioli 11
Alciati A. 35
Alessandri G. 62, 260
Alessandrini L. 151, 363, 364
Alessi F. 125
Alfano M. 156, 176
Aliprandi C. 11
Allavena P. 144
Alleva E. 322, 335
Altavilla G. 57
Amadori A. 157, 369
Ambrosino C. 253
Amici C. 252
Amici R. 227, 335
Ammassari A. . 42, 44, 74, 75, 115, 117, 139, 277
Andreoni M. 30, 36, 37, 38, 60, 309, 328, 330
Andreotti M. 106, 335, 340, 341
Angarano G. 63, 111, 158, 287, 333
Angeloni A. 81
Angeretti A. 290
Angheben A. 36
Annunziato F. 105, 250
Antinori A. 39, 40, 42, 44, 74, 75, 113, 115 277, 279, 342, 344, 346, 348
Antinucci G. 113
Antonelli G. 45
Antonozzi I. 28
Antonucci G. 42, 44, 287, 342, 344, 346, 348
Appice A. 158
Aquaro S. 178, 179
Aquino A. 268
Arancia G. 339, 361, 385, 390
Arcà M. 14
Arcamone F. 217
Arcieri R. 335
Argentiere A. 29
Argnani R. 224
Arici C. 13, 342, 346, 348
Ariotti S. 237
Arrigoni E. 11
Arru G. 197
Arseni S. 300
Artico M. 159, 160, 161
Artioli S. 309
Arya S. 218
Aspiro E. 29
Atzori C. 269, 270
Audero E. 53
Bacci A. 302
Bacigalupo I. 326, 331
Bada M. 3
Bagetta G. 84
Bagnarelli P. 66, 178, 186, 233
Bagnato F. 298
Baiguera C. 15, 16
Baiocchi M. 360
Baj A. 68
Bajetto A. 129, 130
Balboni P.G. 224
Baldanti F. 66, 88, 89, 284, 285
Baldari M. 124
Baldassarri L. 390
Baldini F. 74, 75
Balestra E. 207
Baloni L. 162
Balotta C. ... 3, 47, 66, 86, 111, 112, 196, 233, 259
Bandera A. 286
Banks L. 295
Barasolo G. 102
Barassi C. 219
Barbanti Brodano G. 57, 182
Barbero S. 129, 130
Barbierato M. 63
Barchetta A. 226
Barchi E. 7
Barchiesi F. 304, 305
Bardelli C. 123
Barduagni O. 330

Bargagli A.M. 6
Barillari G. 49, 326, 327, 329, 331, 382
Barnaba V. 162
Baroncelli S. 370, 379, 380, 381
Barp S. 185
Barracchini A. 40
Barracco A. 7
Bartoli G. 24
Bassetti D. 50, 51, 287
Bassetti M. 50, 51, 287
Bastetti G. 119
Battaglia P.A. 355
Battistella L. 29
Battistini A. 356
Bazzaro M. 87
Bedini A. 279, 342, 344, 346, 348
Beghini R. 28
Begnini M. 22
Behr J.P. 218
Belardelli F. 165, 321, 357, 358, 365, 366, 377
Belardinelli V. 29
Belardo G. 252
Bella D. 15, 16
Belli R. 370, 379, 381
Bellocchi M.C. 40, 356
Bellotto E. 29
Beltrami C. 279
Benatti U. 82
Bendinelli M. 90, 163, 164, 243
Benedetti F. 227
Benedetto A. 315
Benelli R. 154
Beninati C. 307
Bensi T. 195, 196, 222
Benvenuto E. 165
Berchialla I. 29
Beretta A. 167
Bergaglia M. 29
Bernabei P. 237
Bernardi D. 135, 137, 138, 140
Bernardi S. 128
Bernasconi S. 225
Berni Canani R. 95, 303
Bertazzoni U. 168, 169
Bertoli A. 40, 111, 112
Bertorelle R. 78
Bertucci R. 29
Bestetti A. 64, 65
Bestetti G. 5
Betti M. 182
Betto P. 325
Bezzi P. 107
Biagini W. 29
Bianchi G. 144
Bianchi N. 202
Bianchi V. 52
Biancolini D. 64, 262
Biancone L. 55
Biasin M. 67
Binelli A. 321, 335, 340
Bini L. 390
Bini T. 273, 274
Binotto G. 256
Biolchini A. 197
Biondo C. 307
Biryahwaho B. 205
Bistoni F. 308
Biswas P. 156, 170, 294
Blasi F. 171
Boanni L. 29
Boarini C. 182
Boccellino M. 68
Boccia S. 280
Boelaert J.R. 306
Boffa L.C. 82
Bogner J.R. 328
Boldorini S. 281
Bolognesi M. 220
Bombana E. 13, 309
Bombonato M. 29
Bona R. 380
Bonaccorsi A. 182, 332
Bonacini M. 15
Bonaventura M.E. 13, 29
Bonavia R. 129, 130
Bonci F. 163, 243
Bonfante S. 309
Bonfanti P. 125
Bonfiglioli B. 29
Bonini R. 29
Bonissoni S. 196
Bonmassar E. 49, 267, 268
Bonvicini D. 29
Borderi M. 10
Bordignon V. 367
Borgatti M. 201, 202
Borghi I. 237
Borghi V. 10, 72, 279
Borgia P. 17
Boros S. 316
Borsetti A. 356, 359, 369, 379, 381
Bortolanza S. 157
Bortolin M.T. 76
Bosch M. 218
Boschini A. 22, 208
Bosco O. 7

Boscolo A.R. 29
Boselli D. 237
Bossi T. 108
Bosticardo M. 237
Botta M. 234, 235
Bottarel F. 195, 196, 222
Bottelli A. 68
Bottone L. 96, 223, 293
Bottura P. 29
Bovolenta C. 156, 169, 237
Boyd M.R. 245
Bozza S. 302
Bozzoni I. 172
Bracci A. 223, 278
Bracci L. 96
Bragardo M. 195
Brambilla S. 275
Branca M. 315
Brenchio B. 175
Brenci S. 97
Brianza E. 11
Bricalli D. 340
Brignolo 11
Brivio B. 11
Brocca Cofano E. 57, 182, 332
Broliden K. 188
Bromuro C. 378, 386
Browning P.J. 59
Bruni C. 11
Brunori L. 390
Bruzzese E. 303
Bruzzone B. 231
Bryant M. 214
Bucci I. 28
Bucciardini R. 335
Bugarini R. 328, 331, 380
Bukrinsky M. 156
Bulgheroni E. 251
Buonaguro F.M. 94, 204, 205
Buonaguro L. 94, 204, 205
Buonfiglio D. 195
Burastero S.E. 173
Buratti A. 11
Burioni R. 174
Burny A. 208
Burrai B. 29
Burroni M. 28
Bussolino F. 53, 54, 175, 176
Butera A. 307
Butini L. 110, 177
Buttò S. 217, 323, 326, 328, 333
334, 379, 380, 381
Cabrelle A. 256
Cadeo B. 309, 311
Cadeo G.P. 60, 86
Cafaro A. 326, 332, 333, 370, 379, 380, 381, 382
Caffarelli E. 172
Cafforio P. 189
Caggese L. 124
Caggiari L. 76
Cagni A.E. 281
Calabrò M.L. 63
Calamandrei G. 322
Calandrella M. 163
Calattini S. 5
Calcabrini A. 361, 385
Calcagno G. 124
Calcaterra S. 21, 39
Caliò R. 178, 179
Calistri A. 118, 238
Callea F. 15, 16
Camisani A. 11
Cammisa A. 253
Campana M. 11
Campanini B. 168
Campanini G. 88
Campelli A. 97
Campione G. 11
Campomenosi P. 83
Camussi G. 55, 56
Cancellieri C. 124, 309
Cancellotti E. 118, 238
Cancrini C. 128, 199, 249
Canella A. 87
Canini I. 365, 366
Cannavò G. 121
Cantaluppi V. 55, 68
Cantarelli L. 29
Capellini R. 29
Capello D. 85, 115
Capiluppi B. 19, 204, 259
Capitani S. 180
Capobianchi M.R. 21, 39, 181, 196
Capobianco M. 217
Capone F. 322
Capone I. 165
Caporaletti T. 177
Cappellacci L. 207
Cappellini A. 312
Cappiello G. 181, 315
Caputo A. . 57, 182, 247, 323, 332, 379, 380, 381
Cara A. 380
Caramello P. 42, 44
Carbonara S. 287
Carbonari M. 131
Carbone A. 59, 85

Carcò F. 124
Cardia A. 11
Cardinali G. 81
Careddu A. 255
Carfora M. 226
Cargnel A. 7, 113, 125, 269, 270
Carle F. 304, 305
Carlei D. 326, 329, 331
Carlini F. 380
Carollo D. 256
Carolo G. 30
Carosi G. 10, 15, 16, 42, 44, 60, 61, 309, 311
Carosi G.P. 86, 287
Carosio R. 264
Carpaneto E.M. 82
Carradori S. 125, 287
Carrera P. 64, 65, 262
Carta S. 28
Carulli B. 11
Caruso A. 62, 260
Caruso U. 28
Carvelli C. 40
Casabianca A. 221
Casanova J.L. 237
Casari S. 10, 309
Casartelli N. 199
Casazza R. 120
Cascone I. 53
Casella A. 13, 309
Caselli D. 95
Caselli E. 278
Caselli F. 304
Casoli C. 149, 168, 169
Cassai E. 278, 332
Cassatella M. 154
Cassone A. 271, 292, 301, 378, 385, 386, 387
Castagnoli L. 183
Castelli F. 89
Castelli Gattinara G. 95, 128, 249
Castelli G. 368
Castelli P. 124
Castelnuovo B. 274
Castelnuovo F. 309
Castrucci M.R. 360
Caterini A. 14
Catone S. 370
Cattaneo E. 88
Cattelan A. 256
Cauda R. 75, 196, 271, 338, 387
Cavel F. 255
Ceddia P.G. 385
Cefis M. 11
Ceglie L. 289
Cenci E. 302
Cerasi A. 221
Cereseto A. 326
Cesareni G. 183
Cesari L. 13
Chang Y. 119
Chelucci C. 368
Chen X. 254
Chersi A. 373
Chiani P. 386, 387
Chiapello N. 142
Chiarantini L. 221
Chiaricò P. 29
Chieco Bianchi L. 24, 63, 77, 78 91, 184, 185, 369
Chiesa A. 11, 89
Chiesa E. 274
Chiesi A. 335, 342, 344, 346, 348, 350
Chiodera A. 29, 124
Chiodo F. 10, 86
Chiorazzi N. 83
Chiovato L. 28
Chiriacò P. 124
Chirianni A. 120, 287, 342, 344, 346, 348
Ciancio B. 74, 115, 117
Cianfriglia M. 324, 325, 361, 385
Ciannamea R. 28
Ciaramella A. 272
Cibella J. 3
Ciccolella C. 329
Cicconi R. 272
Ciminale V. 184, 185, 242
Cinelli F. 125
Cingolani A. 40, 74, 75, 85, 115, 117, 277
Cinque P. 64, 65
Cinquegrana A. 11
Cioffi G. 124
Cioni M. 28
Cirillo P. 303
Citterio P. 251
Ciucci A. 29
Ciuffreda D. 204, 259
Clementi E. 294
Clementi M. 47, 66, 174, 178, 186, 233, 244
Clerici M. 67, 187, 188, 219, 269, 286
Coccia E.M. 356
Cocco M.R. 29
Cocito L. 231
Cogliati M. 286
Cohen M. 35
Colaianni A. 11
Colasante C. 26, 27
Colbacchi B. 80

Colizzi V. 272
Collacchi B. 79
Colleoni P. 11
Colombini P. 13
Colombo E. 192
Colombo M.C. 47, 66
Colucci M. 337
Comandini A. 49, 267
Comini A. 379
Comolli G. 89, 284
Conaldi P.G. 68, 70
Concia E. 10, 30, 36, 86
Contegiacomo P. 29
Conti L. 357, 358
Conti S. 300, 301, 316
Contini P. 97
Corallini S. 134
Corasaniti T. 84
Corbellino M. 5
Corbetta C. 28
Corelli F. 234, 235
Coronado O. 13
Corpolongo A. 145
Correa Leite M. 11
Corrias F. 369, 370
Corsico L. 113
Corvasce S. 3, 66, 86
Corvino E. 297
Cosma A. 167
Cosmi L. 105, 250
Cossarizza A. 10, 72, 132, 152, 279
Costa F. 28
Costa P. 231, 232
Costantini A. 110, 177
Costanzi G. 281
Costi R. 160
Cotroneo L. 124
Cozzi Lepri A. 111, 112, 113
Cozzi M.G. 108
Cozzolino E. 11
Cranage M. 370
Cremoni L. 125
Cremonini L. 279
Creti R. 390
Crisalli M.P. 309
Cristina G. 309
Cristini F. 13
Cristini M. 10
Croce F. 251
Crosato I.M. 124
Cugini P. 29
Cuorti M. 11
Curreli S. 197
Cutrona G. 82, 83
D'Agostini F. 114
D'Agostino C. 101, 145
D'Agostino D.M. 184, 242
D'Aloja P. 363
D'Ambrosio D. 144
D'Andrea E. 369
D'Anna C. 29
D'Arminio Monforte A. 3, 42, 44, 111, 112, 113 273, 274, 275, 277, 279, 284
D'Arrigo R. 40
D'Elia R. 24, 91
D'Ettorre G. 37, 38, 101, 145
D'Offizi G. 40, 73, 181
Daglio M. 29
Dal Cin E. 264
Dal Maso L. 31
Dal Monte P. 288
Dalle Nogare R. 309
Damato V.D. 28
Dammacco F. 189
Davoli M. 6
De Angelis F. 172
De Benedictis L. 258
De Berardinis P. 210
De Bernardis F. 271, 385
De Camillis 11
De Carli G. 29, 124
De Cristofaro M. 79, 80, 152, 333
De Fabritiis P. 106
De Fazio M. 29
De Filippi C. 294
De Flora S. 114
De Forni D. 77
De Francesco M.A. 260
De Gennaro M. 13, 309
De Giuli Morghen C. 190
De Lalla F. 125, 287
De Lerma Barbaro A. 149, 150
De Longis P. 40
De Luca A. 14, 40, 74, 75, 115 117, 178, 277, 279
De Maddalena C. 26, 27
De Magistris M.T. 378
De Maria A. 150, 231, 232
De Martino G. 161
De Martino M. 23
De Micco G. 11
De Palma R. 293
De Panfilis G. 62
De Paoli L. 24
De Paoli P. 76
De Pascalis C.R. 50, 51

De Paulis A. 226
De Romeo A. 24, 91
De Rossi A. 24, 77, 78, 91, 191
De Santis C. 257
Deaglio S. 222
Deambrogi C. 85
Deambrosis I. 55
Declich S. 187, 334, 393
Degener A.M. 197, 298
Degli Esposti A. 286
Dehò L. 3, 233
Dejiang T. 390
Del Borgo C. 7, 14, 124
Del Carpio C. 28
Del Galdo F. 293
Del Mauro D. 164
Del Mistro A. 24
Del Pozzo G. 210
Del Prete A. 189
Del Sorbo F. 108
Del Sorbo L. 55
Del Vecchio C. 118
Delfanti F. 170
Delfino D. 307
Delia S. 42, 44
Dell'Antonio G. 264
Dell'Armi C. 183
Demin F. 17
Desperati M. 29
Dettin M. 191
Dettori 11
Di Bartolomeo S. 239
Di Bello C. 191
Di Bello F. 388
Di Bonito P. 388
Di Cesare S. 128, 199
Di Fabio S. 321, 335
Di Fava R. 351
Di Fazio C. 11
Di Fiore P.P. 192
Di Gaetano M. 98
Di Gennaro G. 50, 135, 136, 137, 138, 139, 140
Di Giambenedetto S. 40, 74, 75
Di Lauro V. 136
Di Leva F. 253
Di Luca D. 278
Di Matteo G. 199
Di Nallo R. 335, 342, 344, 346, 348, 350
Di Nardo V. 29
Di Natale F. 309
Di Paola I. 29
Di Perri G. 10, 86
Di Pietro M. 259
Di Pucchio T. 377
Di Rosolino 124
Di Sano F. 239
Di Santo R. 160
Di Sora F. 104
Di Stefano M. 158
Di Traglia L. 104
Dianzani F. 111, 181, 193, 194
Dianzani U. 195, 196, 222
Difranco D. 196
Dini F. 125
Distefano M. 12
Dodi F. 13, 309
Dolei A. 197
Domercq M. 107
Donadeo 11
Donanno S. 79
Donati E. 309
Donato F. 15
Doratiotto S. 159
Doria M. 199
Dorrucci M. 3, 317
Dossena L. 88
Doublier S. 55
Drago L. 270
Drenaggi D. 124
Dupuis M.L. 324, 325
Duranti R. 335
Egger I. 29
Eggers M. 328
Elba E. 11
Elviri L. 168
Enders G. 328
Ensoli B. 49, 57, 62, 131, 132, 267, 323, 326, 327, 328, 329, 330, 331, 332, 333, 334, 339, 356, 359, 367, 370, 378, 379, 380, 381, 382
Ensoli F. 79, 80, 152, 333
Erb P. 328
Ercoli L. 36, 37, 38
Erpoli P. 11
Esame Esoke F. 24
Esposito R. 42, 44, 72, 279, 342, 344, 346, 348
Fabiani M. 393
Fabietti P. 287
Fabris M. 332
Facchi G. 111, 112, 233
Facchinetti V. 98
Facco M. 256
Fadda G. 280
Faggioni A. 81
Fagion I. 108, 125
Fais F. 83

Fais S. 361, 362
Falchi M. 332, 362
Fallani S. 304
Fanales Belasio E. 379, 380, 381, 382
Fantoni G. 269, 270
Fantoni M. 7, 14, 124
Fantuzzi L. 365, 366
Farchi F. 30
Farina A. 81
Fasan M. 27, 138
Fasoli M.G. 11
Fasulo G. 29, 124
Fattorini L. 268, 282, 390
Fauci A.S. 254
Favia A. 12
Favret M. 15, 16
Fea M. 11
Feasi M. 51
Federici C. 361
Federico A. 373
Federico M. 151, 241, 363, 364, 368
Feligoni M. 127
Fenoglio D. 96, 223, 293
Feriotto G. 201, 202
Ferrandi C. 54
Ferrante P. 281
Ferrara M. 22
Ferrara R. 389
Ferraresi I. 29
Ferrari D. 100
Ferrari M. 64, 65, 262
Ferrarini A. 294
Ferrarini M. 82, 83, 170
Ferrario G. 286
Ferrero E. 222
Ferri F. 13
Ferrini S. 154, 155
Ferro T. 184
Fibbia C. 124
Ficara F. 153
Finazzi Agrò A. 84
Finocchi L. 162
Finzi G. 29
Fiocchi C. 311
Fiore J.R. 12, 63, 158
Fiorelli L. 66, 186
Fiorelli V. ... 79, 80, 152, 323, 327, 333, 334, 378
Fiorentini C. 239
Fiorentini S. 62, 260
Fiorio M. 29
Fiorucci G. 151
Fiorucci G.C. 28
Fisicaro P. 168, 169
Fittipaldi A. 203
Fizzano M.R. 325
Floridia M. 335
Florio G. 226
Florio T. 129, 130
Fogli M. 231, 232
Foli A. 102
Follenzi A. 236
Fonzi S. 11
Forastiere F. 14
Forbici F. 39, 40, 111, 112, 207
Forcina G. 145
Forleo M.A. 61
Forni G. 237
Fortini C. 87
Fortis C. 122, 264
Fortuna P. 125
Fossati S. 188
Fragola V. 335
Fraldi A. 216
Francavilla V. 162
Franceschetti S. 85
Franceschi S. 31
Francesconi M. 29
Franchetti P. 207
Franchini L. 336
Franci 342, 344, 346, 348
Franco M. 330
Franconi R. 388
Franzese O. 49, 267, 299
Franzetti F. 286
Fraschetti M. 299
Fraternale A. 221
Fraticelli P. 144
Fraziano M. 272
Frazzi R. 300
Freer G. 243
Fronza G. 83
Fruzakis N. 229
Fulgenzi D. 359
Fulgheri M. 29
Funaro C. 362
Furci L. 200
Furlini G. 215
Gabarre J. 136
Gabbuti A. 13, 125, 287
Gabiano C. 23, 95
Gabrilovich D. 218
Gaeta C. 297
Gaggiani 11
Gaidano G. 59, 85, 98, 115
Galasso R. 335
Galati V. 73

Galazzi M. 5
Galbo R. 307
Galli G. 105
Galli L. 23, 95
Galli M. 5, 8, 10, 26, 27, 86, 113, 232, 233, 251, 273, 275, 333
Gallicano K. 74
Gallina A. 229, 230
Gallinaro L. 52
Gallo R.C. 208
Galloni D. 309
Galluzzo C.M. 335
Galluzzo T. 227
Galvan M. 278
Gambari R. 201, 202
Gandini L. 134
Garaci E. 299
Garbesi A. 323
Garbuglia A.R. 315
Gardella R. 245
Gariglio M. 290
Garulli B. 360
Garzelli C. 282, 283
Gasperini P. 63
Gastaldi R. 106
Gatti A. 262
Gatti F. 36
Gatti G. 50, 51
Gattuso G. 13, 309
Gauzzi M.C. 357, 358
Gavioli R. 87
Gazzola P. 231
Genini E. 284
Gennari F. 118, 238
Gentile G. 106
Geraci A. 335, 350, 369
Germanà A.M. 28
Germinario E. 321, 335, 340
Gerna G. 88, 89, 229, 284, 285
Gervasoni C. 113
Gessani S. 357, 358, 365, 366
Gherardi L. 29
Ghersi C. 126
Ghezzi P. 225
Ghezzi S. 122, 244
Ghinelli F. 125, 287
Ghio M. 29, 97
Giacca M. 76, 90, 203, 247, 295
Giacomet V. 24, 77, 91, 95
Giacometti A. 305
Giacomini P. 201
Giammarioli A. 362
Giancola M.L. 39, 117, 277
Gianelli E. 8, 86, 113
Giannecchini S. 163, 164
Giannelli E. 274
Giannini G. 321, 335, 340
Giaquinto C. 24, 77, 91, 95
Gibellini D. 215
Gigliani F. 355
Giomarelli B. 245
Giordani L. 372
Giorgi C. 388
Giovannetti A. 152, 153
Giraldo G. 93, 94, 204, 205
Girardi E. 6, 14, 28, 39, 40, 287
Giraudo E. 53
Girolomoni G. 144
Gismondo M.R. 270
Giuliani A. 268
Giuliano M. 315, 335, 340, 393
Giuliodoro S. 177
Gloghini A. 59, 85
Goebel F.D. 328
Golay J. 98
Goletti D. 330, 380
Gomez Morales M.A. 391, 392
Gomez M.J. 378
Gonelli A. 312
Gonnella R. 81
Gori A. 259, 286
Gorza M. 157
Gosselin G. 133, 214
Govindarajan S. 15
Govoni A. 277, 279
Govoni C. 29
Graf M. 303
Graifemberghi S. 62
Grandi P. 224
Grassi F. 206
Grasso M. 100
Graybill J.R. 305
Graziani G. 268
Greco G. 29
Greenberg P. 218
Grelli S. 104
Gribaudo G. 290
Grieco A. 174
Grifantini M. 207
Grilli M. 127
Grinello D. 189
Gringeri A. 155, 208, 262
Grisetti S. 39, 277
Gritti F. 10, 86, 113
Gualandi G. 29
Guardiola J. 210

Guarino A. 95, 303
Guenci T. 178
Gullo D. 28
Gulminetti R. 89
Gutierrez M.I. 203
Haas J. 328
Hahn G. 229
Heeney J. 370
Heydari A. 11
Hinkula J. 188
Hintermaier R. 328
Huebscher U. 133
Hurwitz S.J. 102
Iaccheri L. 141
Iacopino B. 11
Icardi G. 231
Iebba F. 80
Iemoli E. 124, 287
Imbach J.L. 214
Incitti F. 370
Indiveri F. 96, 97, 223
Innocenti F. 335
Introna M. 98
Iona E. 268, 282, 390
Ippolito G. 3, 7, 13, 21, 22, 28, 29, 39
40, 42, 44, 111, 112, 124, 287
Irato L. 13
Isgrò A. 153
Isola P. 163, 164, 243
Iuliucci R. 29
Ivaldi F. 293
Iwanicki M. 254
Izzi I. 7
Izzo M.C. 42, 44
Jacchetti G. 27
Jenner P. 390
Juzbasic S. 135, 136, 138
Kaul R. 188
Kituuka P. 393
Koszinowski U. 229
Kunkl A. 96, 223, 293
La Colla P. 211, 212, 213, 214
La Placa M. 215
La Seta Catamancio S. 251
La Sorda M. 280
Lamanna O. 29
Lamorea M. 360
Lanave M. 29
Landini M.P. 288, 289
Landolfo S. 290
Landonio S. 125
Lanfredi M. 57
Lania L. 216
Lapenta C. 321, 377
Lari N. 282, 283
Larocca L.M. 59, 85, 115
Larussa D. 117
Lattanzi L. 165
Lauria F. 10, 14, 104
Laus M. 217, 323
Laverda A. 24
Lazzari D. 13
Lazzarin A. 64, 65, 99, 100, 122
156, 173, 200, 204, 219, 259
Lazzarin L. 141
Lazzarini A. 170
Lazzarotto T. 288
Le Buanec H. 208
Lembo D. 290
Leoncini F. 105, 125
Leone P. 330, 380
Leopardi O. 306
Levi G. 336, 337
Lewis M. 248, 254
Li Pira G. 96, 223, 293
Libi F. 131, 132
Libianchi S. 6
Libralato C. 29
Licciardo P. 216
Licenziati S. 62, 260
Lichtner M. 104, 145
Lico C. 165
Lilleri D. 284
Lillo F. 100
Limogelli 14
Lipani F. 139
Lisziewicz J. 102, 218
Liuzzi G. 39, 40, 120
Liuzzi M.G. 101
Lo Nigro L. 124
Locatelli G. 291
Loddo R. 160, 161, 211, 213
Lodesani C. 124
Lodi A. 29
Lodini S. 100
Logozzi M. 362, 377
Loi A.G. 214
Lombardi A. 267
Longhi R. 281
Lopalco L. 188, 200, 204, 219, 257
Lopez M. 165
Loregian A. 238, 293
Lorenzani M. 29
Lorenzini F. 57, 182
Lori F. 102, 218
Lortat Jacob H. 247

Lorusso A. 124
Losana G. 237
Loverro P. 200
Lovivu G.F. 8
Lozupone F. 361, 362
Lozzi L. 96, 223
Lucchini A. 11
Luchetti R. 19
Lucia M.B. 196
Luciani F. 361, 362
Lugini L. 361, 362
Lukwiya M. 187, 328, 334
Lusic M. 203
Lusso P. 173, 200, 220, 261, 291
Luzi G. 79, 80
Maccario R. 284
Maccarrone M. 84
Maccarrone S. 29
Macchi B. 104
Macchia I. 370, 379, 380, 381
Macchini A. 355
Macchioni A. 29
Macedonio A. 14
Macino B. 263
Maffezzoni N. 11
Maga G. 133
Magenta L. 99
Maggi E. 250, 292
Maggi P. 13
Maggi T. 245
Maggiolo F. 89, 102
Maggiorella M.T. ... 359, 369, 370, 379, 380, 381
Magliani W. 300, 301
Magnani C. 125
Magnani F. 335
Magnani G. 309
Magnani M. 207, 221
Magnone A. 11
Magoni M. 393
Mai A. 159, 211
Maier J.A.A. 258
Mainardi M. 78
Majello B. 216
Malavasi F. 222
Mallardo G. 303
Malnati M. 291
Malorni W. 338, 357, 358, 362
Mameli G. 197
Mammano F. 255
Mammoliti G. 11
Manca F. 96, 223, 293
Mancini C. 165
Mancini S. 110, 177
Manconi P.E. 42, 44
Mancuso R. 281
Maneschi M. 102
Manetti R. 105, 250
Manfredi A.A. 294
Manfrin V. 287
Manganaro D. 275
Mangili R. 11
Mannucci P.M. 208
Manservigi R. 224
Mantelli B. 170
Mantovani A. 144, 225
Manzari B. 127
Marastoni M. 87
Marcato N. 157
Marceddu T. 159, 161
Marcello A. 203, 295
Marchegiano P. 29
Marchei E. 351
Marchetti G. 29, 286
Marchetti M. 339
Marchiò S. 176
Marchisone C. 246
Marconi P. 75, 224
Maresca B. 296
Marin O. 242
Marinacci C. 17
Marinari B. 240, 241
Marinaro M. 378
Marino M. 309
Marino V. 11
Mariotti M. 258
Mariotti S. 390
Marone G. 226
Marsili G. 356
Martellotta F. 137, 140
Martinelli C. 124, 125
Martini F. 141
Martini L. 13
Martini M. 115
Martiniello R. 29
Martino A. 272
Martino P. 106
Marusic C. 165
Marzi M. 335, 350
Marziali G. 356
Marziali M. 152
Masala P. 29
Mascioli M. 124
Maserati R. 61, 67, 89, 102, 281, 284, 309
Masi A. 124
Masocco M. 316
Massa S. 159, 160, 161

Massari M. 29, 124
Massella M. 335
Massetti P. 38, 145
Massimi P. 295
Mastino A. 104
Mastroianni C.M. 101, 145
Masucci M. 87
Matarese G. 249
Matarrese P. 239, 338, 357, 358, 362
Materazzetti A. 29
Matteelli A. 61, 287
Mattei A. 335
Matteucci C. 104
Matteucci D. 90, 163, 164, 243
Mattia G. 368
Mattiacci B. 29
Mattioli F. 124
Mavilio D. 231, 232
Mazza A. 24, 91
Mazza S. 150
Mazzei T. 304
Mazzetta F. 152
Mazzetti M. 105
Mazzetti P. 163, 243
Mazzi R. 30, 36
Mazzoni M. 180
Mazzotta F. 42, 44, 60, 61, 125
287, 342, 344, 346, 348
Mazzucchelli R. 3, 86
Mcmahon J.B. 245
Meacci F. 245
Meazza R. 155
Medaglini D. 245
Meggio F. 184, 242
Meldolesi J. 107
Mele R. 391, 392
Mellado C. 35
Melucci Vigo G. 332
Memmi G. 297
Mencacci A. 302
Mengoni F. 145
Menin C. 369
Menna A. 28
Mennella V. 324, 325
Menzo S. 47, 66, 186, 244
Meraviglia P. 35, 108
Mercurio V. 29, 124
Merlo M. 11
Meroni L. 273, 275
Mezzanotte C. 11
Mezzaroma I. 131, 153, 389
Mezzatesta C. 196
Miceli A. 307
Micheletti F. 87
Michelini Z. 370, 379, 381
Micheloni G. 29, 124
Michienzi A. 172
Miertus S. 227
Migliore G. 315
Migliorino G. 29
Miglirino M. 102
Milan G. 263
Milan I. 137, 140
Milanese G. 17
Milanesi G. 229, 230
Milani D. 180
Milazzo F. 10, 86, 287
Miletich F. 50, 51
Milini P. 29
Millo E. 82
Miluzio A. 306
Mingari M.C. 232
Minoli L. 60, 113
Miorin M. 256
Mirandola P. 180, 312
Mirra M. 335
Mischiati C. 201, 202
Misserini A. 28
Mitola S. 175
Mochi S. 388
Moioli R. 11
Molinari A. 361, 385
Mollica R. 11
Molteni C. 286
Monachetti A. 66, 186
Monaco M. 94, 205
Mondello F. 385
Mondino A. 171
Mondino F. 309
Monga G. 281
Mongiardo N. 124
Monini P. 59, 62, 131, 132, 328
330, 331, 332, 339
Monno L. 12, 47, 111, 112, 158
Monolo G. 10, 86
Montagnoli C. 302
Montaldo F. 123
Montefiori D.C. 254
Montella F. 10, 14
Monterastelli E. 72
Montesarchio E. 342, 344, 346, 348
Montesoro E. 368
Monti M. 124
Montroni M. 110, 177
Monzoni A. 76
Moore P.S. 119

Morace G. 280
Morandini B. 259
Morelli C. 141
Moretta L. 105, 231, 232
Moretti F. 10
Moretti L. 72
Moretti S. 379, 380, 381, 382
Mori T. 245
Morini M. 114, 123
Moroni M. 3, 5, 26, 27, 47, 86
111, 112, 113, 233, 274
Morra E. 85
Moscardini M. 243, 263
Moscufo N. 356
Muciaccia B. 134
Mugnaini C. 235
Mundo A. 24
Munoz C. 110, 177
Mura M. 159, 161, 211, 212
Muratori C. 363
Murdaca G. 97, 232
Murgioni C. 160, 161, 211, 213
Murone M. 122
Murri R. 7, 42, 44, 74, 75, 117, 277
Musetta A. 303
Musicco M. 8, 10, 208
Musiu C. 211
Mussini C. 72, 279
Musulin B. 246
Muti G. 85
Nacci V. 234, 235
Naim V. 355
Naldini L. 236
Nallira G. 29
Napolitano G. 216
Nappi F. 356, 367
Narciso P. . 37, 40, 42, 44, 73, 342, 344, 346, 348
Nardese V. 220
Nasi M. 72
Nasti G. 135, 136, 137, 138, 139, 140
Nastruzzi C. 202
Natalini Raponi G. 14
Negri D.R.M. 369, 370, 379, 381
Neipel F. 328
Nicastri E. 13, 30, 36, 37, 38, 328, 330
Niccolai V. 29
Niccolini F. 124
Nicolosi A. 11
Niero F. 124
Nigra E. 139
Nigro L. 138, 139
Nisini R. 389, 390
Niutta M.G. 6
Nobile A. 335, 350
Nobili C. 14, 124
Noonan D. 114, 123, 155, 246
Norbedo S. 227
Noris E. 290
Norley S. 370
Noto P. 40, 42, 44
Novelli A. 304
Novelli F. 237
Novi A. 79, 80
Nozza S. 99, 122, 206
Nurra G. 21, 29
Oggioni M. 307
Oggioni M.R. 297
Okong P. 393
Oletto S. 24
Olimpico F. 248
Olivetta E. 151, 363, 364
Ometto L. 77, 78
Onorati A.M. 226
Orani A.M. 60
Orchi N. 7, 21
Orefici G. 268, 282, 390
Oreste P. 85
Origoni M. 100
Orlandi C. 221
Orlandi P. 199
Orlando G. 124
Orlando S. 225
Orr ù G. 297
Orsi N. 298
Ortona L. 42, 44, 115, 117
Pace M. 380
Pacifici R. 351
Paganelli A. 141
Pagani E. 281
Pagani L. 309
Paglia M.G. 277
Paiardini M. 121
Palladino C. 326, 329, 331
Pallavicini F. 13
Palmieri C. 253, 254
Palmieri F. 287
Palmisano L. 106, 227, 335, 340, 341
Pal ù G. 118, 238, 293
Palvarini L. 29, 61, 124
Pan A. 13, 89, 102
Pandiani I. 29
Pandolfi F. 181
Panina Bordignon P. 244
Panni S. 183
Pantaleoni M. 13
Panzeri B. 262

Paolucci S. 66, 88, 89
Papagno L. 3, 233, 259
Pappagallo M. 30
Paradiso C. 29
Parazzini F. 125
Parisi S.G. 30, 36, 37, 38
Parlato G. 28
Parlato S. 362, 377
Paroli M. 162
Parolin C. 118, 238, 359
Parravicini C. 5, 119
Pasqualini M. 207
Pasquato A. 191
Pasquini E. 28
Pasquinucci S. 10
Passamano M. 254
Pastecchia C. 139
Pasticci M.B. 287
Pastore C. 200
Pastore G. 12
Pastori C. 219
Patiri F. 77
Patrizio M. 337
Patrone M. 229
Patroni A. 61
Pattarini R. 126
Paulli M. 85
Pauluzzi S. 42, 44, 287
Pavia C. 21
Pavlakis G.N. 367
Pavone Cossut M.R. 380
Pedemonte P. 51
Pedone C. 202
Pedrali Noy G. 229, 230
Pedrazzani G. 11
Pegoraro G. 203, 295
Pelicci P.G. 192
Pelissero S. 29
Pende D. 232
Penna C. 29
Pennisi 11
Pepe A. 141
Percario Z.A. 151
Percivalle E. 229, 284, 285
Peretti S. 340, 341
Perno C.F. 39, 40, 111, 112, 178, 179, 207
Peroni M. 21
Perosino M. 29
Perotti P. 11
Perucci C.A. 6, 14, 17
Peschle C. 368
Petraroli A. 226
Petrosillo N. 13, 309
Pezzotti P. 3, 42, 44, 111, 279, 316, 317
Piacentini M. 239
Piatier Tonneau D. 191
Piazza M. 42, 44, 120
Piazzi S. 28
Pica F. 299
Picardi V. 106
Piccini G. 29
Piccinini G. 284
Piccinini M. 142
Piccinni M.P. 292
Piccioli P. 129, 130
Piccolella E. 240, 241
Picelli G. 29
Piconi S. 67, 187
Piedimonte G. 121
Pierconti F. 115
Pierdominici M. 131, 152
Pieretti C. 124
Pierotti C. 64, 65
Pierpaoli S. 312
Piersantelli N. 124
Pietrella D. 308
Pietropaolo V. 197, 298
Pignatelli S. 288
Pignero A. 28
Pilati E. 107
Pilotti E. 168, 169
Pinna L.A. 184, 242
Pinter E. 132, 153
Pinti M. 72
Pintor C. 28
Pirazzini M.C. 29
Pirillo M. 321
Pirillo M.F. 106, 335, 340
Pischedda L. 29
Piselli P. 21, 124
Pistello M. 90, 163, 164, 243, 263
Pistoia V. 83
Pittaluga A. 126, 127
Pizzato M. 238
Pizzigallo E. 42, 44
Plebani A. 199, 255
Poccia F. 73
Poggi A. 264
Poggio A. 61
Poli A. 163
Poli C. 29
Poli F. 5
Poli G. 10, 99, 122, 156, 168, 169 170, 176, 237, 244, 294, 365
Polidori M. 29
Polifemo A. 22

Polito G. 303
Polizzi C. 335
Pollini S. 219
Polo S. 220
Polonelli L. 300, 301
Poloni F. 385
Polyanskaya N. 370
Pontali E. 51
Ponzanelli A. 82
Porcasi R. 13
Porcellini S. 206
Porta A. 296
Porta D. 6, 14
Possati L. 247
Posteraro B. 280
Poudrier J. 296
Pozio E. 391, 392
Pozzi G. 245, 297, 301
Prat M. 123
Pratesi C. 76
Pregnolato M. 133
Presciutti R. 6
Presi S. 64, 65
Presta M. 246, 247
Presutti C. 172
Prete A. 11
Prete S.P. 268
Preziosi G. 13
Prigneau O. 296
Primavera A. 231
Primo L. 54
Priora C. 11
Pristerà R. 342, 344, 346, 348
Proietti E. 321
Propato A. 162
Prost P. 179
Provvedi R. 245
Puca A. 248
Pucillo L. 73
Puddu P. 324
Pugliese G. 28
Pugliese K. 363, 364
Pugliese O. 373
Punzi G. 158
Puoti M. 15, 16
Puppo F. 97
Puro V. 29, 124
Pushkarsky T. 156
Pusterla L. 125
Putzolu V. 15, 16
Quaranta G.M. 372
Quaresima S. 11
Quinto I. 248, 253, 254
Quirino T. 125
Racca S. 65
Racchetti G. 107
Racioppi L. 199, 205, 249
Radaelli A. 190
Raineri G. 29, 124
Raise E. 10, 60, 86
Raiteri M. 126, 127
Ramazzotti E. 215
Ramoni C. 361
Rampazzo C. 52
Rampon O. 24, 78, 91
Rapiti E. 14
Raponi G. 29
Rappuoli R. 156
Ratti G. 309
Ravasio L. 42, 44, 113
Re M.C. 215
Regamay N. 328
Regazzi Bonara M. 60, 311
Regnery C.M. 110, 177
Reichard P. 52
Reina F. 11
Renusi R. 29
Renzi M. 336
Renzulli M. 234
Resentini M. 11
Resnati M. 171
Restivo B. 124
Revello M.G. 284, 285
Rezza G. 3, 21, 22, 30, 31, 42, 44, 112
315, 316, 317, 328, 330, 333
Ribatti D. 246, 247
Riboldi F. 11
Ricci E. 125
Riccio P. 101
Ricotti M. 142
Ridolfi B. 356, 359, 379, 381
Ridolfo A. 138
Ridolfo A.L. 113, 274
Riera L. 290
Rigamonti L. 237
Righini V. 108
Rimessi P. 332
Rinaldo F. 176
Rinaudo M.T. 142
Rindi L. 282, 283
Ripalti A. 289
Ripamonti D. 309
Riva A. 275
Riva C. 47, 112
Riva E. 153
Rivas C. 119

Rizza P. 165
Rizzardini G. 60, 187
Rizzi C. 156
Rizzi M. 220
Rizzo M.G. 40, 74, 75, 117
Roberti L. 14
Roberto G. 11
Roca C. 54
Rocchi G. 17
Rodella L. 180
Rodriguez M. 197
Romagnani S. 105, 250, 292
Romagnoli G. 389
Romanelli A. 202
Romani L. 302
Romano C. 28
Romano L. 143
Rombolà G. 108
Romeo D. 227
Romeo G. 151
Romiti L. 249
Romiti M.L. 128
Roncella S. 82, 83
Rosa A. 6
Rosa F. 124
Rosati A. 29
Rosato A. 263
Rosi D. 29
Rossi A. 252
Rossi D. 85
Rossi J. 172
Rossi L. 207
Rossi P. 128, 199, 249
Rossi S. 15
Rosso O. 155
Rosso R. 287
Rossolini G. 219
Rotelli P. 29
Rovere P. 294
Rozzi S. 35
Rubartelli A. 264
Rubino A. 303
Ruco L. 144, 153
Ruga E. 24, 78
Ruggero L. 216
Ruiz Alvarez M.J. 323, 333, 334
Ruocco M.R. 253
Rusconi S. 47, 66, 232, 251
Rusnati M. 246, 247
Russo S. 55
Sabbatini A. 11
Saccani A. 225
Saccani S. 225
Saggioro D. 185
Saitta M. 124
Sala N. 340
Salanitro A. 17
Salassa B. 124, 287, 309
Salati A. 300
Salatiello I. 94
Salemi S. 152
Salvatori F. 255
Samoggia P. 368
Sanglard D. 280
Sanguinetti M. 280
Santagostino E. 208, 262
Santambrogio S. 8, 10, 26, 27, 86
Santarcangelo A.C. 363, 364
Santarelli R. 81
Santini Muratori D. 80, 152, 153
Santini S. 377
Santoni De Sio F. 236
Santopadre P. 145, 342, 344, 346, 348
Santoro F. 291
Santoro M.G. 252
Santucci M.B. 272
Saponiero A. 267
Saracco A. 19, 259
Saracino A. 12, 158
Sarmati L. 30, 36, 37, 38
Sarno S. 242
Sartorius R. 210
Savarino A. 222
Saviano M. 202
Sbarbati 11
Sbardella G. 159
Scala G. 248, 253, 254
Scalamogna C. 5
Scaletti C. 292
Scalise G. 113, 304, 305
Scalone S. 136
Scalzini A. 42, 44, 125
Scapini P. 154
Scarabaggio T. 13, 158
Scaramuzzi D. 131, 132
Scarfì S. 82
Scarinci C. 191
Scarlatti G. 199, 255
Scasso A. 42, 44
Schatz O. 328
Schettini G. 129, 130
Schiaffella E. 162
Schiavano G.F. 221
Schiavone M. 254
Schiavoni I. 363
Schimizzi A.M. 304, 305

Schioppa O. 135, 136, 137, 140
Schols D. 179
Schulz T.F. 328
Sciandra A. 10
Scoglio A. 323, 333, 334, 381
Scognamiglio P. 22
Secchi E. 11
Secchi M. 229
Secchiero P. 180, 312
Segata A. 29
Selva C. 73
Semenzato G. 256
Seminari E. 67
Sempala S.D.K. 205
Senese D. 3, 47, 112, 233, 275
Serafini S. 207
Serafino A. 299
Sernicola L. 369, 370, 379, 381
Serpelloni G. 7
Serra C. 197, 214
Serra I. 160, 212
Serraino D. 21, 22, 115
Sestili P. 321
Sette P. 40
Setti M. 97
Setzu M.G. 160, 161, 212, 213
Sgadari C. 49, 62, 326, 327, 329, 330, 331
Sgarbanti M. 356
Sheldon J. 328
Sica A. 225
Siccardi A.G. 206, 219, 257, 261
Signorini L. 309, 311
Sileo C. 29
Silvestri R. 161
Silvestris F. 189
Simonelli C. 135
Simonelli S. 137, 140
Simonini G. 29
Sinigaglia F. 144
Sinnone M. 200
Sirchia G. 5
Sirianni M.C. 131, 132, 330
Sironi F. 220
Sironi M. 144, 225
Sisto F. 306
Siviero M. 256
Smacchia C. 22
Smith Caruso A. 291
Smithi C. 170
Sodano M.L. 29
Soldani F. 39, 40
Soldini L. 122
Sommadossi J.P. 214
Soprana E. 219
Soranzo M.L. 287
Soria M.R. 258
Soscia F. 14, 29, 42, 44
Sozzani S. 365
Sozzi S. 163
Spada M. 165, 321, 377
Spadari S. 133
Spadaro F. 361
Spaggiari G.M. 264
Spanò A. 28, 181
Sparnacci K. 217
Spazzapan F. 29
Speciale L. 281
Sperati A. 6
Spina M. 135, 136, 137, 138, 139, 140
Spinedi A. 239
Spinetti A. 15, 16
Spreca A. 302
Stagni G. 61
Stagno A. 124
Starace F. 95
Stefanini M. 134
Stefano V. 11
Stegagno M. 28
Stievano L. 157
Stoppacciaro A. 144
Straface E. 338
Stringaro A. 361, 385, 390
Sturtzl M. 326, 328, 330, 331, 332
Sulas D. 29
Superti F. 339
Suter F. 7, 10, 86
Taccagni G. 100
Tacconelli E. 387
Tagliamonte M. 204
Talamini R. 135
Tallarino A. 120
Tambini R. 29
Tambussi G. 19, 99, 122, 200, 204, 206, 259, 264
Tandurella S. 124
Tangenti M. 29
Taramelli D. 306
Tasciotti E. 203
Tavanti L. 29
Tavio M. 135, 137, 138, 140
Tedesco T. 131
Teloni R. 389, 390
Ten Haft P. 370, 379, 381
Teofilova E. 29
Terranova P. 96, 223, 293
Testa L. 7, 277, 284
Testa U. 368

Testini B. 29
Teti G. 301, 307
Thlick A.E. 119
Timillero L. 125
Tinari A. 339
Tinghino B. 11
Tirelli U. 42, 44, 50, 135, 136, 137, 138, 139, 140
Titti F. 359, 369, 370, 379, 380, 381
Toccaceli V. 316
Toffoli G. 50
Tognazioli C. 306
Tognazzo S. 78
Tognon M. 141
Tomaselli G. 307
Tomasetti S. 6
Tomasi M. 360
Tomasoni L. 102
Tomatis R. 87
Tombesi M. 324
Tomino C. 335
Tondelli L. 217, 323
Tonelli C. 29
Tonini G. 11
Topino S. 131, 132, 330
Toriello M. 253
Tornesello M.L. 94, 204, 205
Torosantucci A. 271, 386, 387
Torrisi M.R. 81
Torti C. 16
Toscanini F. 51
Toschi E. 49, 326, 327, 329, 331
Tosi G. 149, 150
Tosini F. 391, 392
Tosoni A. 256
Tossi A. 227
Tossini G. 351
Toti M. 287
Tovo P.A. 23, 142
Tovoli D. 29
Tozzi V. 7, 39, 40, 73
Trabattoni D. 67, 187, 188, 269, 286
Tracconelli E. 271
Tramontano E. 213
Traniello S. 87
Trentin L. 256
Trimboli F. 248, 253
Tripiciano A. .. 217, 323, 333, 334, 378, 379, 381
Tritarelli E. 372
Troiano L. 72, 132
Trotta M.P. 40, 42, 44
Tumbarello M. 271, 387
Tuosto L. 240, 241
Turano A. 260
Turano P. 84
Turbessi G. 14
Turriziani O. 181
Tyagi M. 203
Uberti Foppa C. 100
Ubiali D. 133
Uccella I. 30, 36, 37, 38
Ulivi M. 82
Urbinati C. 246, 247
Urciuoli R. 316
Ursitti M.A. 279
Vaccher E. 50, 135, 136, 137, 138, 139, 140
Vago L. 281
Valdarchi C. 316, 317
Valensin P.E. 143
Valenti S. 217
Valenzi C. 6
Valerio A. 269, 270
Vallanti G. 156, 365
Valori C. 107
Valsecchi D. 11
Valsecchi L. 125
Valvo 325
Valvo L. 351
Van Cott T.C. 254
Van Damme J. 179
Vanacore P. 287
Varaldo P.E. 174
Varano B. 357, 358
Varchetta S. 275
Varga G. 218
Vargiu L. 159, 212
Varia L.M. 124
Vaschetto R. 195
Vecchi A. 144
Vecchiarelli A. 308
Veglia F. 100, 122
Velati C. 11
Vella S. 104, 321, 335, 338, 340, 341
342, 344, 346, 348, 350, 393
Velleca R. 3, 47
Venturi G. 143
Venuti A. 388
Verani A. 261
Verani P. 363, 364, 369, 370, 379, 380, 381
Vercelli D. 261
Vertuani S. 87
Viale P. 13, 309, 311
Vicenzi E. 156, 168, 169, 244, 262
Vichi F. 342, 344, 346, 348
Vichi M. 316
Vicini E. 134
Videtta M. 298

Viganò A. 95, 340
Viganò P. 125
Vigano P. 108
Vigevani G. 7, 124, 125
Viglio A. 85
Vignocchi B. 141
Vignoli M. 215
Villa M. 11
Villani A. 311
Villani P. 311
Vincenzi L. 40, 73, 330
Vincenzo S. 11
Vincenzo V. 139
Violin M. 47, 111, 112, 233
Viora M. 372, 373
Visani F. 29
Visciano M.L. 94
Visco Comandini U. 40
Visentin S. 336
Visonà R. 7
Vitale F. 333
Vitale M. 180
Vitali R. 11
Vivalda A. 29
Vivarelli A. 309
Vivenza D. 85
Vlacos D. 29
Volonterio A. 124
Volpi A. 299
Volterra A. 107
Vullo V. 37, 38, 42, 44, 101, 104, 113 145, 342, 344, 346, 348
Vultaggio A. 292
Vultaggio G. 135, 136, 137, 138, 140
Wagner T. 333
Waltenberger J. 246
Weimer L.E. 335
Wernli M. 328
Xu J. 218
Yaguboglu S. 328
Zaccarelli M. 40, 342, 344, 346, 348
Zaccaro L. 163, 164, 243
Zacchello.F. 24, 91
Zacchini F. 287
Zagury D. 208
Zaltron S. 15, 16
Zamarchi R. 157, 369
Zambelli A. 7
Zanchetta M. 24, 77, 78, 91, 191
Zanetti M. 137, 140
Zangrando D. 29
Zanini B. 15, 16
Zanini M.T. 11
Zaniratti S. 21
Zanotto C. 190
Zanovello P. 263
Zanussi S. 76
Zappa A. 65
Zauli G. 180, 215, 312
Zauli T. 124
Zavattoni M. 88, 284
Zazzara M. 317
Zazzi M. 143
Zehender G. 26, 27
Ziccheddu M. 197
Ziezt C. 326
Zito S. 355
Zocchi M.R. 264
Zonan A. 29
Zoppè M. 90, 203
Zotti L. 184
Zuccaro P. 351
Zulli E. 28

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