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First research project on viral hepatitis: etiopathogenesis and diagnosis (1997-1999) Progress report

Edited by the Scientific coordinator
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First research project on Viral hepatitis: etiopathogenesis and diagnosis (1997-1999). Progress report.
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2000, v, 246 p. Rapporti ISTISAN 00/1

Progress report of the First research project on Viral hepatitis: etiopathogenesis and diagnosis. The project is subdivided into the following subprojects: 1) structural and biological characterization of recently identified viruses and viral variants; 2) pathogenesis of hepatitis viral persistent infections; 3) hepatitis viral infection markers in particular categories of patients.
Key words: Biomedical research, Public health, Viral hepatitis

Istituto Superiore di Sanità
Primo progetto di ricerca sull'Epatite virale: eziopatogenesi e diagnosi (1997-1999). Progress report.
A cura di Maria Rapicetta
2000, v, 246 p. Rapporti ISTISAN 00/1 (in inglese)

Progress report del Primo progetto di ricerca sull'Epatite virale: eziopatogenesi e diagnosi. Il progetto è suddiviso nei seguenti sottoprogetti: 1) caratterizzazione strutturale e biologica di virus di recente identificazione e di varianti virali; 2) patogenesi dell'infezione virale persistente di virus epatici; 3) marcatori di infezione da virus epatici in particolari categorie di pazienti.
Parole chiave: Epatiti virali, Ricerca biomedica, Sanità pubblica

Cinzia Bisegna and Luigia Mauro of the Laboratory of Virology, Istituto Superiore di Sanità, were responsible for the coordination and layout of this report.

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Preface Please note that the title in small capital bold is referred to the title of the Project. The title in italic is relative to the specific subject of the progress report.

SUBPROJECT: STRUCTURAL AND BIOLOGICAL CHARACTERISATION OF RECENTLY IDENTIFIED VIRUSES AND VIRAL VARIANTS

ML = Milioni di Lire

SUBPROJECT

STRUCTURAL AND BIOLOGICAL CHARACTERIZATION OF RECENTLY IDENTIFIED VIRUSES AND VIRAL VARIANTS

FUNDS ALLOCATED ML 860

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GEOGRAPHICAL CHARACTERIZATION OF HEPATITIS C VIRUS SUBTYPES IN POPULATIONS AT DIFFERENT RISK OF INFECTION BY GENOTYPING AND SEROTYPING TECHNIQUES
Coordinator: Prof. Pietro CROVARI

Progress Report

GEOGRAPHICAL CHARACTERIZATION OF HEPATITIS C VIRUS SUBTYPES IN POPULATIONS AT DIFFERENT RISK OF INFECTION BY GENOTYPING AND SEROTYPING TECHNIQUES

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Summary

The second phase of study focused on three groups of subjects infected with hepatitis C virus (HCV): patients with chronic liver disease, haemodialysis patients and intravenous drug users.

A group of 271 patients with different forms of HCV-related chronic liver disease (chronic hepatitis, cirrhosis, hepatocellular carcinoma) were evaluated with regard to clinical information and biochemical, histological and functional parameters in order to evaluate which factors are correlated with degree of liver disease. Age and duration of infection were the only variables independently associated with progression to cirrhosis in a multiple logistic regression analysis, and only age correlated with development of liver cancer. Genotype 1b was detected more frequently in older patients with serious chronic liver disease, but such high prevalence was explained by a cohort effect. No correlation was found between any HCV genotype and evolution of disease.

Twenty haemodialysis patients seroconverted to anti-HCV in the period April 1996 – January 1997 in a single dialysis centre. Seroconverters and patients already anti-HCV positive at the beginning of the outbreak were studied by sequencing the 5'-UTR region of HCV. Viral strains were perfectly overlapping in 5 already positive (source of infection) and 12 seroconverter patients. Most seroconverters used different rooms from their sources of infection, thus strongly suggesting that dialysis apparatus was not involved in transmission, but that lack of compliance to standard operating procedures by ward personnel was most probably the cause of cross infection between patients.

A larger sample of HCV-infected intravenous drug users was collected. Although HCV type 3 is the most prevalent in all geographical areas, its relative importance seems to be higher in northern (58% of isolates) than in central Italy (45% of isolates).

Key words: *HCV, genotypes, sequencing, age, chronic liver disease, clinical evolution, haemodialysis, intravenous drug addiction*

After the first period of research, when a baseline picture of the prevalence of HCV subtypes was drawn in different risk groups and geographical areas, the study aimed at:

- an evaluation of factors influencing the evolution and severity of chronic liver disease in anti-HCV positive patients;
- following-up 20 haemodialysis patients who had seroconverted to anti-HCV in the period April 1996 - January 1997;
- a further collection of samples from intravenous drug users infected with hepatitis C virus, for whom a different geographical distribution of subtypes had been put into evidence in the first phase of the project.

Patients with chronic liver disease

The sample of 100 anti-HCV positive patients with chronic liver disease and cirrhosis that were identified in the first part of the project, was substantially enlarged to include 271 subjects overall. Clinical information and biochemical, histological and functional parameters were collected in order to be able to infer (through appropriate statistical elaborations) which factors best correlate with severity of disease.

In particular, our purpose was to investigate whether any HCV genotype was correlated with particular patterns of clinical, histological or functional parameters and whether age or other demographic characteristics were determinant in the progression of the disease. For this purpose, we also collected information on the presumed source of infection and years of exposure to hepatitis C virus.

The sample consisted of 168 subjects with chronic hepatitis (126 males and 42 females; mean age 44 ± 12 years; range 16-72 years), 61 with cirrhosis (44 males and 17 females; mean age 51± 9 years; range 33-73 years), 42 with hepatocellular carcinoma on cirrhosis (30 males and 12 females; mean age 65 ± 9 years; range 40-84 years). All patients were anti-HCV and HCV-RNA positive and negative for both HBsAg and anti-HIV. Also metabolic causes of liver disease were excluded. No patient was a heavy alcohol consumer.

Investigated risk factors for HCV infection were blood transfusions, infusion of haemoderivates, occupation at risk, tattooing and intravenous drug use. With regard to duration of infection, time of possibly implicated blood transfusion or beginning of drug addiction were traced back. For patients whose date of exposure to HCV was not identifiable (sporadic infections), time of first abnormality of biochemical hepatic parameters was considered.

Data were analysed dividing subjects into two groups: those with certain duration of infection (intravenous drug addicts, transfused patients, recipients of haemoderivates), and those with sporadic infection. Three age groups were also considered: < 35 years; 35-50 years; > 50 years.

Statistical analysis of differences among patients with different diseases or belonging to different age groups was performed by chi-square and Kruskall-Wallis tests, while differences between subjects with genotype 1b vs. genotype non-1b were analysed using Mann-Whitney's test and Fisher's exact test. Finally, a stepwise multiple logistic regression was performed in order to evaluate which, among different variables, were independently associated with an advanced stage of chronic liver disease.

The first result was that patients' age progressively increases with worsening of liver disease stage, starting from simple chronic hepatitis (mean age: 44 ± 12 years), to cirrhosis (mean age: 51 ± 9 years) and hepatocellular carcinoma (mean age: 65 ± 9 years). A similar trend was registered for duration of exposure to the virus, evaluated both according to years since the beginning of infection (chronic hepatitis: 11.2 ± 6.7 years; cirrhosis 12 ± 6.1 years; hepatocellular carcinoma: 19.6 ± 8 years), and to years of elevated transaminases (ALT) values (chronic hepatitis: 7.9 ± 6 years; cirrhosis 9.9 ± 6.6 years; hepatocellular carcinoma: 12.2 ± 6.9 years). Genotypes non-1b were prevalent in cases of chronic hepatitis. Overall, 76% of patients had infections that, with regard to way of transmission, could be classified as 'sporadic'. Functional hepatic staging, measured by production of monoethylglycinexylidide (MEGX) and related to both histology and clinical score, was better in cases of hepatocellular carcinoma (33 ± 22 ng/ml) than in cases of cirrhosis (22 ± 17 ng/ml).

No biochemical, functional or histological change was related to a specific HCV genotype in patients with chronic hepatitis. Similarly, the analysis of Child-Pugh scores in cases of cirrhosis and hepatocellular carcinoma was not influenced by HCV genotype. Therefore, no modification of the parameters we studied seems to be peculiar of any particular viral strain at all stages of chronic liver disease.

However, after dividing patients into two groups only, that is those infected with genotype 1b and those with genotype non-1b, the former showed significantly higher levels of the main indexes of cholestasis (_-GT: p=0.02; ALP: p=0.03), and higher degree of biliar damage, even though at non-statistically significant levels, in patients with simple chronic hepatitis. In subjects affected with cirrhosis and hepatocellular carcinoma such differences were not detected.

When subjects were divided according to age groups, subjects with chronic hepatitis showed an increasing index of histological activity with older age, and differences between older and younger patients were statistically significant. Similar differences were registered for fibrosis and necrosis/inflammation scores (p=0.001 and p=0.04, respectively, between ages ≥50 years and ≤35 years). Also cirrhosis was increasingly serious with older age, while no case of hepatocellular carcinoma was present in younger subjects, and only two were less than 50 years old.

Genotype 1b was progressively more prevalent with increasing age of patients, not only overall, but also in a separate analysis for each level of liver disease divided by three age groups (chronic hepatitis: 18%, 39%, 53% genotypes 1b in subjects ≤35 years,

35-49 years and ≥50 years of age, respectively; cirrhosis: 0%, 44%, 73% genotypes 1b in subjects ≤35 years, 35-49 years and ≥50 years of age, respectively; hepatocellular carcinoma: 0%, 0%, 62% genotypes 1b in subjects ≤35 years, 35-49 years and ≥50 years of age, respectively).

Differently from genotype 1b, genotypes 1a (chronic hepatitis: 20%, 10%, 7%; cirrhosis: 33%, 22%, 0%; hepatocellular carcinoma 0%, 50%, 5% in subjects ≤35 years, 35-49 years and ≥50 years of age, respectively) and 3a 1a (chronic hepatitis: 36%, 21%, 1%; cirrhosis: 0%, 22%, 0%; hepatocellular carcinoma 0%, 0%, 5% in subjects ≤35 years, 35-49 years and ≥50 years of age, respectively) are more prevalent in subjects of younger age, whose way of transmission is more frequently represented by intravenous drug addiction and less connected with transfusions and 'sporadic' cases. This confirms our observation during the first phase of the project about prevalent genotypes according to transmission path of HCV (1).

Multiple logistic regression on demographic parameters associated with evolution of chronic hepatitis towards cirrhosis and hepatocellular carcinoma was performed dividing genotypes into 1b and non-1b. The results show that age and duration of exposure are the only variables independently associated with progression towards cirrhosis, while only age showed an independent effect on the development of hepatocellular carcinoma.

The importance of HCV genotype with regard to severity of clinical evolution and response to antiviral therapy was and is still the object of a lively debate in the scientific community. Our data on a remarkable number of subjects affected by different forms of HCV-related liver disease seem to support the hypothesis that a higher prevalence of genotype 1b in histologically more advanced forms of disease and in older subjects is due to a cohort effect. A wider circulation of HCV type 1b in the past and a more recent spread of other genotypes like 1a and 3a, together with a concomitant change in the prevalent way of transmission would further sustain such view.

Our study did not show an association between different genotypes and different forms of chronic liver disease. Patients with hepatocellular carcinoma over cirrhosis, although of older age, had a better clinical-functional staging than subjects with cirrhosis only. A possible explanation of this phenomenon lies in the fact that patients whose infection is more frequently sub-clinical are also exposed to the virus for longer times, with evolution to cancer development via still unknown mechanisms. In such perspective, the prevalence of HCV type 1b in older patients should be considered an epi-phenomenon of an older infection and a longer survival.

Histological and clinical staging of chronic hepatitis showed significant differences between extreme age groups. Further analysis on clinical staging of cirrhosis and hepatocellular carcinoma cases confirms that no viral genotype in the patients we studied was correlated with more severe forms of disease. This suggests that HCV infection follows a progressive but slow evolution, taking 15-20 years to cause significant clinical/histological changes, and that different genotypes are not responsible for such evolution. Moreover, a sub-division of patients in 15-year age groups better responded to the need of highlighting changes of prevalence of different viral strains and of ways of transmission. Overall, we observed that age of patients is one of the

possible determinants of disease. In our sample, with 76% ‘sporadic’ infections, such parameter resulted a valid surrogate for duration of exposure to the infection. As a matter of fact, age was associated not only with increasing severity of disease, but also reflected modifications of the path of transmission along time. Such conclusions are in line with those of other foreign and Italian researchers (2-4).

Multivariate logistic regression performed following the evidence of an increase of prevalence of genotypes 1b with increasing age shows that patients’ age and duration of chronic infection are the only variables associated with the progression of chronic hepatitis and cirrhosis. Age is the only variable associated with progression to liver cancer. Since only 20-30% of chronic hepatitis cases evolve to cirrhosis, age should not be regarded as the only risk factor. Other factors such as viremia and environmental factors are also likely to be important determinants of disease evolution.

Haemodialysis patients

Twenty patients seroconverted to anti-HCV in a single dialysis centre between April 1996 and January 1997 (attack rate = 35%). Nine cases were ascribable to HCV genotype 1b (45%), 2 to genotype 1a (10%), 7 to genotype 2a/c (35%), 1 to genotype 3a (5%) and 1 to genotype 4 (5%). Such distribution reflects prevalence of HCV genotypes among haemodialysis patients reported in our preliminary study (1).

Genome sequencing (5’-UTR region) was performed on all available samples from patients applying to the centre who were already anti-HCV positive before the outbreak occurred, and from subjects who were initially anti-HCV negative and seroconverted during the 10-month period. Rooms and monitors used by each subject as well as turns were identified. Viral strains were perfectly overlapping in 5 already positive and 12 seroconverting patients. All but 3 cases of seroconversion applied to the dialysis centre in the same turn as the carriers of their homologous virus (source of infection). Most seroconverters used different rooms from their sources of infection, thus strongly suggesting that dialysis apparatus was not involved in transmission, but that lack of compliance to standard operating procedures by ward personnel was most probably the cause of cross infection between patients. Viral strains remained unchanged during the follow-up (two years). ALT elevations were detected in 2 patients only, 1 of which had normal transaminases in the last 6 months.

The group of seroconverter haemodialysis patients, for which time of infection is precisely known, will be further followed-up in the future in order to better elucidate the relative importance of HCV genotype, time of infection, age and viral load in determining the evolution towards more serious forms of chronic liver disease.

Intravenous drug users

Data regarding genotyping and serotyping of samples from anti-HCV positive intravenous drug users during the first phase of the study had shown that a different

prevalence of subtypes existed between areas of the North (Liguria), Centre (Tuscany) and South (Campania) of Italy. In particular, the first two regions had a quite different prevalence of HCV subtypes, since HCV genotype 3 was prevalent in Liguria and HCV genotype 1 was prevalent in Tuscany.

The overall sample of studied subjects was enlarged to about 350 by involving also the Siena area in Tuscany. Although differences still exist, the resulting picture of HCV genotype prevalence is more homogeneous than before, with about 20% of cases of HCV infection in drug addicts living in Tuscany ascribable to genotype 1a, 15% to genotype 1b, 12% to genotype 2a, 45% to genotype 3 and 8% to genotype 4.

The distribution of HCV genotypes in drug addicts living in Liguria was confirmed at values of about 10% genotype 1a, 5% genotype 1b, 58% genotype 3a, 10% genotype 2a and 17% genotype 4.

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PCR AND SEQUENCING IDENTIFICATION OF DIFFERENT STRAINS OF HEPATITIS E VIRUS IN HUMAN AND ENVIRONMENTAL SAMPLES
Coordinator: Dott. Maurizio DIVIZIA

Progress Report

HEPATITIS E VIRUS IDENTIFICATION BY PCR IN HUMAN AND ENVIRONMENTAL SAMPLES

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Summary

In the present study we have evaluated the seropositivity for hepatitis E infection in a population of Alexandria, Egypt; more than 200 sera and stool samples were collected. The analysis showed a large diffusion of hepatitis E infection in this population whereas 4 stool samples were RT-PCR positive. Dual infection were also found in this group, whereas some risk factors and significant differences in the behaviour of two enterically hepatitis were identified. Tentative of HEV cultivation showed the possibility to growth the prototype SAR55 on the PLF/PLC/5 cells. One stool sample out of 4 RT-PCR positive was also positive after passage on the PLF/PLC/5 cells. Different results were obtained on the seroprevalence test using two commercial tests: Abbot and Genelab. The second test overestimated the percentage but the age distribution appeared similar. Preliminary results on multiplex RT-PCR using Poliovirus, Hepatitis A virus and Hepatitis E virus showed the possibility to use this test to evaluate the presence of different enteric viruses in one sample reducing time, expenses and number of samples. All the used environmental samples were positive with the exception of dried sludges.
Key words: *Hepatitis E virus, serology, molecular biology.*

Hepatitis E disease has been characterised by the absence of specific makers as in hepatitis A-B and C infection. Hepatitis E virus (HEV) is a member of Calicivirus family and susceptible subjects are infected trough the oral-faecal route, as hepatitis A virus (HAV), leading to a self-limiting acute episode. HEV and HAV are endemic in developing countries: Africa, East Asia and South America. Anyway, significant differences are present between HEV and HAV: the incubation period (six weeks for HEV and four for HAV), the highest death rate among pregnant women for HEV (10-20%) and the lower degree of infectivity of HEV.

In endemic areas, this enterically transmitted disease accounts for at least 4-50% of acute viral hepatitis in young adults, whereas in the industrialised countries this disease appears as sporadic cases associated with immigrants or tourists coming from endemic areas.

HEV has been transmitted to several primates and human volunteers, but, up to date, there are only three reports in literature about tentative of HEV cultivation.

Seroprevalence study have shown that, while hepatitis E is responsible up to 50 of acute hepatitis cases in young adults in developing countries, in Europe and North America the prevalence of anti-HEV antibody is very low: 0,1 - 3,0%.

In the framework of a Research Development Programme (CMT) at the Medical Research Institute (University of Alexandria, Egypt) were collected 202 serum and stool samples from acute hepatitis patients attending the Fever Hospital in Alexandria. The sera were analysed for anti-HAV IgM and IgG whereas the stool samples were analysed for total cultivable enteroviruses and HEV-HAV-RNA by RT-nested PCR. Serological analysis were performed by Abbott with the exception of anti-HEV IgM by Genelabs.

At the same time, information questionnaire, concerning drinking waters, sewage disposal, medical history and personal data, were obtained from each patients.

The age-range was 1-73 years old and the predominant symptoms were: jaundice, dark urine, nausea and vomit. Anti-HAV IgG (Table 1) was found in all the patients with the exception of a 32 years old female, anti-HAV IgM were found in 21 patients with an overall incidence of 10,4%.

Table 1. Class age and seropositivity for HAV and HEV

Class Age	IgM HEV	IgG HEV	IgM HAV
0-9	23,5	17,6	64,7
10-19	39,4	54,5	13,3
20-29	30,0	60,0	5,0
30-39	21,0	42,1	2,6
40-49	16,0	36,0	4,0
>=50	6,1	53,1	3,1

Anti-HEV IgG were found in 44,5% of all the patients with no significant difference for gender bias. Anti-HEV IgM were present in 49 sera (24,2%).

Significant differences were also observed in the class age distribution: the highest incidence for HAV IgM was in the class age 0-9 (64,7%) whereas HEV IgM

were better observed in the class age 10-19 (39,4%) with a progressive decline in the following class age reaching the lowest value (6,1%) in the class age >=50. This difference has been previously shown by other Authors.

A closer inspection of the age distribution revealed that HAV was contracted during the early years of the life with a mean age attack of 15,5 years whereas the mean age attack of HEV was 25,4 years.

The risk factor analysis (Table 2) of the 21 anti-HAV IgM positive patients indicated the contact with jaundiced family members (28,5%) and dental care (28,5%) showing a possible inter human (poor level hygiene) and blood transmission. All the other risk factors concerning drinking waters ad sewage disposal were non-significant maybe because of the early attack of the disease. Risk factor analysis indicated only two possible common sources of infection for both enteric viruses: the contact with jaundiced family members (28,5% and 17,7% for HAV and HEV respectively) and dental care (28,5 and 15,5% for HAV and HEV respectively). HEV positive patients got more frequently than HAV positive ones water from a common village tap source (12,2%) and indoor dry pit (13,3%) than HAV patients: 4,7% and 4,7% respectively.

Table 2. Comparison of potential risk factors of transmission of enteric hepatitis.

Potential risk factors	Hepatitis A	Hepatitis E
Contact with family members	28,5%	17,7%
Dental care	28,5%	15,5%
Village tap water	4,7%	12,2%
Indoor dry pit	4,7%	13,3%

No positive correlation was evident for HAV whereas a positive association was present between HEV and water pollution, showing a possible contamination of drinking water. The Alexandria sewage system consists of canals flowing into an inland lake (Lake Mariout) and, after a partial sedimentation, directly in the sea. These canals are also present in rural areas and the water is frequently used for irrigation purpose and for animal care. The statistical analysis confirmed that the highest risk to acquire hepatitis E disease is in the rural area versus the urban area ($p<0,01$, OR: 2,34, 95% CI: 1,17 - 4,74). Positive correlation was found between HEV positivity and common village water ($p<0,05$, OR: 5,13, 95% CI: 0,96-13,41), indoor dry pit ($p<0,01$, OR: 5,57, 95% CI: 2,14 - 15,08).

Given the large circulation of the two enterically transmitted hepatitis viruses and a common faecal-oral route of transmission, dual infection can be encountered. One sample (anti HAV IgM and HAV PCR positive) was also positive for Coxsackievirus, whereas another one (anti-HEV IgM and HEV PCR positive) was positive for an

enterovirus characterised by electromicroscope as Reovirus. One patient positive for HEV PCR and IgM was also HAV IgM positive.

Fifteen sample out of 21 anti-HAV IgM positive were also analysed for RT-PCR, 7 were positive (46,6%), whereas 4 out of 75 IgG/IgM HEV positive patients were RT-PCR positive (5,3%). The low number of RT-PCR HEV positive patients versus RT-PCR HAV positive patients can depend on the mean time of stool collection: 18,9 days (range 3 - 40 days) for HEV, whereas 9,89 (range 3 - 20 days) for HAV.

The preliminary sequence analysis of the 4 HEV-RNA positive samples showed that all nucleotide sequences were very similar to one another, and the comparative analysis showed maximum homology with the Burma strain. About 20 point mutations were found in the 4 different isolates without changes in the deduced aminoacid sequences.

As previously indicated HEV can be transmitted to several primates and human volunteers. In addition the virus has also been transmitted to laboratory rats and pigs. Liver cells from infected chimpanzees were maintenance in vitro for few months, but only three reports about in vitro cell infection were reported in literature.

We obtained stool samples from chimpanzee infected with the international SAR 55 strain (Pakistan prototype) from Pasteur Institute.

Different cell lines were tested to evaluate the possibility to support HEV replication: human fibroblasts, CaCO2, HT, RC and PLF/PLC/5 (Table 3). The stool samples were prepared in 10% suspension in PBS (phosphate buffered salts) and decontaminated by filtering through 0,22 um filters pre-treated with cell media containing 10% inactivated foetal calf serum.

Table 3. HEV (SAR55) growth on cell line

Cell line	**Passage and day post-infection**			
	1°	**2°**	**3°**	**4°**
PLC/PLF/5	+	+	+	+
HT	-	-	n.d	n.d
CaCO2	-	-	n.d	n.d
Vero	-	-	n.d	n.d
RC	-	-	n.d	n.d
Human Fibroblasts	-	-	n.d	n.d

Human fibroblasts, RC cells, CaCO2 and HT were scored as negative, only PLC/PLF/5 cells permitted HEV replication (as confirmed by RT-PCR) and were scored as positive until the 4th passage after 15-10-10 and 9 days post-infection respectively.

To confirm HEV replication on these cell lines, experiments were performed to reveal the presence of the negative replicative strand of HEV-RNA. These strands are produced only during the replication cycle and used as template for the polymerisation of the positive strand RNA to obtained new viral genome copies. Detection of the negative RNA strand in cell culture is indicative of viral replication. The cDNA product obtained after RT reaction was digested by Pancreatic RNase to destroy the viral RNA and avoiding non- specific amplification before employing the cDNA in the PCR test. A specific RT-PCR product was evident at 2,5 and 3,5 h post-infection.

To confirm the possibility of PLF/PLC/5 cells to support HEV replication, 75 stool samples, obtained from Fever Hospital in Alexandria (Egypt) and IgG/IgM positive, were used to infect the cells. Four samples, 3 IgM and 1 IgG anti-HEV positive, were scored positive by RT-PCR but just one was positive on cell culture. The sequence analysis (Table 4) of the PCR products obtained from stool and infected cells show sequences very similar to each other and the Burma strain. About 10-20 point mutations were observed but they do not provoke alterations of the deduced aminoacid sequences. Overall, we have observed one conservative substitution of aminoacid 56 (valine $\rightarrow$ isoleucine); for the other two isolates and the first passage of the only replicative isolate, the insertion of an Ant at the position 115 was observed.

Table 4. Sequences analysis of four HEV RT-PCR positive samples
Seq 1/2/3/4,

115	118	136	142	159	163	166	173	175	nt	
A	C	T	C	A	C	C	C	G		Seq 1
	T	C			T					Seq 2
	T	C			T					Seq 2/1
-	T					T		A		Seq 3
-			T	G		T	A	A		Seq 4

187	193	194	199	202	235	238	241	244	nt	
T	T	G	C	C	T	C	T	T		Seq 1
C				T	C	T	C	C		Seq 2
C				T	C	T	C	C		Seq 2/1
C	C	A	T		C	T				Seq 3
C	C	A	T		C	T				Seq 4

Seq 1/2/3/4= RT-PCR positive stool, Seq 2/1= First passage on cell

No differences were observed comparing the nucleotide sequences of the amplification products obtained directly from stool samples and from the first passage on cell culture.

No cytophatic effect was observed on cell culture infected by SAR55 strain and positive stool sample.

The possibility to have a susceptible cell line offers appropriate tool to evaluate the environmental distribution of HEV and to shed light on the molecular biology of this virus.

Hepatitis E diagnosis is still difficult to determine. The recovery of RNA genome by RT-PCR in sera is difficult to perform during an outbreak. We used two different commercial tests: Abbot and Genelabs for IgG and only Genelab for IgM. Both tests are prepared using recombinant antigens obtained from the ORF 2 and 3, that show the maximum homology among the different prototypes. Sera were collected during several months in Alexandria (Egypt) and kept frozen until the analysis.

Both tests show a large diffusion of HEV infection in the population even with a high difference: 46,1% Abbott and 82,9% Genelab. The seroprevalence obtained with Genelab appear too high but the Abbott value agree with the percentage found by several researchers. In the class-age 0-9 the Abbott test shows the lowest value (17,6%), these percentages increase to reach the maximum values in the class-age 20-29; for Genelab the lowest value is in the class-age 40-49 whereas the maximum value is in the class-age 10-19 (100%).

The overall positivity with the Genelab test is clearly over-valued whereas the class-age distribution and the ratio sample/cut off are similar to the Abbott data. Overall, 59,6% of sera (112 out of 188) agree in both tests versus 40,4% of difference 76 sera out of 188).

Forty-seven sera (25%) were positive for anti-HEV IgM, with the highest percentage found in the age group 10-19 (39,4%). In the IgM positive sera, the agreement for the anti-HEV IgG was 61,7%. The class-age distribution of the 47 positive sera for anti-HEV IgM was similar to the distribution of IgG positive sera.

Several enteric viruses can be identified in the environmental samples, whereas the cell culture isolation is time-consuming and expensive. For this reason we decided to evaluate a multiplex RT-PCR for testing different enteroviruses. Different samples, including seawater, dried sludge, faecal suspension and mussel homogenate, were sperimentally infected using several dilution of Poliovirus, Hepatitis A virus and Hepatitis E virus.

In the first part of our experiments, we evaluated different set of primers to show any possible cross-reaction. Each set of primers was found specific for each virus without any cross contamination. No particular difference were evident after RT-PCR using both random primers or specific primers, only the dried sludges were constantly negative with both primers showing the presence of aspecific or specific inhibitor of the RT reaction.

The multiplex RT-PCR offers a specific tools to study the virological environmental contamination evaluating the presence of more viruses in one reaction test reducing time, expenses and number of samples to evaluate.

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SELECTIVE LIVER DELIVERY OF RIBAVIRIN TO INCREASE ITS CHEMOTHERAPEUTIC INDEX IN CHRONIC HEPATITIS C
Coordinator: Prof. Luigi FIUME

Progress Report

SELECTIVE LIVER DELIVERY OF RIBAVIRIN TO INCREASE ITS CHEMOTHERAPEUTIC INDEX IN CHRONIC HEPATITIS C

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Summary

Ribavirin, (RIBV), when administered to patients with chronic hepatitis C in association with α-interferon (IFN), produces a sustained biochemical and virological response in a significantly higher number of patients than in the group treated with IFN alone. A drawback of RIBV is its accumulation in red blood cells (RBC), which produces haemolysis and causes the withdrawal of the drug in some patients. In order to obtain a liver targeting of RIBV, thus reducing haemolysis, this drug was conjugated to lactosaminated poly-L-lysine (L-Poly(LYS)), a galactosyl terminating peptide which selectively enter hepatocytes. L-Poly(LYS)-RIBV injected intramuscularly (IM) in mice was selectively taken up by the liver, where the drug was released in a pharmacologically active form. This was demonstrated in mice infected with murine hepatitis virus, in which coupled RIBV produced a 50 % inhibition of intrahepatic MHV replication at a daily dose 1.7 times lower than that of the free drug. Haemotoxicity of L-Poly(LYS)-RIBV was studied in primates. Free and conjugated RIBV were administered IM at the same dose (30 mg/kg/day) to cynomolgus monkeys. Both groups of monkeys developed anaemia, which was significantly more severe in animals treated with the free drug.

Assuming that RIBV enhances the efficacy of IFN in HCV infection treatment by acting within hepatocytes, these results suggest that conjugated RIBV may exert the same therapeutic effect as the free drug, but with a significantly reduced haemotoxicity.
Key words: *Ribavirin, chronic hepatitis C, liver targeting of drugs.*

Introduction

Ribavirin (RIBV) is a broad spectrum antiviral nucleoside analogue (NA) (1) which, when administered to patients with chronic hepatitis C in association with α-

interferon (IFN), produces a sustained biochemical and virological response in a significantly higher number of patients than in the group treated with IFN alone (2, 3). The mechanism of the beneficial effect of RIBV in this infection is not known. A drawback of RIBV is its accumulation in red blood cells (RBC) (4) which produces haemolysis (2, 3). This side effect causes symptomatic anemia in some patients and constantly raises the iron concentration within hepatocytes to levels (5) which can reduce the response to IFN (6).

We considered the possibility that a selective delivery of RIBV to the liver could lower the haemolysis, thus improving the therapeutic value of IFN and RIBV association. Since a liver targeting of NAs can be obtained by conjugation with galactosyl terminating peptides, which specifically enter hepatocytes through the asialoglycoprotein receptor (7), we coupled RIBV to lactosaminated poly-L-lysine (L-Poly(LYS)) (8-10). This galactosyl terminating peptide enables the preparation of conjugates injectable by intramuscular (IM) route (7) which in subchronic and chronic (26 weeks) toxicity studies in rats and monkeys displayed a good tolerability (RBM Exp. n. 970152 and 971057. Data on file at Laboratori Baldacci, Pisa, Italy).

Here we describe:

a) the synthesis and chemical characterisation of the conjugate (L-Poly(LYS)-RIBV).
b) its biological properties in mice (distribution in organs and erythrocytes, antiviral activity in liver, concentration of an active metabolite of the drug (RIBV triphosphate) in hepatocytes).
c) the effect of free and conjugated RIBV on haematological parameters in cynomolgus monkeys.

Materials and Methods

Preparation of the conjugate. - The conjugate was prepared as described in (8). Coupling of lactose was obtained by reductive amination. RIBV was conjugated via the imidazolide of its 5'-monophosphate derivative. [^{14}C]L-Poly(Lys)-RIBV was obtained by using [D-glucose-1-^{14}C] lactose. In order to prepare a conjugate labeled in the drug moiety, the commercially available radioactive RIBV could not be used because tritium was completely lost during the coupling procedure. Therefore, tritium was introduced into the RIBV molecule with a stable bond. The primary OH group of the sugar moiety of RIBV was oxidised and the nearly formed aldehyde was reduced with tritiated borohydride. The [^{3}H]RIBV preparation procedure is described in detail in (9). The chemical structure of [^{3}H]RIBV was confirmed by the ^{13}C NMR spectrum which showed the resonances of the parent compound. Tritiated RIBV was 98% pure, assessed by HPLC, and had a specific activity of 49,000 dpm/µg.

Organ distribution of the conjugate. - Distribution in liver, intestine, kidney and spleen of mice after intramuscular (IM) administration of the conjugate was measured by using the preparation labelled in the lactose moiety. The study of the penetration of the conjugate in mouse, human and monkey red blood cells in vitro was performed using a preparation labelled in the drug moiety. Experimental details are given in (8).

Determination of hepatic [^{3}H]RIBVTP. - The pharmacologically active metabolites of RIBV are the 5' mono-, di- and triphosphate derivatives (11). In mice injected with free or conjugated [^{3}H]RIBV liver concentrations of the triphosphate ([^{3}H]RIBVTP) were measured. L-Poly(LYS)-[^{3}H]RIBV was IM injected; free [^{3}H]RIBV was given by oral gavage (PO). Liver perchloric extracts, prepared as described in (9), were analysed by HPLC using an anion exchange column. A RIBVTP standard, synthesised according to (12), was added to each sample of liver extract; the amount of [^{3}H]RIBVTP was determined by counting the radioactivity eluting in the same position as the standard.

Study of antiviral activity. - It was performed using the Friend-Braunsteiner strain of Murine Hepatitis Virus (MHV), which was found to be sensitive to RIBV by Sidwell et al. (13). The virus was titrated on NCTC clone 1469 cells. Balb/C mice were inoculated intraperitoneally (IP) with approximately 100 CLD_{50} (lethal dose for 50% cultures in the titration assay). Free and conjugated RIBV were IM administered twice daily beginning 2 h before virus inoculation. Ten animals were used in each treatment group. Mice were killed 48 h after virus inoculation. Titration of virus in liver homogenates was performed on monolayers of NCTC clone 1469 cells, as described in (8).

Results

Chemical characteristics of conjugate. - The contents of Poly(Lys), lactose and RIBV in 1 mg conjugate ranged between narrow limits among the different preparations; they were 286-322, 238-260 and 312-327 μg, respectively. Considering that 1 mg Poly(Lys) contains 7.8 μmoles of lysine residues and that the molecular weights of lactose and RIBV are 342 and 244.2, respectively, it can be calculated that the ε-NH_2 groups of Poly(Lys) were substituted by the sugar and by the drug in the percentage ranges of 27-30 and 54-57, respectively. The solubility of coupled RIBV in NaCl 0.9 % was 64 mg/ml (=200 mg/ml of conjugate); however, by adding a dose of D(-)ribose equimolar to RIBV, the coupled drug can be dissolved to 150 mg/ml (=450 mg/ml of conjugate) maintaining a good fluidity. Moreover, ribose allows the conjugate to easily dissolve after lyophilisation.

Biological properties. - No clinical signs or behavioural alterations were observed in any of the five female Swiss mice (28-30 g) which received a single i.v. injection of the conjugate at the dose of 2 mg/g. The body weight gain, measured 3, 7 and 14 days after conjugate injection, was similar in treated mice and controls i.v. injected with saline.

The stability of the bond between RIBV and L-Poly(Lys) in blood was studied using L-Poly(Lys)-[^{3}H]RIBV, following the procedure described by Di Stefano et al. (8). L-Poly(Lys)-[^{3}H]RIBV incubated in mouse, monkey or human blood at 37°C for up to 6 hr did not release the drug.

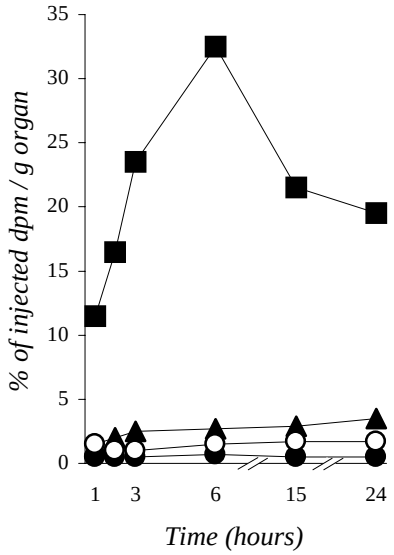

Figure 1 - *Distribution of radioactivity in liver (■), kidney (▲), spleen (●) and intestine (○) of female Swiss mice IM injected with [^{14}C]L-Poly(LYS)-RIBV (6.5 µg/g; specific activity 750 dpm/µg). Each entry represents the mean value of results from two animals. SE ranged from 1% to 7% of mean values. From Biochem Pharmacol 1997, 54: 357-363, with permission.*

Figure 1 shows that after IM administration to mice, [^{14}C]L-Poly(Lys)-RIBV was selectively taken up by the liver. Since the main toxic effect of RIBV is haemolysis caused by drug accumulation in RBC (4), the penetration of free and coupled [^{3}H]RIBV into human, monkey and mouse erythrocytes incubated in vitro was studied (8, 10).

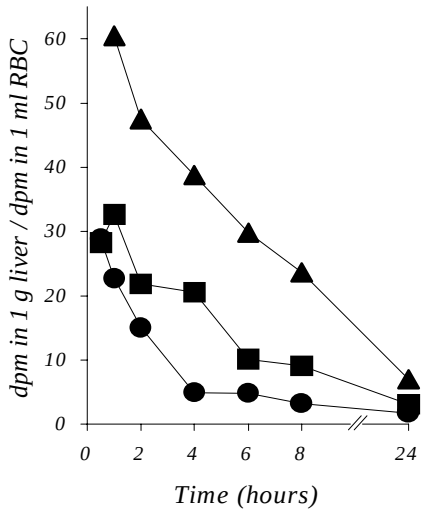

Figure 2 - *Ratios dpm in liver/dpm in RBC after administration of free [^{3}H]RIBV given PO (●) or IM (■) and conjugated [^{3}H]RIBV (▲) given IM. The AUC (0-24 h) were 114.20 (RIBV, PO), 241.15 (RIBV, IM) and 539.88 (conjugated RIBV). From Ital J Gastroenterol Hepatol 1997, 29:420-426, with permission.*

Moreover, the levels of radioactivity in liver and RBC of mice IM administered with free and conjugated radioactive drug were measured. In the experiments in vitro, free [^{3}H]RIBV entered in erythrocytes in large amounts. On the contrary the conjugate did not penetrate in RBC. Figure 2 shows the ratios dpm in liver / dpm in RBC in animals administered with the conjugate or with the free drug. In mice treated with the conjugate the trapezoidal Area Under Curve (AUC) (0-24 h) of this ratio was 2.2 or 4.7 times higher than in animals which received the free drug given IM or PO, respectively. Since the conjugate does not enter RBC in vitro and the bond between RIBV and L-Poly(LYS) is stable in mouse blood, the radioactivity detected in RBC of conjugate treated animals is probably due to [^{3}H]RIBV and/or its metabolites which partly went out from liver into the bloodstream after release from the carrier inside hepatocytes.

Liver concentrations of [^{3}H]RIBVTP (an active metabolite of RIBV (12)) were measured in mice which received L-Poly(LYS)-[^{3}H]RIBV IM or free [^{3}H]RIBV PO the route of drug administration commonly used in patients). The AUC (0-24 h) of [^{3}H]RIBVTP concentrations were found to be 2 times higher in mice injected with conjugated RIBV than in animals PO treated with the same dose of the free drug.

Antiviral activity. - The antiviral activity of the conjugate was studied in Balb/C mice with hepatitis caused by MHV (Friend-Braunsteiner strain). This virus, which replicates in mouse hepatocytes and causes liver necrosis, has been found to be sensitive to RIBV (14). Figure 3 shows the relation between the virus titer in liver and the daily

doses of free and conjugated RIBV administered to mice.

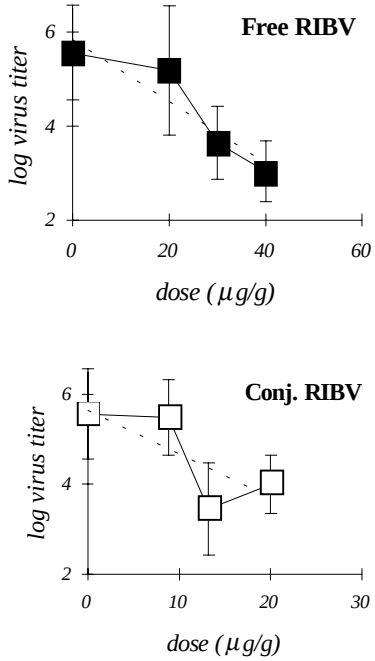

Figure 3 - *Relationship between the daily doses of free (■) and conjugated RIBV (□) and MHV titer (mean value ± SD) in liver. From Ital J Gastroenterol Hepatol 1997, 29: 420-426, with permission.*

The regression equations of titer versus dose, using all available individual data in the estimation, is: titer = 5.55 (±0.22) - 0.101 (±0.021) x dose with conjugated RIBV, and titer = 5.65 (±0.22) - 0.061 (±0.010) x dose with free RIBV. The estimation of the dose yielding a 50% decrease of virus titer yields, respectively, 27.4 and 47.2 µg/g. Thus, the antiviral potency of conjugated versus free RIBV, as either ED_{50} or slope ratio, is 1.7.

These results indicate that conjugated RIBV is released from the carrier inside hepatic cells in a pharmacologically active form. Moreover, they show that conjugated RIBV can display an antiviral activity in liver stronger than the free drug. To confirm the pharmacological equivalence in liver of doses of free and conjugated RIBV with comparable antiviral activity, the hepatic [^{3}H]RIBVTP concentrations were measured in mice administered with free or conjugated drug given by two daily IM injections at the dosages of 30 and 13.2 µg/g, respectively. Twenty-four hours after the first injection (and six hours after the second) in mice treated with 13.2 µg/g/day of conjugated [^{3}H]RIBV and in those administered with 30 µg/g/day of free [^{3}H]RIBV, the nmol of [^{3}H]RIBVTP per g liver were 10.3 and 10.5, respectively.

Haemotoxicity of free and coupled RIBV in cynomolgus monkeys. - Haemotoxicity of RIBV cannot be studied in mice since it is observed only in Primates. The study was

therefore performed in cynomolgus monkeys. Two groups of four age and weight matched females were treated for 15 consecutive days with the same dose (30 mg/kg/day) of free or coupled RIBV. Both groups of monkeys developed anaemia,

Table 1. Effect of free and coupled RIBV on monkey haematological parameters

	Days							
	0		15		21		49	
	Free	Coupled	Free	Coupled	Free	Coupled	Free	Coupled
RBC ($x\ 10^6$ / µl)	5.9 ± 0.3	6.5 ± 0.4 p = 0.275	3.3 ± 0.4	4.7 ± 0.3 p = 0.031	3.3 ± 0.5	5.1 ± 0.4 p = 0.031	5.5 ± 0.2	6.6 ± 0.4 p = 0.049
Hgb (g / dl)	11.8 ± 0.5	11.7 ± 0.5 p = 0.892	6.7 ± 0.9	8.5 ± 0.3 p= 0.107	6.7 ± 0.8	9.0 ± 0.2 p = 0.032	11.1 ± 0.5	11.5 ± 0.3 p = 0.518
Hct (%)	38.4 ± 1.4	39.1 ± 1.9 p = 0.777	22.0 ± 2.9	29.4 ± 1.4 p = 0.061	22.5 ± 3.1	31.4 ± 1.8 p = 0.029	38.2 ± 1.5	40.0 ± 0.7 p = 0.319
Platelets ($x\ 10^3$ / µl)	471 ± 52	28 ± 58 p = 0.116	683 ±70	451 ± 88 p = 0.086	744 ± 77	429 ± 89 p = 0.029	399 ± 22	302 ± 34 p = 0.054

Monkeys were maintained in an animal facility at the Istituto Superiore di Sanità (Rome) according to European Guidelines (EEC, Directive 86-609 Nov. 26, 1986). Drug administration was performed in two daily i.m. injections at ten hour intervals. For each injection the administered volume was 0.4 ml / kg. Drugs were dissolved in NaCl 0.9 %. Results are given as mean values ± SE.
(From J Hepatol 1998, 29: 1032-1033, with permission)

which was significantly more severe in animals treated with the free drug (Table 1). As observed in humans (3), RIBV increased the platelet counts, which were greater in free drug treated monkeys. Thirty-five days after the end of treatment haematological parameters returned to normal values in both groups of animals. Since the conjugate does not penetrate into monkey red blood cells and the bond between RIBV and L-poly(LYS) is stable in monkey blood (10), the residual toxic effect of L-poly(LYS)-RIBV on RBC is probably due to a partial release of RIBV (and its metabolites) from the hepatocyte into the bloodstream after the drug is set free from the carrier inside the cell. Actually, such a release was observed also in mice injected with L-poly(LYS)-[3H]RIBV (see above).

Conclusions

Assuming that RIBV enhances the efficacy of IFN in HCV infection treatment by acting within hepatocytes, the results reported here suggest that conjugated RIBV may exert the same therapeutic effect as the free drug, but with a significantly reduced

haematotoxicity. The conjugate might be the appropriate form of administration to patients where RIBV has to be withdrawn because of symptomatic anaemia or a decreased haemoglobin level associated with cardiovascular disease.

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EPIDEMIOLOGICAL, CLINICAL AND MOLECULAR CHARACTERIZATION OF THE HBeAg MINUS MUTANT IN ITALY
Coordinator: Prof. Giovanni Battista GAETA

Progress Report

EPIDEMIOLOGICAL, CLINICAL AND MOLECULAR CHARACTERIZATION OF THE HBV HBeAg MINUS MUTANTS IN ITALY

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Summary

The identification of HBeAg minus variant of HBV has led to the association of this virus to a severe form of chronic hepatitis B (HBsAg/anti-HBe positive chronic hepatitis, with continuous or intermittent presence of HBV-DNA in serum) (1-3). More recently, HBeAg minus variants of HVB have been detected also in cases with a benign outcome. This finding reinforced the concept that different factors which may influence the progression of the disease beside the presence or absence of the pre-core mutation. Some studies in patients with chronic hepatitis B showed that the wild type population increased before the ALT flare-up, while the HBeAg minus HBV was selected after this event (4). Thus, the relative amount of mutant and wild type virus (mt/wt) may vary in the different phases of the disease.

The impact of the HBeAg minus mutants in Italy may be substantial. In recent years, most of the Italian trials using IFN in chronic hepatitis B dealt almost exclusively with anti-HBe positive chronic hepatitis B; again, about 13% of liver cirrhosis and similar proportion of hepatocellular carcinoma cases are HBsAg positive, mostly with anti-HBe antibodies (5, 6). This may bring about economical and social costs by

striking the income producing ages and by inducing long term costly therapy with anti-viral agents.

These considerations have given rise to this research program, designed for strictly related objectives, i.e. 1) to assess the relative prevalence and the epidemiological profile of HBeAg minus variants in chronic hepatitis B in Italy; 2) to perform a molecular characterisation of the HBV e-minus variants; 3) finally, to make up a bank of sera from patients with chronic hepatitis B, which could be available for further studies. During the development of these main objectives, some cooperations gave rise to some ancillary studies.

Key words: *HBV, chronic hepatitis B, e-minus HBV, anti-HDV.*

Phases and methods of the research

The first phase of the research was a transversal, multicentre study during which all HBsAg patients observed over a 12 month period in the participating centres were enrolled. For each patient, a form was filled containing demographic data, risk factors, clinical and laboratory data and liver biopsy results (if available). For each patient, a serum sample was aliquoted, stored at -30°C and sent in dry ice to the coordinating laboratory.

Laboratory procedures. - Anti-HBc IgM antibodies were detected using Core M Kits (Abbott Labs). Quantitative HVB-DNA determinations were performed using Amplicor HVB-DNA Monitor Kits (Roche Diagnostic Systems).

Pre-core region characterisation. - After phenol/clorophorm extraction, HVB-DNA was amplified by polymerase chain reaction using commercial amplification kit (Cetus Corp., Emeryville, CA) and primers encompassing the precore and core region. The primer sequences were 5'GGGGAGGAGATTAGGTTAA3' (1744-1761) and 5' GGCAAAAACGAGAGTAACTC3' (1940-1959), respectively. Thirty cycles of amplification were carried out in a thermal cycler (9600 Perking Elmer Cetus, Emeryville, CA): 95C for 45'' (2'for the 1 st cycle), 55 C for 45'', 72 C for 45'' and final extension at 72 C for 5'. Amplicons were spotted on nitrocellulose filters, incubated with P32 5' end-labeled oligonucleotides without mutantions (5'TGGCTTTGGGGCATGGAC3') or without 2 nucleotide mutations (5' TGGCTTTAGGACATGGAC3') under stringent hybridisation and washing conditions and autoradiographed.

HBV genotyping. - After phenol/clorophorm extraction, HBV-DNA was amplified by polymerase chain reaction using commercial amplification kit (Cetus Corp., Emeryville, CA) and primers encompassing the pre-s [primer sequences were

5'GGAACAAGAGCTACAGCATG3' (2833-2852) and 5'GGTTGAAGTCCCAATCTGGAT3' (2955-2935)] and core [5'ACCACCAAATGCCCCTATCT3' and 5'CGTCTGCGAGGCGAGGGAGT3' (2399-2380)] regions. Thirty-six cycles of amplification were carried out in a thermal cycler (9600 Perkin Elmer Cetus, Emeryville, CA): 95C for 30" (1'for the 1st cycle), 50C for 20" 72 C for 20" and final extension at 72 C for 5'. Amplicons were detected by ethidium bromide staining and allocated to genotype A, B-C, D-E according to their size.

Analysis of the data. - The data were elaborated utilising BMDP and SPSS softwares.

Results and Comments

872 consecutive HBsAg positive patients were enrolled. On the basis of the hepatitis virus markers, the patients were initially classified as: 1) HBeAg positive carriers; 2) anti-HBe positive carriers; 3) Chronic HBV carriers with anti-HDV antibodies; 4) Chronic HBV carriers with anti-HCV antibodies; 5) Chronic HBV carriers with anti-HDV and anti-HCV antibodies (Table 1); data refer to 842 patients with no missing data. Anti-HBe positive chronic hepatitis B is, by far, the prevalent form of chronic hepatitis B in Italy, accounting for 76% of the cases; HBeAg positive cases were 9.4%. Interestingly anti-HDV antibodies were found only in 7.4% of the cases and anti-HCV antibodies in 6.5%. Males predominated in all etiological cathegories; HBeAg positive cases were younger than anti-HBe positive patients ($p<0.001$).

HBV-genotype was determined in 97 sera, randomly selected among all participating centres. Genotype D-E was present in 94% of the cases.

In a subgroup of patients, the relative amount of mutant and wild type HBV was determined (Table 2). About one third of the patients carried a mixed population (mt/wt) of HBV. Patients with mixed or prevalent e-minus HBV population had higher anti-HBc IgM index values (.575 and .539, median) vs patients carrying wild type HBV or with indeterminate result (.395 and .330, respectively) ($p<0.001$). Liver cirrhosis was present in 31% of the cases with prevalent e-minus population and in none of the patients with prevalent wild type virus or with indeterminate results.

Sequencing of pre-core and core-promoter region is in progress.

Table 1. Main characteristic of HBsAg chronic carriers.

	No. (%)	**Age (years) mean±DS median**	**Males No. (%)**
HBeAg pos.ve	79 (9.4)	28.2±14.4 25	61(77.2)
anti-HBe pos.ve	639 (76)	42.6±12.8 43	49.9(78.1)
anti-HDV pos.ve	62 (7.4)	42.3±10.5 43	44(77.2)
anti-HCV pos.ve	55 (6.5)	49.3±14.6 51	47(75.8)
anti-HDV and anti-HCV pos.ve	6 (0.7)	53.5±16.1 53	4 (66.6)

Table 2. HBV genotype and mt/wt in chronic HBV carriers.

Genotype (tested=97) • DE • A	 91 (93.8%) 6 (6.2%)
mt/wt (tested=53) • wild type 100% • HBeAg minus 100% • mixed • indeterminate	 10 (18.8%) 13 (24.5%) 17 (32.2%) 13 (24.5%)

Mutations in the HBV polymerase during famciclovir therapy. - The study investigated whether failure in famciclovir (FCV) therapy was associated to particular mutations in the HBV polymerase.

Seven patients with primary non-response and two with a breakthrough during therapy were analysed. YMDD motif and B region were conserved; V $\rightarrow$ I change at position 555 was observed in some patients, in whom lamivudine rescue therapy efficiently reduced viremia.

Prevalence and risk factors of HDV infection. - The overall prevalence of subjects with anti-HD antibodies was 8.2% (69/841); age specific prevalence were 5.9% in the age class 0-29 years; 9.5% in 30-50 and 7.9% over 50 years.

A univariate analysis of the associated risk factors of HDV infection showed that the presence of anti-HD antibodies was associated to residence in the South of the Country (OR=6.4; CL=2.6-16.2), having on anti-HD positive household member (43.7; 16.3-118), i.v. drug addiction (9.8; 2.9-33.1), sexual intercourse with on intravenous drug addict (15; 3.3-68.6), the presence of liver cirrhosis (3.7; 2.2-6.1). Conversely, blood transfusion, a low education level and the number of household members were not associated to the presence of anti-HD antibodies. The adjusted odd ratios obtained by a multiple logistic regression analysis (Table 3) showed that, when each variable was

adjusted for the confounding effects of all the other variables, the risk factors associated to HD infection were having an anti-HD positive household member, being an i.v. drug addict, to reside in the south of the Country. Moreover, a significant association was found to the presence of cirrhosis.

Previous Italian survey in 1987 and in 1992 showed a prevalence of anti-HD antibodies among HBsAg carriers of 23.3 and 14.4%, respectively. The study documented the decline of HDV endemicity in Italy.

Table 3. Adjusted odd ratios (OR) for presence of anti-HD antibodies

	OR	**C.I. 95%**	**p**
Age>40 years	1.18	0.59-2.4	0.63
Sex male vs female	1.07	0.51-2.28	0.85
Residence in the South	11.11	2.89-42.77	0.005
Anti-HD positive household	33.66	11.7-96.8	<0.0001
i.v. drug	8.07	1.42-45.87	0.0185
Sexual intercourse with TD	1.51	0.13-16.87	0.73
Cirrhosis	3.89	1.95-7.77	0.0001

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GENOTYPE CHARACTERIZATION OF DELTA VIRUS IN DRUG ADDICTS. HEPATITIS VIRUSES INTERACTION
Coordinator: Dott. Grazia Anna NIRO

Progress Report

GENOTYPE CHARACTERIZATION OF DELTA VIRUS IN DRUG ADDICTS. HEPATITIS VIRUSES INTERACTION.

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Summary

This study a) characterised, by direct sequencing, delta virus in drug addicts; b) evaluated the role of HDV in triple hepatitis infection, either in HIV +ve or -ve; c) determined HBV and HCV genotypes associated to HDV genotype I. Phylogenetic tree generated by both Parsimony and Maximum Likelihood programs showed two groups of HDV sequences: the first included 24 HDV sequences with a mean identity of 5.43%, the second 11 sequences with a mean identity of 5.41%. These clusters were not well defined when bootstrap analysis was performed. Mean percent difference among IVDU was 7.03% (range 2 -11.08%). When HDV sequences were compared, in IVDU subjects and controls, the total mean identity was unchanged (7.08 %). Among 35 HDV-RNA positive patients, antibodies to HCV were present in 85,7%, HCV-RNA in 22.9%, HBV-DNA in 68.5%, and antibodies for HIV in 60%. In 19 out of 24 HBV-DNA positive patients HBV sequence analysis has been performed. Mean distance among HBV sequences was 98%. Based on homology with the previously described 6 genotypes, in all but one HBV genotype D has been detected. HCV genotyping showed genotypes 1b and 3a in the 8 HCV-RNA +ve patients.
Key words: *Delta Virus, HDV subtypes, HBV/HCV genotypes.*

Introduction

Hepatitis delta virus (HDV) infection is a major problem among intravenous drug abusers. Being the more relevant group at risk of exposure to the HBV (1), drug addicts provide also the reservoir for HDV infection, particularly in low endemic areas (2). According with recent data the prevalence of HDV in Italy amounts to 24-37.5 % in drug addicts (3). Three genotypes of HDV have been identified world-wide, genotype I being the predominant one. Differences among genotype I, II and III were calculated, respectively, as 8.8% 23.3% and 30.7%. Inside genotype I, initial findings suggested the existence of subgroups IA and IB; however no subtypes appeared when several Italian and HDV strains collected from different parts of the world were compared (4).

Phylogenetic analysis of HDV sequences showed some correlation between intravenous drug use and clusters of HDV sequences, suggesting the hypothesis that variants of HDV might circulate among these subjects.

Previous studies described a pattern of rapidly progressive delta disease in intravenous drug users (IVDU) with death at a relatively young age. The relationship between HDV genetic variants and disease behaviour is constrained, particularly in drug addicts, by many factors, such as triple viral infection (HBV, HDV, HCV), AIDS development, and delta variants, each of them by itself affecting the outcome of the disease. Moreover, in multiple hepatitis virus infection, the viral interplay is complex and poorly understood (5). In the present study we have sequenced delta virus from intravenous drug addicts to establish whether particular genetic subtypes or strains prevail in this population.

Patients and Methods

Patients. - Thirty-eight anti-HDV positive patients with a history of drug abuse were included. Their mean age was 31 years, range 20-45 (3 females and 33 males), 26 patients came from different towns in the Apulia region, 11 from Northern Italy, and the last one from Sardinia. Mean duration of drug addiction was 9 years (range 2-20 years). They were hospitalised for evaluation of liver disease. A control population of 35 anti-HDV positive patients, without a history of drug addiction were also examined for comparison of sequence variations.

Serology. - All drug addicts were HBsAg positive anti-HBe positive. 33 of them were anti-HCV +ve and 21 HIV +ve, respectively 11 patients at stage A of the CDC classification, 5 at stage B and 5 at stage C.

HDV-RNA Detection and Sequencing. - HDV-RNA was extracted, reverse transcribed, and amplified using primers highly selected among genotype I isolates. A 357 nt segment between nucleotides 908-1265 was directly sequenced (4). Phylogenetic analysis was performed by using Parsimony and Maximum Likelihood programs.

HBV, HCV: PCR Detection and Genotyping. - HBV-DNA was also detected by PCR method. The amplified fragment included a 1123 bp fragment encompassing the major surface antigen-coding region (single PCR) and an internal 497 bp fragment (nested-PCR), as described (6). By direct sequencing a 380 nt fragment was obtained using the nested PCR primers.

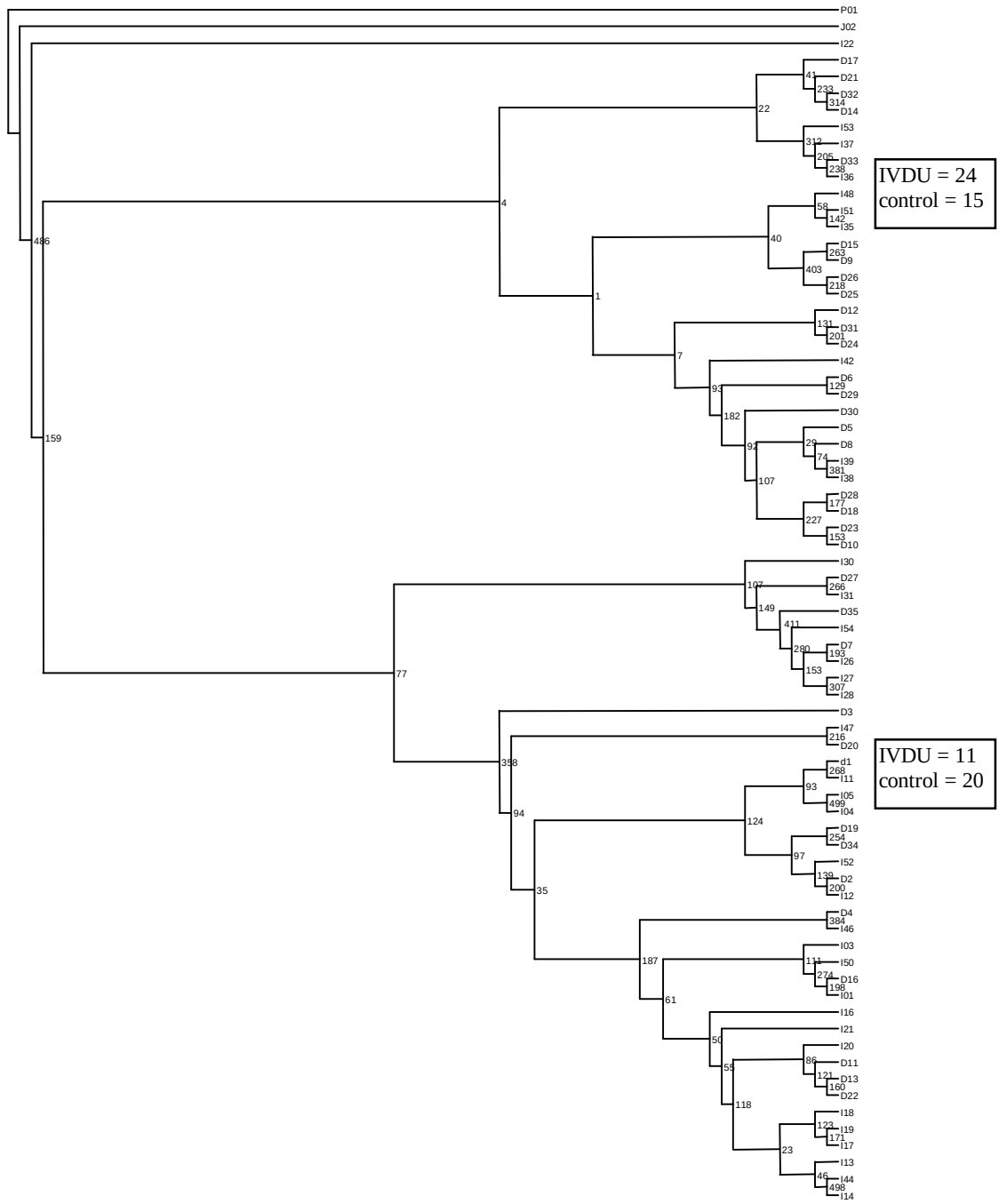
HCV-RNA was determined by reverse transcription and PCR amplification of 5' untranslated region. HCV genotyping was carried out using a probe-specific hybridisation assay, as described (7).

Results

Sequence Analysis of HDV-RNA. - Thirty-five out of thirty eight (92%) patients tested positive for HDV-RNA positive and underwent further analysis. All sequences were closely related to HDV genotype I; mean percent difference among IVDU was 7.03% (range 2 -11.08 %). Although both Parsimony and Maximum Likelihood analysis showed two groups of HDV sequences, these clusters were not well defined when bootstrap analysis was performed; mean identity inside each subgroup was, respectively, 5.43% and 5.41%. When HDV sequences were compared in IVDU subjects and controls, the total mean identity was unchanged (7.08%). Sequences from IVDU and controls were not evenly distributed among these two groups: the first one included 24 IVDU and 15 controls and the second 11 IVDU and 20 controls (Figure1). The segregation of IVDU and controls in distinct groups was statistically significant ($p = 0.03$) by Maximum Likelihood analysis. According to a previous study (8), a distance cut-off of $\leq 3.5\%$ between HDV sequences was able to determine the same viral strain of HDV. Among 2380 base pairs calculated between sequences, only 86 (3.6%) showed distances $< 3.5\%$; pair components were similar in 42 drug addicts and in 9 not drug addicts. In the remaining 35 couples a high similarity was found between sequences belonging to a drug addict and a control patient.

No significant correlation was found between sequence similarity and residence of patients; out of 42 pairs of highly identical sequences, only 11 belonged to subjects living in the same town.

Parsimony tree: HDV IVDU + Controls.



PCR Analysis of Viral Nucleic Acids. - Among 35 HDV-RNA positive patients, antibodies to HCV were present in 30 (85,7%), HCV-RNA in 8 (22.9%), HBV-DNA in 24 (68.5%), and antibodies for HIV in 21 (60%). Viral markers in these patients are given in Table 1.

Table 1. Profile of viral markers in HBsAg positive HDV-RNA positive intravenous drug abusers.

PATIENT	Age (yrs)	Anti-HCV	HCV-RNA	HIV	STAGE	HBV-DNA
D1	34	Neg	Neg	Neg		Pos II
D2	25	Pos	Pos	Pos	C3	Pos II
D3	24	Pos	Pos	Pos	B2	Pos II
D4	31	Pos	Neg	Pos	A2	Pos II
D5	34	Pos	Neg	Pos	B3	Pos II
D6	26	Neg	Neg	Neg		Pos II
D10	37	Pos	Pos	Neg		Neg
D11	24	Pos	Neg	Neg		Neg
D12	35	Pos	Neg	Pos	C3	Pos I
D13	28	Neg	Neg	Pos	A1	Pos I
D14	32	Pos	Pos	Pos	C3	Pos I
D15	33	Pos	Neg	Neg		Neg
D17	32	Pos	Neg	Pos	A2	Pos I
D19	39	Pos	Pos	Neg		Neg
D21	39	Pos	Neg	Pos	C3	Pos I
D24	28	Pos	Neg	Pos	A2	Pos I
D22	37	Neg	Neg	Pos	B3	Pos II
D23	33	Pos	Neg	Pos	A2	Neg
D26	33	Pos	Neg	Pos	C3	Pos I
D27	30	Pos	Neg	Pos	A2	Pos II
D28	39	Pos	Neg	Pos	B3	Pos II
D29	33	Pos	Neg	Pos	A1	Pos II
D30	20	Neg	Neg	Neg		Neg
D31	38	Pos	Neg	Neg		Neg
D32	20	Pos	Neg	Neg		Pos II
D33	27	Pos	Neg	Neg		Neg
D34	26	Pos	Neg	Neg		Neg
D7	21	Pos	Pos	Pos	B2	Pos I
D8	24	Pos	Neg	Pos	A1	Pos I
D9	35	Pos	Neg	Neg		Neg
D16	45	Pos	Neg	Neg		Pos II
D18	26	Pos	Neg	Neg		Pos I
D20	25	Pos	Pos	Neg		Pos II
D25	22	Pos	Pos	Pos	A2	Neg
D35	40	Pos	Neg	Neg		Pos I

In the 17 HIV positive patients with triple infection (HDV, HBV, HCV) 5 were HCV-RNA positive and 15 HBV-DNA positive. In the remaining 13 HIV negative, 3 were HCV-RNA positive and 5 HBV-DNA positive (Table 2).

Table 2. Viremia in pts HDV-RNA +ve with triple infection.

	N° PZ	**HBV-DNA**	**HCV-RNA**
HIV+	17	15	5
HIV-	13	5	3

Genotype Analysis of HBV and HCV. - In 19 out of 24 HBV-DNA positive patients HBV sequence analysis has been performed. Mean distance among HBV sequences was 98%. Based on homology with the previously described 6 genotypes, in all but one HBV genotype D has been detected; in pt D32 HBV genotype A was identified.

Genotype 1b has been detected in 5 out of 8 HCV-RNA positive patients, genotype 3a in the remaining 3 patients.

Discussion

In the past few years several studies have analysed HDV genetic variability. More than 200 isolates have been identified and sequenced, 93% of them belonging to Genotype I and 63% of them coming from European patients. A mean genetic distance of 8 % inside Genotype I has been reported, with a wide range from 0 to 23.2% (4). These data show the overwhelmingly predominance of genotype I and the dispersion of sequences inside Genotype I. This last aspect opens the discussion about the definition of HDV sub-types.

When HDV genotype distinction was first proposed, at least 2 subgroups within genotype I were identified (IA and IB). The work of Zhang (9), who collected sera from 13 HDV patients, defined three distinctly separated clusters within genotype I. The first cluster corresponded to HDV Genotype IA and consisted of samples from Italy, France, Sweden, the United States and Kuwait (the western branch); the second cluster corresponded to HDV Genotype IB and consisted of isolates from mainland China, Taiwan, Nauru, Moldavia, and Bulgaria (the eastern branch); the third cluster, designated HDV Genotype IC, contained the six Ethiopian HDV isolates and the isolates from Jordan, Lebanon, and Somalia (the African-Middle East branch). The mean genetic distance between African and eastern branch was 0.14, and that between the African and western branches was 0.15. The western and eastern branches were calculated to have a mean genetic distance of 0.10.

Subsequent studies, involving a higher number of sequences, while confirming the 3 HDV genotypes, were unable to distinguish major subgroups within genotype I. The major studies (10, 4, 11) with respectively 141, 46 and 33 sequences evaluated identified clustering of sequences, a result that could be supported by bootstrap analysis. Suggestive correlations between HDV-RNA sequence similarity, geography, and transmission route have been found (4). Geographic clustering, beside, was not absolute. Our study points towards a possible HDV subtype, also in a population homogeneous for route of transmission, such as those of drug addicts. Beside clusters of sequences with higher similarity among drug addicts and controls, we could exclude the existence of phylogenetically distinct subtypes within HDV genotype I. Based on wide dispersion of HDV sequences in Italy, we can postulate that HDV infection has been persisting since long time and /or HDV was introduced by multiple inocula over times. Among drug addicts, sharing contaminated paraphernalia, a rate of higher identical isolates could be expected; however the sequence distribution, could not be related to a particular HDV sub-type.

Many studies, attempted to evaluate the viral interplay in patients with HBV, HCV and HDV infection.

HCV infection may inhibit the replication of HDV, as HDV-RNA and IgM anti-delta antibodies were undetectable in patients with dual HDV and HCV infection (12). On the contrary, HCV-RNA absence, both from serum and liver, in HDV positive showed the inhibitory effects of HDV on HCV (5). The mode of acquisition of HDV infection (whether coinfection or superinfection), the sequential of infections, the immune status in coinfected individuals can all account for such disparate data.

In our patients with triple infection (HCV, HBV, HDV) a low rate of HCV replication (22.9%) has been observed independently of the HIV status. HBV-DNA, instead, was detectable in 88% of HIV +ve but only in 31% of HIV-ve. Data on suppression of HCV replication have been previously reported in HIV infected haemophiliacs with chronic hepatitis B and C (13).

The mechanism by which HDV inhibits HCV is unknown but it seems of great interest for therapeutic implication; the persistent inhibition of HDV on HCV in HIV patients could exclude the prevalent role of immune-system.

In patients chronically infected with HBV and HDV, HBV-DNA levels are usually suppressed, although HBsAg production continues. Either in tissue culture or in infected animals it is unclear whether the suppression of hepadnavirus replication results from a direct interaction between HDV and HBV functions. The inhibition of HBV replication could indirectly result from HDV-induced cytotoxicity during the acute phase. Alternatively, the HDV core might be in direct competition with that of HBV for budding envelope particles, therefore inhibiting the production of the helper virus (14).

Severe immunosuppression in progressive HIV disease could theoretically ameliorate the manifestations of an immune-mediated hepatic damage. We could speculate that the decrease of inflammation can lift the inhibitory activity of HDV over HBV. Such a mechanism, however, appears ineffective on HCV.

HBV genotype A and D are predominant in Europe. In our area studies on HDV genotypes which coinfect HBV are lacking. Available data showed an association, in Northern-South America, between HDV genotype III and HBV genotype F (13). Our findings could mirror either the epidemiological distribution of genotypes in specific geographic areas or a better viral interaction.

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HEPATITIS B VIRUS (HBV) REINFECTION IN LIVER TRANSPLANT PATIENTS RECEIVING HEPATITIS B IMMUNE GLOBULIN: ROLE OF S MUTANTS

Coordinator: Prof. Giuseppe PASTORE

Progress Report

HEPATITIS B VIRUS (HBV) REINFECTION IN LIVER TRANSPLANT PATIENTS RECEIVING HEPATITIS B IMMUNE GLOBULIN: ROLE OF S MUTANTS

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Summary

HBV reinfection of transplant livers occurs frequently even in the presence of anti-HBs immunoglobulins. In this study we retrospectively evaluated 209 HBsAg positive patients liver-transplanted for chronic end-stage liver disease and continuously treated with polyclonal anti-HBs (HBIg) in order to investigate the type and role of S variants in HBV infection of the graft. According to transaminase and HBV-DNA hybridisation analysis, 50/209 (24%) liver transplant patients had HBV and hepatitis recurrence during anti-HBs therapy. In 12 patients with HBV reinfection for whom both pre-transplant and reactivation serum samples were available, the S region of the viral genomes was amplified by PCR, and directly sequenced. Overall, 10/12 patients (83%) were found to be infected with S mutant viruses during reinfection, whereas the remaining two patients had an unchanged S sequence. In particular, in 9/10 patients HBV S-gene mutants were identified as the dominant population at reinfection but not pre-transplantation.

These results indicate that HBV reinfection is mainly due to selection under the pressure of polyclonal anti-HBs therapy of S-gene escape HBV mutants which then became the dominant virus population in the grafts. However, it appears that occasionally S mutants can be present before starting immune-prophylaxis. According to previous reports the G145R S-gene escape variant was that most commonly found.

Key words: *HBV variants, S-gene mutant, hepatitis B immunoglobulin, graft infection, liver transplantation, HBV reinfection.*

Introduction

Recurrence of HBV infection in patients who undergo liver transplantation for end-stage chronic hepatitis B is a frequent complication which can result in progressive liver disease and in some cases in graft failure. The use of peri- and post-operative hepatitis B immunoglobulin has reduced this risk and has led to an increase of patient survival. Nevertheless, in some liver transplant patients, HBV reinfection occurs despite immune-prophylaxis. In some of these subjects, HBV variants with mutations in the S gene have been found, resulting in amino acid changes in the “a” determinant.

Although various escape variants have been found in some transplant patients treated with mono- and/or polyclonal anti-HBs antibodies, the type and role of S variants in HBV infection of the graft remains to be studied more extensively in order to clarify the epidemiological and clinical aspects of these mutants. This is a prerequisite for the improvement of strategies to avoid HBV reinfection.

We therefore retrospectively evaluated 209 HBsAg positive patients liver-transplanted for chronic end-stage liver disease and continuously treated with HBIg in order to investigate the type and role of S variants in HBV graft infection.

Patients and Methods

A retrospective study was conducted of 209 patients who underwent liver transplantation for HBsAg-positive end-stage liver disease. Of these, 167 patients were male and 42 female; the mean age was 43 years (range 21 to 61). Delta co-infection was present in 97 patients, whereas 19 and 17 patients showed serological evidence of HCV and HDV/HCV infection, respectively.

Patients intravenously received 10.000 IU/l of commercial polyclonal HBIg during the anhepatic phase and daily during the first 6 post-operative days. The level of anti-HBs was periodically assessed and anti-HBs were again administered intravenously or intramuscularly when the anti-HBs titre was less than 200 U/l. All patients were followed for at least 6 months (range 6-48), and periodically underwent liver function tests. In addition, HBV, HDV and HCV serological markers were repeatedly tested.

HBV, HDV and HCV serological markers were detected by commercial immunoenzymatic kits (Abbott Laboratories, Chicago, Ill., USA; Sorin Biomedica, Saluggia, VC, Italy). HBV-DNA was quantified by a sandwich capture molecular hybridisation assay (Digene Diagnostics, USA). Viral DNA from serum samples obtained before transplantation and during HBV infection from 12 patients with recurrent hepatitis B was extracted with phenol/chloroform, and the S region was amplified by polymerase chain reaction using synthetic oligonucleotide primers 66 (plus) (5´-GCTCCAGTTCAGGAACAGTAAACCC) and 1121 (minus) (5´-AGAAAGGCCTTGTAAGTTGGCG). After purification of the amplification products, both strands were directly sequenced on an automatic sequencer (Perkin Elmer 377).

Results

During post-transplantation follow-up, HBV reinfection, characterised by the appearance of HBsAg and HBV-DNA in the serum and by clinical and/or biochemical symptoms of liver disease, was observed in 50/209 patients (24%) after a mean period of 11 months (range 1-84 months). Before liver transplantation, HBV DNA was positive by molecular hybridisation in 30% of reinfected patients, while only 4% of the patients who were never reinfected were viremic. Among patients with HBV reinfection, end-stage liver disease was due to HBV infection in 29 patients (58%), to HBV/HDV co-infection in 12 (24%), to HBV/HCV co-infection in 7 patients (14%), and to HBV/HDV/HCV infection in 2 (4%).

In 12 patients with HBV reinfection for whom both pre-transplant and reactivation serum samples were available, serum HBV DNA was extracted and the region S was amplified by PCR and directly sequenced. Overall, 10/12 patients (83%) were found to be infected with S mutant viruses during reinfection, whereas the remaining two patients had an unaltered S sequence. The type of mutations observed are shown in Table 1.

Table 1. Amino acid changes in the S region during HBIg treatment in 12 liver transplant patients with HBV reinfection

Patients	Pre-Transplantation	Post-Transpantation
1	G145R	G145R
3	Wt	G145R
1	Wt	G145A
1	Wt	D144A G145A
1	Wt	D144E G145R
1	Wt	D144K G145E
1	Wt	G145R W163G
1	Wt	L162H W163G
2	Wt	wt

In particular, in 9/10 patients, the S region of viral genome showed mutations only in the sera obtained during reinfection, but not in the samples taken before transplantation. In the remaining patient, S mutant HBV was already present before liver transplantation.

Discussion

Of the 209 HBsAg-positive patients liver transplanted for end-stage chronic liver disease and treated continuously with polyclonal HBIg, 50 patients (24%) experienced HBV reinfection. This occurred more frequently in patients with HBV- or HBV/HCV-related cirrhosis than in HBV/HDV co-infected patients (38% and 37% respectively vs 12%). This finding is in agreement with the fact that HBV is suppressed by co-infection with HDV and suggests that transplant patients with HBV/HDV have a lower risk of HBV liver graft reinfection when appropriately treated with HBIg.

According to our preliminary results (1, 2), the amino acid at position 145 changed from glycine to arginine (G145R) in the majority of reinfected patients studied. This mutation is located in the so-called “a” determinant of HBs which represents at least part of the major neutralising epitope of all HBV isolates known thus far. Interestingly, in three patients, the HBs protein of the viral population during reinfection had an additional amino acid mutation at position 144 in the “a” determinant.

If S-gene mutant viruses are present at undetectable levels before liver transplantation or if they are randomly produced during viral replication, it is only a matter of time before the new liver becomes infected. Since the S-gene escape variants found in our study are recognised to a lesser degree by human anti-HBs antibodies and HBIg, it is very likely that they escaped neutralisation by HBIg, and consequently could amplify and infect the graft. This hypothesis is supported by other recent studies and by the fact that the same and similar S-gene escape mutant viruses have been found in breakthrough infections of anti-HBs positive vaccines. Another possible mechanism to explain the HBV reinfection in the two patients with wild-type virus both in the pre- and post-transplant serum sample is to assume reactivation of viral replication under immunosuppressive therapy. In this case, failure of passive immunisation would be mainly due to the exceedingly high levels of viremia present at some point in time. If the HBIg were not be able to neutralise all the virus present, reinfection of the graft would be triggered by “wild-type“ virus.

In conclusion, these results indicate that HBV reinfection is frequently associated with selection under the pressure of polyclonal anti-HBs therapy of S-gene escape HBV mutants which became the dominant virus population in the grafts. However, it appears that S mutants can occasionally be present before starting immune-prophylaxis. According to our study and previously published reports, the G145R S-gene escape variant was that most commonly found.

As the S gene overlaps with the polymerase gene, mutations in the amino-acid sequence of the HBsAg can affect the major catalytic region of the viral polymerase protein and viceversa. Therefore, considering that inhibitors of viral polymerase such as lamivudine and famciclovir are increasingly used in liver transplant patients to prevent or treat HBV reinfection, it is important to study the potential influence of drug-induced mutations and resistance in order to transfer this knowledge to therapy management.

HUMAN HEPATITIS A VIRUS: MOLECULAR MECHANISM(S) RESPONSIBLE OF THE HOST-CELL DEATH

Coordinator: Prof. Raul PEREZ BERCOFF

Progress Report

HEPATITIS A VIRUS: MOLECULAR MECHANISM(S) RESPONSIBLE FOR THE HOST-CELL DEATH

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Summary

Human Hepatitis A is an infectious disease hyperendemic in numerous affluent and in all developing countries as well. Systematic immunological surveys reported by W.H.O. indicate that in Southern Europe (including Italy, Spain, Portugal and Greece) ca. 80% of the population over the age of 20 is seropositive for Hepatitis A virus (HAV) (against 3% in Scandinavian countries).

In South America 90% of the low income population bear serological evidence of past infection. In both continents, Hepatitis A accounts for 50% of all acute Hepatitis reported and presents an **ascertained mortality** of 0.5%, i.e: one fatal case every 200 infected persons, and a recent report from French Polynesia indicate that this figure may be **7 fold higher in children**.

The **ethiological agent** of Hepatitis A is a **picornavirus** of the hepatovirus group which, in contrast to all members of this family, replicates very slowly in tissue culture (several days instead of hours), with little production of viral proteins.

Accordingly, we proposed:

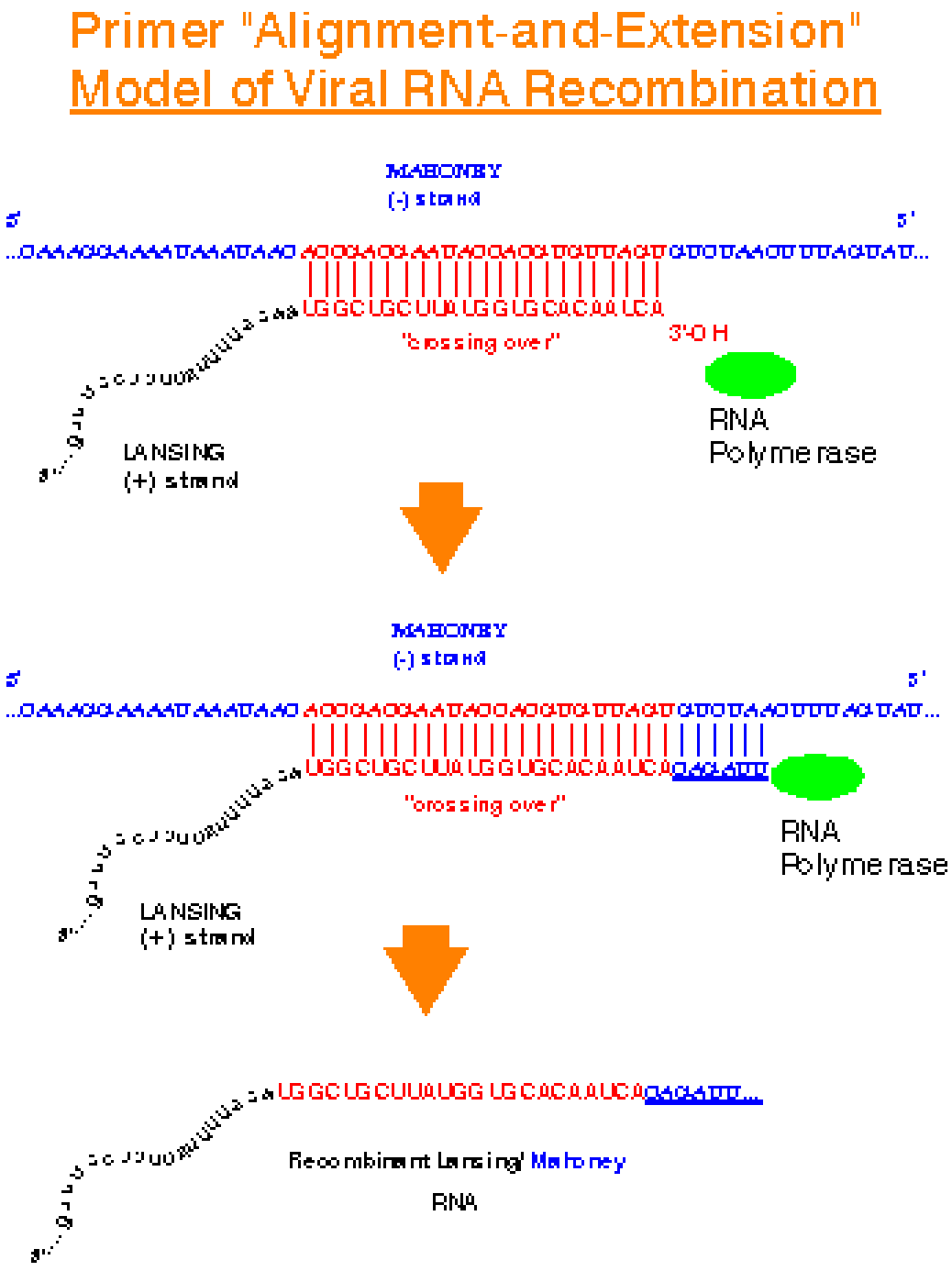
a. to ascertain whether the cellular translation initiation factor eIF-4G is cleaved as a result of HAV infection;
b. to establish the function(s) of the viral protein 2A, and to characterise its functional domains by mutational analysis;
c. to investigate whether the delayed inhibition of host-cell protein synthesis was due to a down regulation of translation of HAV-RNA;
d. to determine whether the short ORFs started by alternative translation initiation codons and present in the 5'-terminal extra-cistronic region of HAV RNA play a role in infectivity and viral tropism.

Key words: *Hepatitis A virus, molecular virology, viral replication.*

Final Activity Report

The work conducted by this Unit within the frame of the project led to the following results:

a. we **uncovered the molecular mechanism responsible for the recombination of viral RNAs,** a mechanism that we called of *"primer alignment-and-extension"*. The novel model comes to explain how RNA viruses (positive or negative strand) recombine in nature (a process of outmost importance in viral evolution) and predicts the limits of this process. The model is depicted in Figure 1 (cf. "Expanded Report"). The implications of the model did not pass unnoticed (especially in view of the well-known fact that recombinant Sabin/wild type poliovirus has been isolated in the course of recent outbreaks in Israel, Rumania and Albania), and following the publication of this paper we have been invited to illustrate the model in seminars at the Pasteur Institute of Paris and Brussels, and at Universities in Berlin, Poznam, Warsaw, Montréal, Buenos Aires, Versailles, Nantes, and to lecture as keynote speaker at international conferences of virology (Sydney and Budapest);
b. in the course of these studies, we **identified** a short Open Reading Frame (ORF) started with the alternative translation initiation codon ACG (**a "hidden" gene ?**) at position 157-159 of poliovirus RNA, whose integrity was indispensable to secure the infectivity of poliovirus cDNA clones. The implications of these findings were extensively discussed in the paper reporting these results.
   Work is in progress to check whether the genomic RNA of HAV carries also a similar "cripto-gene", and its role in infectivity and tissue tropism of HAV;
c. we proved that (in open contrast to previous claims by others) the two Pyrimidine-Rich Tracts (PRTs) present in HAV-RNA play a major role in regulating the translation ability of the viral RNA and, therefore, the viral infectivity;
d. we were able to establish that the too short sequence extending between the 3'-PRT of HAV-RNA on the one side, and the AUG initiation codon on the other, (the *"spacer"*) is responsible for the down regulation of translation and consequently for the slow replication and scarce infectivity of the virus;
e. we constructed cDNA clones of HAV of increased infectivity in which the *"spacer"* in the 5'-ECR sequence had been properly elongated;
f. we demonstrated that (in open contrast to previous claims by others) that the expression of peptide 2A of HAV abrogates cap-dependent (but not internal) initiation of translation by a mechanism reminiscent of that used by entero-, rhino-, or aphtho-viruses;
g. we firmly established that the boundaries of peptide 2A of HAV are located at amino-acids 791 and 981 of the viral polyprotein;
h. mutational analysis of this peptide, allowed us to identify the triad Ser_{916}, His_{927}, and Asp_{931} stringently required for the inhibitory effect, suggesting that the inactivation of the cellular translation initiation factor eIF-4G may be involved in the process;
i. in collaboration with Drs. Jean COHEN and Didier PONCET (Jouy-en-Josas, France) work is in progress to provide direct experimental evidence of the physical cleavage of the cellular factor as a result of the expression of HAV 2A;



j. we have constructed a series of expression vectors that upon induction with IPTG are able to over express the peptide 2A of Polio- or HAV in E. coli. In collaboration with Drs. Jean COHEN (Jouy-en-Josas) and Félix REY (Gif-sur-Yvette, France)

work is in progress to purify the over expressed proteins 2A of these viruses, crystallise them in an attempt to establish their tertiary structure;

k. Under the auspices of the European Community and in close collaboration with the laboratories directed by:
Sylvianne BILLAUDEL, Nantes, France
Esteban DOMINGO, Madrid, Spain
Albert BOSCH, Barcelone, Spain
Juán CRISTINA, Montevideo, Uruguay
Cristina MOGDASY, Montevideo, Uruguay
Victor ROMANOWSKI, La Plata, Argentina
Hugo FAINBOIM, Buenos Aires, Argentina
Eugenio SPENCER, Santiago, Chile
Inés VEGA-RODRIGUEZ, Valdivia, Chile
Maureen TAYLOR, Pretoria, South Africa,
Jane YEATS, Cape Town, South Africa

We are currently directing a molecular epidemiological program to ascertain the presence of serological variants of HAV circulating in extra-European countries.

Expanded Report

A. - "Primer Alignment-and-Extension", a novel model of viral RNA recombination responsible for the rescue of lethal mutants of poliovirus cDNA clones. - We had proposed to ascertain whether the 11 short open reading frames (ORFs) initiated by alternative initiator codons GUG (Val), ACG (Thr), or AUA (Ile), present in the long 5'-terminal extra-cistronic region (5'-ECR) of HAV-RNA (nts. 1-734) were indeed devoid of coding capacity, or else they may be translated into small peptides (6 kDa). To that end, studies were first conducted on poliovirus, the paradigm of enteroviruses, as a first step towards the identification of a putative coding function of the 5'-ECR.

The genomic RNA of poliovirus type 1 (Mahoney strain) contains 10 short open reading frames (ORFs) starting with the alternative translation initiation codons GUG, AUA, and ACG in the sequences 5'-distal to the main ORF that codes for the viral polyprotein. Mutations introduced in all but one of these mini-cistrons had no effect on the infectivity of full-length cDNA clones, except when they modified a "hidden frame" spanning nucleotides 157-192 (starting triplet: ACG).

The minicistron 157-192 is conserved in position, length and sequence in the genome of all types and strains of poliovirus. Adaptation to rat (Lansing) or mouse (variant of Sabin 2) is accompanied by a consistent pattern of changes in the primary sequence of this *"hidden frame"*. The substitutions that abrogated the infectivity of cDNA clones were not expected to modify the predicted secondary structure of the 5'-terminal extracistronic region (5'-ECR), and they did not alter the ability of the IRES to direct internal initiation of translation in bi-cistronic mRNAs.

The infectivity of the mutated poliovirus cDNAs could be rescued in *trans* by co-transfecting the target COS-1 cells with an expression vector containing just the 5'-ECR of poliovirus type 2 (Lansing strain). Direct sequence analysis of the viral RNA revealed that the infectious virus recovered by co-transfection were recombinants Lansing/Mahoney, with points of *"crossing-over"* that varied from one experiment to another. The infectivity of poliovirus cDNA could be restored by co-transfecting short RNA transcripts of the wt 5'-ECR (Lansing), suggesting that both the complementation in *trans*, and the recombination of poliovirus genomes observed in nature involve a novel mechanism of RNA recombination that we propose to call *"primer alignment-and-extension"*.

These results open new ways to understand the peculiar replication cycle of HAV, its hepatotropism and possibly **how the circulating variants and strains of HAV may undergo genetic recombination in nature.**

B. – HAV-RNA: Role of the 5'-most and 3'-distal Pyrimidine-rich Tracts (prts) in Regulating Internal Initiation of Translation. - We proposed to investigate whether the delayed inhibition of host protein synthesis induced by HAV was due to down regulation of translation of its genomic RNA, and more specifically, to investigate the role played in this process by the pyrimidine-rich tracts (PRTs) present in the 5'-terminal extracistronic region (5'-ECR).

Protein synthesis directed by HAV-RNA is mediated by a mechanism involving the recognition of internal sequences. Two in-frame AUG codons initiate the long open reading frame (positions 734-6 and 740-2). The extra-cistronic region extending between the uncapped 5'-end and the ORF contains two PRTs: one 12 nucleotides in length in the close vicinity of the initiators AUG, and a longer one between bases 94-140.

cDNA representations of the 5'-terminal extra-cistronic region (5'-ECR) of HAV-RNA were inserted in the intergenic region of the bi-cistronic plasmid pSV- GH/CAT, between the genes coding for the human growth hormone (GH) and the bacterial enzyme chloramphenicol-acetyl transferase (CAT), and following transfection of COS-1 cells, the transient expression of both genes was quantified.

Mutational analysis of the 5'-distal PRT (nts 94-140) identified 2 functional domains (nts 100-106 and 113-119). Substitutions introduced in these hexanucleotides abrogated the ability of HAV 5'-UTR to direct internal initiation of translation.

The role the 3'-distal PRT, appears to be strongly conditioned by the length of the "spacer" sequence extending between this structure and the translation initiation site: Placed 45 nucleotides upstream the initiator codon of a reporter gene, its integrity is stringently required for initiation to occur. Shortening the "spacer" reduces considerably the overall rate of internal initiation of translation, and the relative contribution to this process of the 3'-distal PRT becomes marginal.

Concomitantly, the role of two functional domains previously identified in the 5'-most PRT undergoes drastic changes. While the activity of domain 113-118 paralles that of the 3'-distal PRT, the opposite applies to domain 121-126, whose contribution becomes relevant only after switching off the 3'-distal PRT.

Systematic mutations introduced in the *"spacer"* sequence, suggest that this region may be responsible for the down regulation of translation of HAV-RNA and, possibly, for its lengthy replication cycle.

C. - Inhibition of Cap-dependent Protein Synthesis Induced by Protein 2A of HAV. - We have proposed to study the molecular mechanism(s) responsible for the inhibition of cellular protein synthesis in cultures infected by HAV and to investigate whether the *"shut off"* of the cell protein synthesis occurs via the inactivation of the cap-binding complex, a mechanism known to be operative in other picornavirus/cell systems.

The viral protease 2A of entero- or rhinoviruses, is responsible for the inactivation of the cellular factor p220 and the consequent abrogation of cap- dependent initiation of translation of cellular mRNAs. Since protein 2A of HAV, lacks the 18-aminoacid sequence present in trypsin-like proteases, it was not clear whether the *shut off* of host protein synthesis induced by culture-adapted strains of HAV (1) was due to 2A-mediated cleavage of p220, or was mediated by out competition of cellular mRNAs or de-phosphorylation of a translation initiation factor.

Accordingly, a cDNA representation of the sequences coding for HAV 2A was linked to the 5'-UTR of either the Lansing strain of poliovirus, or its inactive mutant S1, and inserted downstream the gene coding for the human GH. The bi-cistronic mRNAs transcribed from these constructs carried a first cistron (GH) whose translation was entirely cap-dependent, and a second one (HAV-2A) expressed by cap-independent internal initiation of translation driven by Lansing 5'-UTR.

Following transfection of COS-1 cells, the levels of GH present in the supernatants of the cultures were determined. Expression of HAV 2A caused the inhibition of the cap-dependent translation of GH, whereas the cap-independent expression of a reporter gene (CAT) directed by the 5'-UTR of poliovirus RNA was unaffected. Point mutations that destroyed the potential nucleophilic sites of HAV 2A abolished its effect on cap-dependent translation, suggesting that the HAV-induced cythopathic effect may be mediated by a mechanism similar to that of entero- and rhino-viruses.

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HEPATITIS B VIRUS (HBV) VARIANTS AND FULMINANT HEPATITIS: EVALUATION OF HBV GENOMIC HETEROGENEITY IN PATIENTS WITH ACUTE LIVER FAILURE AND IN PATIENTS WITH SELF-LIMITED ACUTE HEPATITIS

Coordinator: Prof. Giovanni RAIMONDO

Progress Report

HEPATITIS B VIRUS (HBV) VARIANTS AND FULMINANT HEPATITIS: EVALUATION OF HBV GENOMIC HETEROGENEITY IN PATIENTS WITH ACUTE LIVER FAILURE AND IN PATIENTS WITH SELF-LIMITED ACUTE HEPATITIS

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Summary

We analysed the complete nucleotide sequences of HBV genomes isolated from serum samples of a surgeon and of his mother, who was accidentally infected by the son, both of whom died because FH. The infecting viruses were genetically almost identical in both patients; all the clones examined carried a double nucleotide mutation in the start codon of the preS2 region preventing the synthesis of the corresponding protein. Analyses of different serum samples from the son revealed only wild-type precore sequences in a high viremic serum, while HBe antigen (HBeAg)-defective strains were prevalent when the viremia had decreased. Subsequently, we extended the analysis to the viral genomes isolated from 18 additional patients with acute HBV infection and different clinical behaviours: 3 of 5 patients with FH and without previous liver disease resulted infected with viruses carrying preS2 start codon mutations preventing the preS2 protein synthesis, while none of 13 control cases had similar genomic rearrangements. Summarising, these results indicate that: 1) preS2 protein is not essential for HBV infectivity; 2) infection by preS2-defective virus is frequently associated with FH, suggesting that this variant might play a pathogenetic role in cases of acute liver failure; 3) emergence of HBeAg-defective viruses might be a late event in the course of FH, occurring when HBeAg-producing viruses have been mostly cleared.

The following step of our study was to examine HBV isolates from HBsAg/anti-HBe positive mothers and their infants who developed either fulminant or self-limited acute B hepatitis. Our findings show that HBeAg defective viruses may infect babies

both with benign and fulminant hepatitis. In contrast, HBVs from fulminant hepatitis cases carry missense mutations at level of the preS and the core-promoter regions which might interfere with the host' immunological response and might enhance the virus replication.
Key words: *Hepatitis B virus, HBV mutants, HBV infection.*

Pre-S2 defective hepatitis B virus infection in patients with fulminant hepatitis

Hepatitis B virus (HBV) infection is the main cause of fulminant hepatitis (FH) in several geographic areas, including the Mediterranean basin. Traditionally the fulminant course of an HBV infection has been ascribed to an enhanced host immune response against viral antigens. There is some evidence, however, indicating that viral factors may also be involved in the pathogenesis of this disease. A number of papers have recently reported a significant association between FH and infection by HBV variants unable to produce HB e protein (HBeAg) because of a translational stop codon in the precore region of the viral genome. Nevertheless, the possible role of HBeAg-defective variants in the development of FH is controversial, since a) an association between these viral mutants and the acute liver failure was not found in all the studies; b) evaluation of HBV genomic heterogeneity was generally limited in cases of FH to examination of the precore/core gene; c) HBV molecular analysis was performed on serum samples collected late in the course of the infection, when the viremia had already decreased to levels detectable only by the polymerase chain reaction (PCR) amplification technique.

We had the opportunity to retrospectively examine serum samples collected from two fatal cases of type B FH in a surgeon and, two months later, in his mother. Neither patient had a history of previous liver disease, and in both of them other causes of liver damage were excluded, comprising hepatitis C and Delta infection. The index case, a 38-year old surgeon, was found to be HB surface antigen (HBsAg) positive in the absence of other serological HBV markers 15 days after accidental exposure to a possibly infected blood. Twelve days later the first detection of HBsAg, he became symptomatic and was hospitalised. At this time laboratory analyses showed the presence of HBsAg, IgM anti HB core antigen (anti-HBc) and anti-HBe and high levels of alanine aminotransferase (ALT, 4990 IU/l); ten days later this patient died, with a diagnosis of "hyperacute" type B FH.

About 7 weeks after the death of this patient, his mother developed fever (38°C) and asthenia. She spoke of exposure to the blood of her son after an accidental needle-stick the day before the son's hospitalisation. One week after the onset of symptoms, she was hospitalised, had high levels of ALT (4000 IU/l) and was HBsAg positive. She died 3 days later from FH type B with gastrointestinal haemorrhage.

The availability of stored (-20°C) serum samples collected during both high and low viremic phases of the disease from the son and from the mother the day of her death gave us the opportunity to compare the sequences of the infecting viruses present in the 3 samples. Quantitation of HBV-DNA showed a sudden drop of viremia levels between

the 1st (32 pg/ml) and the 2nd serum (5.7 pg/ml) of the son, while level of viral DNA in the mother's serum was unknown.

The HBV genomes isolated from the 1st sample of the son were entirely amplified, cloned into a pUC19 vector through an enzyme-free cloning procedure, and sequenced with the SequiTherm Cycle Sequencing kit (Epicentre Technologies, USA). Analogously, the entire viral DNA isolated from the mother and from the 2nd sample of the son were amplified and directly sequenced. In addition, the amplification products comprising the precore and the preS regions were also cloned and sequenced.

The nucleotide sequence of 15 clones for each amplified genomic region of the HBV from the 1st serum of the son showed that the infecting viral populations were genetically almost identical to each other and very similar to the ayw prototypes. The evaluation of the preS region revealed the presence, in all the clones, of a double mutation in the preS2 start codon (ATG → ACA) able to inhibit the production of the preS2 protein. Lack of serum preS2 protein was confirmed by a radioimmunoassay technique performed using monoclonal antibodies specifically directed against envelope proteins. The sequencing analysis of the precore region showed no mutation preventing the synthesis of HBeAg, even when the screening was extended to 15 additional clones. The nucleotide sequencing of the HBV isolated from the mother and from the 2nd sample of the son revealed that: a) the viral strains infecting the mother were almost identical (99.3% of homology) to those isolated from the son, thus confirming the intrafamilial spread of the infection; b) the preS2 start codon carried the double mutation (ATG → ACA) in all the clones from both cases; c) 9/15 clones from the 2nd sample of the son and 5/15 from the mother's sample had the mutation at nucleotide position 1896, producing a precore stop codon and preventing the synthesis of HBeAg.

The uncommon results obtained in the examination of viral strains from these two patients suggested to us to extend our retrospective analysis to viral genomes isolated from serum samples of 18 additional patients with acute HBV infection and different clinical behaviours. Seven of these 18 patients developed FH, but 5 of them (group "A") had had no previous liver disease, while for 2 patients (group "B") the HBV infection was superimposed on alcoholic or HCV related chronic liver disease; 11 cases (group "C") had self-limited acute hepatitis. Very few missense mutations were detected in the HBV nucleotide sequences of isolates from the 7 cases of fulminant hepatitis (5 cases of group "A" and 2 cases of group "B") when compared with the ayw prototypes of the National Center for Biotechnology Information data bank. No mutation known to be capable of influencing the core promoter activity or inducing aminoacid substitution in the core antigen epitopes recognised by cytotoxic-T-lymphocytes was found in any case. The analysis of the precore region showed only wild-type sequences in 3/5 and 2/2 cases of groups "A" and "B", while the contemporaneous presence of wild-type and HBeAg-defective viral populations was found in 2 "A" cases which carried a double nucleotide (G + A) at position 1896. Viral genomes from 3 of the 5 cases of group "A" carried a single (cases 1 and 5: ATG → ATA) or a double (case 3: ATG → ACA) nucleotide substitution in the preS2 start codon preventing the synthesises of the correspondent protein, while neither of the 2 "B" cases had genomic rearrangements able to inhibit the preS2 production. The sequencing analyses of precore-core and

preS1-preS2 regions of HBV isolated from 11 patients with self-limited acute hepatitis (group “C”) showed only wild-type precore sequences in 8 cases and the contemporaneous presence of wild and HBeAg-defective viral strains in 3 cases. No mutation inducing aminoacid substitution in the core antigen epitopes recognised by cytotoxic-T-lymphocytes or capable of influencing the synthesis of preS1 and preS2 proteins was found in any case.

The pathogenetic mechanisms involved in case of FH are poorly understood. In fact, because of the quick development of the massive hepatocellular necrosis, virological studies of fulminant hepatitis B are usually performed “late” in the course of the disease, when virus replication is already strongly suppressed, viremia decreased to very low levels and HBsAg often not longer detectable. Consequently, it is difficult to reconstruct the sequence of virologic and immunologic events leading to the liver failure. In this study we had the rare chance to analyse the viral genomes isolated from two members of the same family who both died from HBV-related fulminant hepatitis. Our results, showing that a patient with type B FH transmitted the HBV to another subject who also developed acute liver failure, provide strong evidence that viral factors may play a key role in the severity of the acute HBV infection.

As mentioned above, controversial data concerning the association between FH and infection by HBeAg-defective HBV have been published. In our study we detected HBeAg-defective strains only in a minority of the cases, independently of the clinical behaviour, and always in association with viruses carrying a wild-type precore region, while no mutation was detected in the core promoter region in any of the cases. Moreover, we had the unique opportunity to examine and compare the HBV populations isolated from serum samples collected during both the high and the low viremic phases of the infection in a patient with FH. The precore region of the HBV from the high viremic sample presented a wild-type sequence in all 30 clones examined, while the analyses of HBV genomes isolated when viremia levels had fallen showed that the prevalent viral population was the HBeAg-defective. These data might lead us to hypothesise that type B FH can be associated with infection by viruses normally producing HBeAg. During the disease, these viruses may be quickly cleared by the host’s immune hyperresponse to the viral antigens, the viremia decreases and the HBeAg-defective viral populations may then emerge and be detectable.

The evaluation of HBV heterogeneity in cases of acute liver failure by sequencing the complete viral genomes showed that 5 of 7 individuals with FH and without previous liver disease were infected with preS2-defective virus, while this variant was not found in any of 13 subjects with acute infection from 2 different control groups (11 self-limited acute hepatitis and 2 FH occurring in subjects with previous chronic liver disease). Detection of HBV unable to produce preS2 protein is not a rare event in chronic HBsAg carriers, above all in patients with the most severe forms of liver disease, such as cirrhosis and hepatocellular carcinoma. We are now reporting the detection of this variant in patients with acute infection and liver failure. In addition, one of these patients was the source of infection for his mother, who also developed FH, and all the viral clones isolated from both these subjects carried the mutated preS2 start

codon. These results seem to demonstrate “in vivo” that HBV variants unable to produce preS2 protein may be transmitted, confirming previous data from “in vitro” experiments which indicated that lack of expression of this protein does not interfere with viral replication nor infectivity. Finally, the association between infection with preS2-defective HBV and acute liver failure observed in most of our patients tempts us to speculate about the possibility that preS2-deficiency of the infecting strains may have an etio-pathogenetic role in cases of acute liver failure. We might hypothesise that, since the T- and B-cell immunities specific for the preS2 protein are important early events in the human immune response to HBV infection, the absence of this protein may result in inefficient neutralisation of the virus, favouring the more severe course of the infection. In addition, the incapacity of the infecting virus for encoding “middle” S protein might lead to overproduction and accumulation of “large” S protein into the hepatocytes, a phenomenon that is associated with fulminant hepatitis in the transgenic mouse model. Further experimental studies are needed to verify the reliability of such hypotheses.

Heterogeneity of HBV genomes isolated from newborn babies with fulminant hepatitis

Transmission of HBV infection from HBsAg/anti-HBe mothers to newborn babies is an unfrequent occurrence which usually induces self-limited acute hepatitis and, very rarely, acute liver failure. These rare cases of fulminant hepatitis occurring in babies born to anti-HBe positive mothers are considered to be related to the perinatal transmission of defective viruses, although the HBV genomic analysis has not been completely convincing in any study reported so far.

We had the opportunity to analyse serum samples collected from 4 HBsAg/anti-HBe positive mothers and from their infants. The babies developed acute hepatitis that evolved in a fatal liver failure in 2 cases.

HBV-DNA was isolated from each sample and entirely amplified, cloned and sequenced.

Virus populations carrying a mutation at nucleotide position 1896 which produces a precore stop codon preventing the synthesis of HBeAg were detected both in infants with self-limited and fulminant hepatitis and in their mothers. On the contrary, missense mutations in the preS regions and at the level of the core promoter located in the X gene (nucleotide 1742 to 1849) were detected only in babies with FH and in the corresponding mothers. In particular, the finding of mutations in the core promoter is of great interest since it has recently reported that rearrangements at that level may enhance the virus replication.

Further “in vitro” studies are necessary to verify whether viruses with the above mentioned modifications may play a role in inducing liver cell damage.

"IN VITRO" EXPRESSION SYSTEMS FOR THE STUDY OF HAV AND HCV PROTEINS AND THEIR FUNCTIONS

Coordinator: Dott. Maria RAPICETTA

Progress Report

CHARACTERISATION OF CHANNEL ACTIVITY OF HCV E1 PROTEIN

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The E1 protein of HCVcontains a C-terminal hydrophobic region (aa 341-383) which is probably involved in membrane association (1-3). Attemps to express the full-length E1 in E.coli have failed. In contrast, when the C-terminal region was truncated, the protein was synthesised to high level (4).

According to the membrane-associating properties of this region, we suggest that the insertion of this domain into membranes may produce modification in membrane permeability that at last cause cell lysis. To test this hypothesis, the E1 gene has been cloned and expressed in BL21(DE3)pLys E.coli strain by pET3a vector (5, 6). This is an inducible expression system specifically designed for the production of toxic protein. We showed that the expression of E1 (aa 192-383), as well as of two C-terminal fragments (aa 331-383 and aa 341-383) corresponding to the transmembrane (TM) region of this protein, was tolerated only for a limited period of time and induced a rapid lysis of cells. The kinetic of expression clearly showed a gradual decrease of E1 synthesis in the four hours after induction. Similarly, the analysis of expression of the two C-terminal fragments revealed that these products were expressed to a very low level and only for the first hour after induction. On the contrary, the expression of a mutant of E1 (aa 192-340), lacking the last 40 aminoacids, was tolerated for several hours after induction and did not cause cell lysis. This fragment was synthesised at high level in the four hours after induction analysed.

To explore the possibility that the insertion of E1 into membrane increases membrane permeability, we analysed membrane selectivity of recombinant clones by four different assays. We first test the entry of Hygromycin B in cells. Hygromycin B acts by blocking protein synthesis, bu the entry of this antibiotic was detected only in cells in which membrane permeability has been modified. We observed inhibition of protein synthesis, caused by Hygromycin B, only in cells expressing E1 or C-terminal fragments (5).

In a second step, we analysed the entry of ONPG into cells. ONPG is a substrate of B-galactosidase that is normally excluded by membrane of intact cell. We tested B-galactosidase activity of recombinant cultures by measuring the appearance of ONPG cleavage product (O.D. 420 nm) at different times after induction. After one hour of induction synthesis of the C-terminal fragments, as well as of the E1 protein, caused an increase of ONPG entry into cell as compared with negative control (5).

To test the possibility that expression of E1 leads to the exit of nucleotides, we loaded recombinant clones with labelled uridine and measured the release of radioactivity at different times after induction. The release of uridine to the medium was first observed after thirty minutes of induction of E1 and after one hour of induction of the C-terminal fragments. Clones expressing the C-terminal deleted mutant, as well as clones bearing the parental plasmid, did not acuse exit of uridine even after longer post-induction times (5).

The effect on membrane permeability produced by E1 expression was further analysed by testing the entry of propidium iodide (PI) into cells at different times after induction. Binding of PI to DNA is followed by a fluorescent emission. Because the entry of PI into cells required alteration of membrane selectivity we used Facs analysis to test PI incorporation in recombinant clones. The uptake of PI by cultures expressing the C-terminal fragments was detected after one hour post-induction. We observed a strong increase in PI fluorescence as compared to cells before induction used as control. Even cells expressing E1 showed a modification of membrane selectivity to PI but the fluorescence signal was lower. Incorporation of PI was not detected in cells expressing the C-terminal deleted mutant as well as in control (5).

The analysis of permeability changes by these assays revealed that modification of membrane permeability to several compounds were observed only in clones expressing E1 and C-terminal fragments, while the synthesis of the C-terminal deleted mutant had little or no effect on permeability (3).

These findings demonstrate that the TM domain of E1 protein has membrane-active properties which may be involved in some aspects of virus-cell interaction (7).

To further characterise other regions of the protein that could be involved in channel-activity we cloned and expressed different deletion mutants of E1 in E.coli system. In addition, we introduced some amino acid substitutions in the C-terminal region, in order to identify residues that are critical for permeability changes. The alteration of membrane permeability in cell expressing E1 mutants were evaluated by three different assays that detect the flux of small compounds through E.coli membrane (8-10). The results demonstrated that the interaction of an internal hydrophobic region (aa 259-298) with membranes induces modification of permeability, suggesting that this region could cooperate with the C-terminal domain in forming ion-channel structure. Furthermore, mutations in the C-terminal region revealed that amino acids W 368 and K 370 play a critical role in protein function. The identification of domains and amino acids functionally related to the channel-activity of E1 represents the first step toward the elucidation of the E1 channel-structure and the design of new antiviral therapies blocking such activity (11).

In conclusion, this study demonstrates for the first time, a biochemical function of the E1 protein of HCV, which may play an important role in specific steps of HCV replication and/or pathogenesis.

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SENSITISATION TO FAS-MEDIATED APOPTOSIS BY HCV CORE PROTEIN IN A HUMAN HEPATOBLASTOMA CELL LINE: ROLE OF *C-MYC* AND *BCL-2* FAMILY PROTEINS

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The studies on the hepatitis viruses life cycles has been hampered by the lack of a cellular system for efficient viral propagation *in vitro*. This led to development of heterologous expression systems as experimental approach to study viral replication cycle and molecular mechanisms underlying the virus-cell and virus-host interactions.

With regard to the pathogenicity of Hepatitis C virus (HCV), either virus-induced or immuno-mediated mechanisms have been reported. However the viral gene product(s) responsible for liver cell damage are not as yet definitely known.

To examine virus-cell interaction between HCV and liver cells we established human hepatoblastoma cell line (HepG2) constitutively expressing various genomic regions of HCV (core, E1-E2, NS2-NS5b). The stable expression system in a human cell line of liver origin was studied as suitable expression system for analysing pathogenic mechanisms of HCV infection, in particular the molecular interactions of HCV proteins and liver cell. Although cell lines of hepatic origin are generally characterised by low transfection efficiency, we obtained a high level of expression for HCV structural proteins and an efficiency of transfection near to 90% in stable HepG2 cells, by expression of viral genes under the control of mammalian promoter EF1-α (1, 2). The constitutive expression of HCV core protein in HepG2 cell line enabled us to study its role in the apoptotic cell death following treatment with anti-Fas monoclonal antibody. Recent evidences reported a role of Fas system in apoptosis of hepatocytes in hepatitis patients and in liver cell injury associated to HCV infection (3, 4). We found that HCV core protein, constitutively expressed in human hepatoblastoma cell line, sensitised cells to apoptosis induced by anti-Fas monoclonal antibody.

Stable cell lines expressing HCV core protein showed morphological and nuclear changes (rounding, shrinkage, detachment from the substrate) and typical DNA fragmentation was also detected. Conversely, same cells expressing HCV envelope proteins (E1-E2) or non-structural proteins did not undergo apoptotic changes in response to anti-Fas. The analysis of Fas receptor indicated that Fas level was not upregulated on the surface of HepG2 cells expressing HCV core protein. Apoptotic cell death in core expressing HepG2 cells was prevented by treatment with a specific inhibitor of the caspase 3 (the cysteine protease CPP32), suggesting that caspase 3, rather than caspase 1 plays a part in the apoptotic effector pathway of HepG2 cells expressing core protein. The results of this study suggests that intracellular expression

of HCV core protein renders cells prone to apoptotic death and that the core protein may have a role in immune-mediated liver cell damage (5).

Apoptosis is reported to be the result of an interaction between initiating stimulus, which can be physiological or result from an injury, and cellular factors which determine cellular susceptibility to activation of the molecular cascade, ultimately leading to activate effector events of cell death pathway. Virus associated citopaticity is often the result of activation of cell death pathways by one or more viral gene products, which modulate or mimick the expression of cellular proteins involved in regulation of apoptosis (6).

With regard to HCV core protein, it has been reported to regulate the expression of several cellular proteins and functions, such as trans-activation of the c-myc promoter and p53, interaction with the cytoplasmic tail of TNF receptor and with lymphotoxin-β receptor thus modulating host immune response (7-9). With referring to the above, we examined the mechanism by which HCV core protein could induce sensitisation to Fas-mediated apoptosis, with the aim to obtain informations about its role in the pathogenicity of HCV infection.

Much of the recent understanding of the molecular regulation of apoptotic cell death were derived from the analysis of the role of oncogenes and tumour suppressor genes in the control of this process (10). In this regard, cellular oncoproteins as *c-myc* and some members of the Bcl-2 family are of particular interest as they are involved in the signalling pathways of apoptosis (11). In particular, a number of observations have suggested that levels of *c-myc* and Bax proteins correlate with the susceptibility of cells to apoptotic death (12, 13). It was recently reported that *c-myc* promotes apoptosis by sensitising cells to the Fas mediated death signal (14). Bcl-2 family of proteins consists of both anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic proteins (Bax, Bak). Relative abundance of both series of proteins determines the ultimate sensitivity or resistance of cells to various apoptotic signals (15). Bcl-2 members as well as *c-myc* protein levels may be modulated by the expression of viral gene products. Therefore we tested the possibility that HCV core protein modulates expression of c-myc and some Bcl-2 members in stable HepG2 cell line. A 60% increase of c-myc levels were reproducibly found in core expressing cells compared to parental HepG2 cell lines. The up-regulation of c-myc resulted from the prolonged half-life of the c-myc protein in HCV core expressing cell lines. In addition, expression of the pro-apototitc Bax protein was found 4-5 times increased compared to the mock transfected and to parental HepG2 cell lines. In contrast, Bcl-2 and Bcl-X_L, which negatively regulate cell death, were not up modulated in cells expressing core protein. The ratio of Bcl-2 to Bax, which has been reported to be an inverse index of susceptibility to apoptotic death, was found to be significantly lower in cell lines expressing HCV core protein than in vector transfected cell. This result is consistent with susceptibility of core expressing cells to apoptosis (16).

Our evidence that intracellular expression of HCV core protein induces accumulation and stabilisation of *c-myc* expression suggests that modulation of *c-myc* by HCV core protein may be relevant not only as a possible mechanism of liver cell

damage, by sensitisation to Fas induced apoptosis, but also as indirect transforming potential mechanism.

The results obtained so far support the evidences of an active role for the core protein in inducing cell death in HCV infected liver cells and in the pathogenesis of liver disease in HCV infected patients.

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ANALYSIS OF THE FUNCTION OF HAV P3 PROTEINS IN VIRAL REPLICATION

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Few data exist with regard to the involvement of hepatitis A virus (HAV) non-structural proteins in viral replication. Replication of most HAV strains in infected cells is delayed, noncytolytic and takes several days to be manifested by the production of viral antigen and RNA (1). In contrast to poliovirus-infected cells, where dramatic morphological changes seem to be induced by the action of viral proteins 2B and 3A (2, 3), only very few strains of HAV induce cytopathic effect in the infected cell (4).

Previous studies to determine the basis of the cytopathogenicity of the fast-growing Italian strain FG (5) had suggested that its protein 3A, carrying the deletion of 3 amino acids at the N-terminus and a different secondary structure, could be involved in this feature (6). Further expression studies, carried out within this project, allowed us to show that protein 3A of FG induces the formation of pores into the bacterial membrane and that the noncovalent association of the negative charges present in the N-terminal amphipathic helix, characteristic of $3A_{FG}$, with the positive charges at its C-terminus is necessary for the protein to assume the pore-forming conformation (7). Some nuclear proteins of the host cell are used during the replication of picornaviruses, after their relocalization to the cytoplasm (8). Pore formation into the nuclear membrane induced by 3A of FG could account for its accelerated growth by promoting a more efficient relocalization of nuclear proteins to the cytoplasm, with respect to other strains. This could lead to a depletion of nuclear proteins needed for cell replication and, as a consequence, to cell death (cytopathic effect).

Several steps in the HAV life cycle have been proposed to determine the slow replication rate, including protein synthesis and processing.

To define the roles of the P3 region proteins in HAV replication we first focused on the determination of the functions of protein 3AB, that has been demonstrated to be a multifunctional protein, with multiple roles, in poliovirus replication (9). Previously, we had demonstrated that 3A is a membrane-interacting protein acting as an anchor of 3B to the membranes of the vesicles during HAV replication cycle (6). In this project we examined the quality of the interaction of HAV 3AB with the membrane. The results of the interaction experiments using the wild-type protein and some deletion mutants, expressed in bacteria as fusion proteins carrying a His-tag at their N-termini and purified by affinity-chromatography on a nickel-Sepharose resin, demonstrated that 1) 3AB can associate post-translationally with membranes as an integral protein, 2) its hydrophobic domain is the transmembrane (TM) segment and 3) the most C-terminal 7 amino acid residues of this domain give probably the main contribution to membrane association of 3AB, through their interaction with the lipid bilayer (10). This study

showed also that HAV 3AB is able to dimerize in the presence of membranes and that dimerization can occur through the α-helical pairing of four glycine residues placed on the same face of the helix formed by the TM domain (10). It could be hypothesised that the ability of protein 3AB to dimerize into the membranes is required for some function(s) in the viral replicative cycle.

Formation of a ribonucleoprotein complex (RC), composed by the viral RNA and various viral non-structural proteins, seems to be crucial for the initiation of RNA replication of picornaviruses (11). As a first assessment of the composition of the HAV RC, we investigated the ability of proteins 3ABC and 3AB to interact with RNA secondary structures formed at the 5' and 3' end of the HAV genome. Using His-Tag fusion proteins and RNA transcripts we demonstrated that 3ABC and, less efficiently, 3AB, are RNA-binding proteins specifically interacting with both termini of the HAV genome (12). We could speculate that those proteins, and in particular 3ABC, a relatively stable intermediate of HAV polyprotein processing, might play an essential role in the initiation of HAV replication.

In a further assessment of the possible role of HAV 3AB, and to extend our studies on the 3AB-RNA binding properties, we examined the interaction of HAV proteins 3A and 3AB with other proteins of the P3 domain that might be essential to the formation of the viral RC. To this end, different P3 proteins were expressed in vitro, and/or in vivo as His-tagged proteins and the interaction of 3C, 3CD, and 3D with 3A and different forms of 3AB was tested. The results demonstrated the formation of stable complexes by 3AB and 3CD involving both electrostatic and hydrophobic forces, and that both moieties of 3AB, and 3C are required for efficient interaction (13). In particular, mapping of the 3A domains of 3AB showed that the hydrophobic region is essential for both the homologous and the heterologous protein interaction. Furthermore, analysis of the primary sequence of protein 3C revealed that it contains two hydrophobic domains that could contribute to the binding of 3CD to 3AB though their interaction with hydrophobic domains or amino acid residues in 3AB (13).

Interaction of proteins 3AB and 3CD was also tested in the yeast two-hybrid system. However, for unknown reasons, negative results were obtained. It has been already reported that interaction of proteins in the two-hybrid system can be influenced by several factors, and that failure to observe an interaction in this system does not necessarily mean that the two proteins do not interact in their normal environment (14).

To improve our understanding of the RNA-binding of HAV 3AB, we analysed the 3AB protein domains involved in RNA-binding specificity and obtained clear evidence that the specificity of the 3AB interaction is determined by the cluster of charged amino acid residues located near the C-terminus of 3A. It can be hypothesised that the C-terminus of HAV 3A is needed for 3AB to assume a conformation suited to recognise specifically the HAV RNA secondary structure formed at either end of the genome (13).

The role of HAV 3CD or 3D in RNA-binding and the formation of the RC could not be assessed since neither protein could be prepared in a functional conformation and in sufficient amounts, which is most likely due to the insolubility of 3D and 3CD (unpublished observation; and, 15).

In conclusion, analysis of the interaction of proteins 3A and 3AB of HAV with other polypeptides of the P3 domain and with RNA allowed us to map the protein domains within 3AB which participate in membrane binding, protein-protein and RNA-protein complex formation. Our observations indicate that HAV 3AB is a multifunctional polypeptide, similar to the corresponding protein of poliovirus. However, our observation that 3A of HAV can specifically interact with the HAV RNA in the absence of other viral or cellular proteins and that the specificity of the interaction itself relies on the highly charged amino acids at its C-terminus implicates that HAV 3A possesses some distinct biological properties whose effect on the virus life cycle remains to be determined.

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EVALUATION OF THE ETIOLOGICAL ROLE OF HEV AND GBV-C IN VIRAL ACUTE AND CHRONIC NONA-D HEPATITIS IN ITALY AND STUDY OF THEIR SPREAD IN INDIVIDUALS AT RISK AND IN BLOOD DONORS

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Progress Report

EVALUATION OF THE AETIOLOGICAL ROLE OF HEV AND GBV-C/HGV IN VIRAL ACUTE AND CHRONIC NONA-D HEPATITIS IN ITALY AND STUDY OF THEIR SPREAD IN INDIVIDUALS AT RISK AND IN BLOOD DONORS

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The main objectives of our research were to study: a) the etiological role of hepatitis E virus and GBV-C/HGV among patients with acute nonA-D hepatitis; b) the prevalence and significance of GBV-C/HGV among patients with chronic liver diseases, among key risk populations and donors; c) the rate and clinical importance of mother-to-infant transmission of GBV-C/HGV.
Published and unpublished results are reported as follows:

1. Identification of a novel variant of hepatitis E virus in Italy

Hepatitis E infection is typically associated with areas in which hepatitis E virus (HEV) is endemic. Except for a few cases in Europe and in the United States, acute hepatitis E is usually associated with travel to endemic areas. We set out to determine the etiologic role of HEV in acute non-A-C hepatitis in Italy. The presence of HEV-RNA and antibody was determined in 218 patients diagnosed as having acute viral non-A-C hepatitis. Acute hepatitis E infection was defined by the presence of HEV-RNA in sera and positivity for IgM anti-HEV and seroconversion to IgG anti-HEV. Acute hepatitis E was diagnosed in 10.1% of the patients with acute non-A-C with 95.5% exhibiting a benign course. A more severe course was observed in a patient co-infected with HAV and HEV. The majority of cases were travellers to endemic areas with 18.2%

reporting no travel. One patient was from a household with an infected patient. Sequence analyses of the PCR product derived from a patient who never visited endemic areas, identified an isolate that is significantly divergent from all reported isolates of HEV (79.5-85.8%). From this study it emerges that HEV accounts for approximately 10% of acute non-A-C viral hepatitis in Italy, generally diagnosed in travellers returning from endemic areas. However, the identification of a new HEV variant in an individual who never indicated travel or contact with individuals associated with endemic areas, provides evidence that this virus may be native to Italy.

2. GB virus C/hepatitis G virus in patient with acute nonA-E hepatitis and in patients with acute hepatitis of defined aetiology

The role of GBV-C/HGV in the aetiology of acute nonA-E hepatitis and its impact on the course of acute hepatitis of defined aetiology were investigated by detecting viral RNA by RT-PCR and antibody to the E2 proteins of GB virus C (anti-E2) by EIA.

Ninety-eight patients with acute nonA-E hepatitis, 35 patients with acute hepatitis A, 63 with acute hepatitis B, 29 with acute hepatitis C and 270 controls were enrolled in this study. The prevalence of GBV-C/HGV RNA was similar among patients with acute nonA-E hepatitis (3.1%), with acute hepatitis A (2.9%), and controls (3.7%), but significantly higher ($p<0.05$) among those with hepatitis B or C (19.0 % and 48.3%, respectively). Similar figure was obtained considering the total rate of GBV-C/HGV exposure (viral RNA and/or anti-E2 positivity). The majority (24/30 or 80%) of GBV-C/HGV RNA positive patients reported a parenteral source of exposure while the remaining 20% denied having known risk factors. The liver function test values and the rate of hepatitis B and C chronicisation were similar in patients co-infected and in those not co-infected with GBV-C/HGV

In conclusion, our study excludes a significant role of GBV-C/HGV infection in the aetiology of acute nonA-E hepatitis in Italy. Neither GBV-C/HCV or GBV-C/HBV co-infection does not worsen the clinical course of illness among patients with acute hepatitis.

3. GBV-C/HGV in patients with chronic liver disease, in population at risk and in blood donors

3.1 Patients with chronic liver diseases - We have analysed 272 patients with biochemical (ALT values more than twice the normal upper limit for at least one year) and histologic evidence of chronic liver diseases. Of these patients, 95 (34.9%) had chronic cryptogenic nonA-C hepatitis (negativity for HBV, HDV and HCV markers and exclusion of autoimmunity), 17 (6.3%) had chronic hepatitis B and 68 (25%) patients had chronic hepatitis C. The study enrolled 54 (19.8%) patients with hepatocellular carcinoma (HCC) (26 with anti-HCV alone, 23 positive for both anti-HCV and markers

of HBV and 5 positive for HBV markers alone), 22 (8.1%) patients with primary biliary cirrhosis (PBC) and 16 (5.9%) patients with a diagnosis of autoimmune hepatitis (AIH). GBV-C/HGV RNA was found in 4 of 95 (4.2%) patients with chronic cryptogenic nonA-C hepatitis. The percentage of GBV-C/HGV RNA positivity rose to 17.7% in patients with chronic hepatitis B and to 40% in those with chronic hepatitis C. Among patients with chronic hepatitis C, the rate of GBV-C/HGV positivity was much higher in IVDU (52%) than in non-IVDU (10%). No difference was seen in the mean ALT level or in liver histology findings between patients who had chronic hepatitis due to HBV or HCV alone and those with GBV-C/HGV co-infection.

GBV-C/HGV RNA was detected in 1 of 54 (1.9%) patients with HCC, in 1 of the 16 (6.3%) patients with AIH and in none of the 22 patients with PBC.

The prevalence of anti-E2 antibody increased from 22.1% among patients with chronic cryptogenic nonA-C hepatitis to 83% among those with HCC. This antibody trend seems to be correlated primarily with the age of the patients and with their exposure to main parenteral source of infection.

3.2 Key risk population. – We have studied the presence of markers of GBV-C/HGV infection in 33 thalassemic patients, 53 haemophiliacs and in 50 patients on dialysis maintenance.

Signs of ongoing (RNA) or past (anti-E2) GBV-C/HGV infection were found in about 82% of thalassemics, in 57% of haemophiliacs and in 72% of haemodialysed.

Retrospective analysis of sera stored at –20°C from 4 individuals (2 thalassemics, 1 haemophiliac and 1 haemodialysed) with GBV-C/HGV RNA alone, without HBV or HCV, showed that two thalassemic patients had normal ALT for periods ranging between 2 and 4.5 years; however, the patient with haemophilia and one thalassemic showed slightly elevated ALT value persistently over a period of 18 and 36 months respectively.

3.3 Healthy individuals and blood donors. – As control, we studied 970 healthy individuals subdivided by age and 428 periodic blood donors with normal ALT.

In healthy individuals the seroprevalence of GBV-C/HGV RNA and anti-E2 was respectively 2.7% and 21.9%. The highest rate of positivity from GBV-C/HGV RNA was observed among individuals aged 35-54 years (5%). We observed a trend of positivity of the prevalence of anti-E2 correlated with the age: from 4% among the group of <15 years of age to 46.6% among those with >65 years of age.

7 of 428 (1.6%) donors examined were found GBV-C/HGV RNA positive in presence of repeatedly normal ALT. Retrospective analysis carried out on sera stored at –20°C from 3 periodic blood donors showed that they had normal ALT despite having persistent GBV-C/HGV infection for periods ranging between 2 and 4.5 years.

Conclusion

- The high seroprevalence of anti-E2 antibodies in patients with chronic liver diseases, in key risk populations, in healthy individuals and in donors indicates that GBV-C/HGV infection is wide-spread and that the majority of infected individuals can clear the infection.
- The high frequency of GBV-C/HGV infection in patients with chronic hepatitis B or C, in IVDUs, in haemodialysed and in multitransfused patients indicates that the virus is mainly transmitted by parenteral routes.
- GBV-C/HGV can clearly cause persistent infection but usually without biochemical evidence of liver disease.
- Despite the relatively high frequency (1.7%) of GBV-C/HGV RNA in periodic blood donors and although the virus can easily be transmitted from donor to recipient, transfusion-associated hepatitis is approaching zero risk in Italy.

In conclusion, these data indicate that GBV-C/HGV is not a major hepatotropic virus.

4. Mother-to-infant transmission of GBV-C/HGV

Evidence indicates that the GBV-C or hepatitis G virus can cause persistent infection in humans, but little is known on the importance of vertical transmission. To assess the risk of mother-to-infant transmission and the clinical outcome of infected babies, we investigated 175 anti-HCV positive mothers and followed-up their children for 3-33 months. GBV-C/HGV RNA was detected by RT-PCR and anti-E2 antibody was assayed by EIA. Thirty-four (19.4%) women were GBV-C/HGV RNA positive and transmission occurred to 21 (61.8%) babies; 20 (95.2%) acquired GBV-C/HGV alone, and one (4.8%) GBV-C/HGV and HCV. Maternal factors such as intravenous drug use, HIV co-infection, HCV-RNA positivity and type of feeding were not correlated with GBV-C/HGV transmission. GBV-C/HGV RNA remained persistently positive in all infected babies but one baby who seroconverted to anti-E2. Seven (35%) babies with GBV-C/HGV alone developed marginally elevated ALT; the baby with HCV and GBV-C/HGV co-infection had highest ALT peak value (664 IU/l). Seven of the 141 (5%) babies born to the GBV-C/HGV RNA negative mothers acquired HCV and six (85.7%) had abnormal ALT. The mean ALT peak value was significantly higher ($p<0.05$) for babies with HCV than for those with GBV-C/HGV. None of the children with GBV-C/HGV or with HCV became icteric. GBV-C/HGV is frequently present in anti-HCV positive women. The infection is transmitted efficiently from mother to baby and rate of transmission is much higher than that for HCV. GBV-C/HGV can cause persistent infection in babies but usually without clear evidence of liver disease.

SUBPROJECT

PATHOGENESIS OF HEPATITIS VIRAL PERSISTENT INFECTIONS

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IMMUNE RESPONSES TO VIRAL AND SELF ANTIGENS INDUCED BY HEPATITIS C VIRUS OR HEPATITIS DELTA VIRUS (HCV AND HDV)
Coordinator: Prof. Vincenzo BARNABA

Progress Report

IMMUNE RESPONSES TO VIRAL AND SELF ANTIGENS INDUCED BY HEPATITIS C VIRUS OR HEPATITIS DELTA VIRUS (HCV AND HDV)

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Summary

Aim of the present project has been that: 1) to identify the HDV epitopes recognised by $CD4^+$ T cells derived from patients with chronic HBV/HDV infection; 2) to study the extracellular processing of HDV epitopes; and 3) to identify the HCV epitopes recognised by $CD8^+$ T cells.
Key words: *Hepatitis C virus, hepatitis D virus, HCV immunology, HDV immunology.*

Human $CD4^+$ T cell response to hepatitis delta virus: identification of multiple epitopes and characterisation of T helper cytokine profiles

T cell-mediated immune response plays a crucial role in defence against hepatotropic viruses as well as in pathogenesis of viral chronic hepatitides. However, very little is known on the role of specific T cells during hepatitis delta infection in humans. In this study, T cell response against hepatitis delta virus (HDV) in chronic hepatitis B virus (HBV) carriers with HDV superinfection was investigated at different levels. Analysis of peripheral blood mononuclear cells (PBMC) proliferation to a recombinant form of large hepatitis delta antigen (HDAg) revealed that 8 out of 30 patients studied (27%) specifically responded to HDAg. By employing synthetic peptides spanning the entire HDAg sequence, we found that T cell recognition was directed against different antigenic determinants, with patient to patient variation in the pattern of response to peptides. Interestingly, all responders had signs of inactive HDV-induced disease, while neither any of the patients with active disease or any of the control subjects showed any significative proliferation. More accurate investigation of specific T cell response was obtained at clonal level. A panel of HDAg-specific $CD4^+$ T

cell clones from three HDV-infected individuals, and fine specificity analysis revealed that the clones tested individually recognised four epitopes corresponding to amino acids (aa) 26-41, 50-65, 66-81 or 106-121 of HDAg sequence. The study of human leukocyte antigen (HLA)-restriction revealed that peptides 50-65 and 106-121 were presented to specific T cells in association with multiple class II molecules. In addition, peptide 26-41 was efficiently generated also after processing of HDAg through the endogenous processing pathway. Cytokine secretion analysis showed that all the $CD4^+$ T cell clones assayed were able to produce high levels of interferon-γ (IFN-γ), belonging either to T helper-1 (Th1) or Th0 subsets, and some of them were cytotoxic in a specific assay. This study provides the first evidence that detection of specific T cell response to HDAg in the peripheral blood in individuals with hepatitis delta is related with the decrease of HDV-induced disease activity. The HDAg epitopes identified here and particularly those recognised by $CD4^+$ T cells in association with multiple MHC class II molecules may be potentially exploited for preparation of a vaccine-prophylaxis and -therapy of HDV infection.

Generation of a MHC class II-restricted hepatitis delta antigen T cell epitope by extracellular processing

Hepatitis delta virus (HDV) is the causative agent of severe acute or chronic hepatitis affecting hepatitis B virus (HBV) carriers. Here we found that presentation of soluble hepatitis delta antigen (HDAg) to a $CD4^+$ T cell clone TB238 specific for the $HDAg_{(106\text{-}121)}$ epitope was surprisingly unaffected by inhibition of the antigen presenting cells (APC) processing machinary. On the oher hand, glutaraldheyde-fixed APC retained their ability to present HDAg to TB238 clone, but not to control clones, only when they were previously Ag-pulsed for at least five hours, demonstrating that some form of extracellular processing had occurred. Moreover, when HDAg was previosly incubated with either foetal calf serum or human AB serum for 5 h., pulsing of fixed-APC for only 1 h was sufficient to provide a good T cell stimulation, suggesting that HDAg had been processed during incubation with serum. These results show that HDAg may undergo extracellular processing by serum proteases, and suggest that prompt availability of HDAg immunogenic peptide generated by this processing mechanism particularly during the early phase of infection could play an important role in the immune response against HDV.

Presence of effector $CD8^+$ T cells in hepatitis C virus-exposed healthy seronegative donors

Cytotoxic T lymphocyte (CTL) responses against multiple hepatitis C virus (HCV) epitopes were detected in 7 out of 29 (24.1%) healthy family members (HFM) persistently exposed to chronically HCV-infected patients (HCV-HFM). These precursors CTL were at very low or undetectable frequencies, as determined by limiting

dilution analysis (LDA). However, when HCV-specific effector $CD8^+$ T cells, freshly isolated from PBMC of HCV-HFM, were assessed by a sensitive enzyme-linked immunospot (ELISPOT) assay, their frequencies were severalfold higher than those of precursor CTL. These results indicate that the two assays detect two functionally distinct T cell populations, and that the effector cells are not assayed by the ^{51}Cr-release assay. Furthermore, the combination of cell depletion and ELISPOT analyses showed that the effector cells were confined into a $CD8^+$ $CD45RO^+$ $CD28^-$ population. The persistence of effector $CD8^+$ T cells specific for both the structural and non-structural viral proteins in uninfected HCV-HFM, suggest that: i) an immunological memory is established upon a subclinical infection without any evidence of hepatitis, in a larg cohort of HCV-exposed individuals; ii) since these cells required neither restimulation nor the addition of particular cytokines in vitro for differentiating in effectors, they should be capable of prompt HCV-specific effector function in vivo, possibly providing anti-viral protection; iii) the maintenance of effector T cell responses may be sustained by persisting low-level stimulation induced by inapparent infections.

HOST FACTORS IN THE PATHOGENESIS OF INFECTION AND DISEASE INDUCED BY FLAVIVIRIDAE (HCV, HGBV-A, -B, -C/HGV)

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Progress Report

HOST FACTORS IN THE PATHOGENESIS OF FLAVIVIRUS INFECTION AND DISEASE (HCV, HGBV-C/HGV)

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Summary

Immunohistochemistry to detect HCV antigens within hepatocytes is a useful tool to identify and quantify the number of HCV-infected cells. We demonstrated a positive correlation between the proportion of hepatocytes positive for HCV antigens and the hepatic viral load. HCV immunohistochemistry has been applied in the setting of orthotopic liver transplantation (OLT). In post-OLT livers, at least in the early phase and in the presence of a massive infection, HCV may be directly cytopathic. Later HCV may induce a liver damage through immunological mechanisms. In other clinical conditions, such as LKM1-positive chronic hepatitis C, we observed a reduced synthesis of HCV proteins. Even patients with ANA and or SMA had a significantly lower number of HCV-antigens positive hepatocytes. In another study involving 290 HCV-infected patients we showed that autoantibody-positive patients had higher levels of transaminase and IgG and a more compromised liver histology. HCV plus HGV coinfection is relatively common, but the impact of concomitant HGV infection seems to be negligible in the natural history of the liver in HCV-RNA positive patients. We investigated the humoral reactivity to CYP2D6, the target antigen of LKM1 and reported the reactivity to conformational epitopes on the recombinant CYP2D6 molecule in all LKM1-positive patients with chronic hepatitis C. We described a significant association of the HLA haplotype B8-DR3 and HCV-related cryoglobulinemic syndrome.

Key words: *HCV, HGV, immunohistochemistry.*

Detection of HCV antigens in liver biopsies

Immunohistochemistry to detect HCV antigens within hepatocytes is a useful tool to identify and quantify the number of HCV infected cells. We have recently demonstrated that there is a positive correlation between the proportion of hepatocytes positive for HCV antigens and the hepatic viral load assessed by a competitive RT-PCR and that their clinical implications are one and the same. Namely, higher number of HCV-infected hepatocytes and higher viral loads have been found in patients infected with genotype 1b and, generally, in patients non-responders to interferon therapy (1).

HCV reinfection in orthotopic liver transplantation. - HCV immunohistochemistry seems a promising tool to evaluate the extent of HCV infection also in the setting of orthotopic liver transplantation (OLT). The number of cells positive for HCV antigens modifies rapidly in the liver of transplanted patients (2) in contrast with chronically infected patients (3). There is no correlation between the number of positive cells observed in the graft and their number in the explanted liver. In transplanted liver when conventional histology shows a hepatitis-like morphology, the majority of hepatocytes (40-90% -median 80%) are infected by HCV. The number of HCV-infected hepatocytes usually decreases with time. A positive staining is frequently found in livers with rejection injury, chronic hepatitis or even «normal» liver, but the number of infected cells is usually less than 30%. These data indicate that after an OLT HCV immunohistochemistry may help the clinician in distinguishing between liver rejection and HCV reinfection of the graft. Furthermore, our data suggest that at least in the early phase and in the presence of a massive infection, HCV may be directly involved in liver damage (4). In a series of 33 liver biopsies with different patterns of liver damage, obtained from HCV RNA positive patients 4 days to 4 years post-OLT and from 10 chronic HCV hepatitis patients, we studied by immunohistochemistry the relationship between HCV antigens, CD8+ lymphocytes, NK cells, apoptosis and regeneration markers (5). HCV positive staining was apparent in 50% biopsies obtained within 20 days and 90% within 21 days to 6 months after OLT. Hepatocyte apoptosis was higher in acute cases of post-OLT hepatitis, but it was also increased in «normal» transplanted livers in comparison with HCV-related chronic hepatitis patients. The number of putative cytotoxic T cells (CD8 and NK) was very high and similar in «acute» hepatitis, chronic hepatitis and rejection. This supports the hypothesis of concurrent direct HCV hepatocellular damage in the phase of acute massive infection, when hepatocellular death by apoptosis is maximal. A good correlation was observed between proliferation markers and apoptosis (6, 7). The magnitude of these phenomena suggests that a complete turnover of the hepatic mass may occur within two weeks in patients with «acute» pattern of liver damage. Counting of the Councilman bodies, a late marker of apoptosis, significantly correlates with the entity of apoptosis detected by evaluating the number of nuclei with DNA fragmentation and appears to be a reliable and useful tool in the routine setting.

The attempts to optimise the HCV-antigens staining protocol on paraffin-embedded sections were unsuccessful. Various fixation times, fixatives and antigen

retrieval techniques employed on paraffin-embedded biopsies gave poor results in comparison with unfixed cryostat sections. The viral antigens seem to be extremely sensitive to formalin and ethanol fixation, thus precluding a more widespread use and applications.

Autoantibodies and hepatic expression of HCV antigens. - HCV immunochemistry applied to other clinical conditions such as autoantibody-positive chronic hepatitis C suggests the hypothesis that in the presence of concomitant autoimmune reactions a better control of HCV infection is obtained. The progression of liver damage in such conditions is possibly mediated by immunomediated mechanisms not strictly related to active HCV infection.

We studied 105 consecutive HCV RNA positive patients, of whom 34 with circulating anti-nuclear (ANA) and/or anti-smooth-muscle (SMA) autoantibodies. Patients with autoantibodies had a significantly lower number of HCV-antigens positive hepatocytes (8). We conducted a similar study with 14 LKM1-positive patients and detected a significantly lower number of HCV-infected hepatocytes, a higher numbers of CD8+ lobular lymphocytes and higher transaminase levels. This observation suggests that, in addition to the HCV-mediated liver damage, alternative immuno (or autoimmune) mediated mechanisms may play a role in the liver damage of LKM1-positive patients (9). In another study involving 290 HCV-infected patients, of whom 87 were HCV RNA/autoantibody positive, we showed that autoantibody-positive patients had higher levels of transaminases and IgG and a more severe liver histology (10).

Mixed viral infections (HCV+HBV and HCV+HGV). - A small number of HCV-infected hepatocytes has been observed in the liver biopsies of patients double-infected with HCV and HBV. The finding of low HCV antigen positivity in the hepatocytes of HBsAg, HBcAg and anti-HBc-positive patients is possibly due to interference at the replicative level between the two different viruses.

The study of HCV+HBV coinfection in chronic and OLT patients showed that the areas of infection were in general mutually exclusive (11). In one single case of massive acute double infection of a transplanted liver, 60% hepatocytes were HCV-antigens positive and 20% HbcAg-positive. Only rare hepatocytes were positive for both HCV and HBV antigens (12).

HCV plus HGV coinfection is relatively common. In a series of 83 HCV-RNA positive patients, 22 (26.5%) were also HGV RNA positive (13). The comparison of the two groups subdivided according to the HGV status showed the close association between HGV infection and genotype 3, younger age at presentation, past intravenous drug use and a good response to interferon treatment (13). These differences are most likely due to epidemiological rather than viral factors. HCV/HGV coinfection did not seem to modify the biochemical and histologic indices (except steatosis) typically observed in single HCV infection, the prevalence of cryoglobulinemia and of autoanitbodies (14). Immunohistochemical staining of HCV hepatocellular antigens did not reveal HGV infection differences in the two groups (13). In summary, the impact of concomitant HGV infection seems to be negligible in HCV-RNA positive patients.

In an attempt to explore the role of HGV infection further, we investigated the possibility of producing a reagent to detect HGV antigens in the liver. Purified, fluorescein-labelled IgG from HGV-RNA positive sera were tested on liver biopsies obtained from HGV-RNA positive patients. Unfortunately, this approach did not yield positive results (unpublished data), possibly because HGV-RNA viremic patients lack neutralising antibodies to HGV. Thus, we used sera from subjects who were HGV-RNA negative and anti-HGV positive to the envelope protein of HGV, but still without any result. The appearance of several reports indicating that HGV is probably unable to infect the liver led us to discontinue this area of investigation, and to dismiss additional studies aiming at comparing tissue, PBMC and plasma levels of HGV-RNA, as originally planned.

Host factors in chronic hepatitis C: autoantibodies, cryoglobulins and the genetic background

Identification and cloning of an autoantigen in chronic hepatitis C: the liver kidney microsomal antibody type 1 (LKM1) model. - As far as the host factors are concerned, we investigated the nature of the target antigens of the circulating autoantibodies frequently seen during the course of flavivirus infection. The use of random phage display technology allowed the selection of several promising phages with LKM1-positive sera obtained from HCV-infected patients, but the selected mimotopes were also recognised by negative control sera. A human liver cDNA library was screened with a LKM1-positive serum obtained from a patient with HCV infection. A clone was isolated coding for nearly the complete sequence of cytochrome P4502D6 (CYP2D6), the known target of LKM1 positive sera. We cloned CYP2D6 in a bacterial expression vector and produced the recombinant CYP2D6 fusion protein in E. Coli. Since only a proportion of LKM1/HCV-positive sera react with prokaryotically expressed CYP2D6, in collaboration with Professor Diego Vergani we produced recombinant CYP2D6 metabolically labelled with 35S methionine by «in vitro» transcription/translation, and used this recombinant protein in an immunoprecipitation assay. Antibodies that bound radiolabelled CYP2D6 were immunoprecipitated and their levels assessed as cpm. Sera from 50 LKM1-positive patients (26 with AIH; 24 with HCV infection), 128 LKM1-negative patients and 57 normal controls were tested. Reactivity to 35S labelled CYP2D6 was observed in all LKM1-positive sera from patients with AIH and HCV infection, but in none of the controls. The cpm in both conditions were significantly higher than in normal controls and were correlated with the immunofluorescence titres of LKM1. Reactivity to 35S labelled CYP2D6 was inhibited by addition of an excess of eukaryotically expressed CYP2D6. LKM1 reactivity in patients with chronic hepatitis C is mostly directed to conformational epitopes on CYP2D6 (15).

LKM1 is conventionally detected by the subjective immunofluorescence technique. We established a simple and objective enzyme-linked immunosorbent assay (ELISA) that measures antibodies to cytochrome P4502D6 (CYP2D6), the target of

LKM1. An indirect ELISA using eukaryotically expressed CYP2D6 was designed. Absorbance values obtained against a reference microsomal preparation were subtracted from those obtained against a microsomal preparation over-expressing CYP2D6, thus removing non CYP2D6-specific reaction. Sera from 51 LKM1 positive patients (21 autoimmune hepatitis and 30 with HCV infection), 111 LKM1 negative patients with chronic liver disease (including 20 with HCV infection) and 43 healthy controls were tested. Of 51 patients positive by immunofluorescence, 48 were also positive by ELISA, while all the 154 LKM1 negative subjects were also negative by ELISA. The absorbance values by ELISA correlated well with immunofluorescence LKM1 titres both in autoimmune hepatitis and HCV infection. This simple, objective ELISA has the potential to replace the standard immunofluorescence technique (16).

To establish the potential pathogenic role of LKM1, we investigated by confocal laser microscopy the reactivity of LKM1-positive sera obtained from patients with chronic HCV infection on isolated rat hepatocytes. LKM1-positive sera reacted with discrete clumps on the outer plasma membrane surface of non-permeabilised, freshly isolated rat hepatocytes. A similar pattern was observed using a polyclonal anti-CYP2D6 antibody. The antigenic target of LKM1 reactivity on the liver cell membrane is indeed CYP2D6. The accessibility of this antigen to anti-CYP2D6 autoantibodies and CD8+ cells substantiates the hypothesis that such an autoreactivity may play a role in the progression of LKM1-positive liver disease (17)

ANA, SMA and pANCA autoantibodies in chronic hepatitis C. - Anti-nuclear (ANA) and anti-smooth-muscle (SMA) autoantibodies, typically present in type 1 autoimmune hepatitis, are also detected in up to 30% patients with HCV-related hepatitis (10). The immuno-morphological characterisation of ANA and SMA may help in the differential diagnosis between primary autoimmune disease and HCV-related autoreactivity (10).

Another autoantibody detected in HCV-infected patients is pANCA; whose target is an unknown antigen present in the cytoplasm of neutrophilic granulocytes. pANCA is frequently found in association with type 1 autoimmune hepatitis, but also in a considerable number of ANA/SMA-positive patients with chronic hepatitis C (18).

Genetic background in HCV-related cryoglobulinemia. - Our studies conducted on cryoglobulinemic patients has elucidated further the role of host factors in the clinical expression of HCV infection. The cohort comprised 111 patients of whom 50 were cryoglobulinemic including 17 symptomatic cases. Preliminary data shows an increased number of HCV infected hepatocytes in cryoglobulinemic patients, especially in those who are also symptomatic, irrespective of the HCV genotype (19). No correlation was found between hepatic viral load, presence of the cryoglobulinemic syndrome and histological and biochemical parameters of disease severity. Preliminary data indicates that cryoglobulinemia may be a favourable prognostic indicator as far as liver involvement is concerned (20).

We recently described a statistically significant association of the HLA haplotype

B8-DR3, which is commonly encountered in autoimmune diseases, and HCV-related cryoglobulinemic syndrome (21). HLA B8-DR3 is always associated with a heterozygote deletion of a C4A allele. Low levels of complement are frequently found in mixed cryoglobulinemia. Thus we hypothesised that this may be due not only to an increase in complement activation by immunocomplexes, but also to a genetic defect. We studied the C4A and C4B complement isotype in 28 patients with cryoglobulinemic syndrome and 150 healthy controls. C4AQ0 findings were similar in the two groups, while C4BQ0 and the C4Q0 phenotype (presence of either C4AQ0 or C4BQ0) was significantly more frequent in cryoglobulinemic subjects. This would suggest a role for complement genes is the pathogenesis of the clinical manifestations of the cryoglobulinemic syndrome (22).

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INTERACTIONS AND GENETIC HETEROGENEITY OF HEPATITIS VIRUSES (HBV, HCV, HDV): MOLECULAR BASIS OF PATHOGENESIS

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Progress Report

INTERACTION AND GENETIC HETEROGENEITY OF HEPATITIS VIRUSES (HBV, HCV, HDV): MOLECULAR BASIS OF PATHOGENESIS

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Summary

During Hepatitis viruses infection the pathogenesis of liver damage depends on the interplay between viral replication and host-immune response. Therefore, dynamic studies of the overtime variations of viral load or mutants viremia within the overall viral population in relation with the host-virus interactions are extremely important to better understand the biological and pathogenetic role of a virus or virus mutant. In our project we addressed some of these aspects studying the pathobiologic implications of pre-C region mutations. We showed that the variations of the ratio between wild-type and mutant correlate with significant events in the host-virus interactions, suggesting that the suppression of HBeAg secretion plays a major role in the pathogensis of liver damage during HBeAg minus HBV infection. Furthermore, we studied the role in liver damage of 2 recently described viruses (GB C/ hepatitis G virus and TTV virus), which had been hypothesised to be responsible for some cases of acute and chronic nonA-nonC hepatitis.

Key words: *HBeAg minus HBV, GBVC/HGV, TTV.*

Introduction

Major Hepatitis viruses are not usually cytotoxic and the pathogenesis of liver damage observed during their infection is sustained by the aggression of the host's immune system against viral antigen expressing hepatocytes. In spite of this evidence, when mutant viruses are identified in association with specific histopatological or clinical patterns we have to rule out the possibility of a direct cytopathic effect of the new strain. Furthermore, when multiple viral infections or new putative hepatitis viruses are detected in a patient with evidence of liver disease it is mandatory to use surrogate

markers of virus induced liver damage or to perform dynamic studies to correlate the variation of viral replication and liver disease activity to reach an ethiological diagnosis. In our project we addressed some of these aspects studying the implications of heterogeneity of the pre-core region of the Hepatitis B Virus genome and the role in liver damage of 2 recently described viruses (GB C/ hepatitis G virus and TTV virus), which had been hypothesised to be responsible for some cases of acute and chronic nonA-nonC hepatitis.

Heterogeneity of the pre-core region of HBV-DNA: pathogenetic implications

The pre-C\C region of the HBV genome encodes for both the nucleocapsidic protein (HBcAg) and Hepatitis B «e» antigen (HBeAg) the non-structural, secretory form of the nucleocapsid protein. The most frequent virologic explanation for the HBeAg negative profile of hepatitis B are mutations occurring within the pre-core region of HBV-DNA which block translation of the protein. Among several mutations so far described, the one responsible for more than 90% of defective HBeAg secretion is a point mutation, G-A switch at nucleotide 1896 (HBeAg minus HBV) that changes a tryptophane (UGG) codon into a translation stop codon (UAG) in the corresponding mRNA.

In preliminary reports infection with HBeAg minus HBV was found to be associated with severe forms of acute and chronic hepatitis B and a direct cytopatic effect of the mutant was suggested. However, more specific studies of the epidemiology of pre-core mutants revealed that HBeAg defective HBVs were also present in HBV carriers with mild or without liver disease and the pathogenetic implications of HBeAg defective mutants were questioned.

Indeed, the discrepancy between the different findings is only apparent and not surprising, as the severity of liver disease is determined not only by virologic factors, but it depends also on other factors including number of infected cells, competence and genetic heterogeneity of the immune system, vigor and extent of non-specific inflammatory response and magnitude of the killing of hepatocytes endangered by metabolic or storage diseases or infected with other hepatotropic viruses.

Furthermore, additional factors should be considered in the analysis of the results: the characteristics (sensitivity, qualitative or quantitative analysis) of the assays used to identify the mutation in the virus genome; the methodology of the study (single point versus dynamic observations and timing of specimen collection during patient follow-up). Last but not least, the understanding of the pathogenetic role of HBeAg minus HBV can not leave out of consideration the biological implications of the lack of HBeAg secretion. The expression of HBeAg is non-essential for virus viability and its precise biological role in the life cycle of the virus remains to be elucidated. However, several experimental and clinical observations suggest that HBeAg secretion may have a pivotal role in the strategy of HBV persistence by inducing immunologic tolerance in the infected host. Serum HBeAg would act as immune modulator through different mechanisms: crossing the placenta HBeAg could establish Th-cell tolerance to HBeAg

and HBcAg in the new-born with perinatal infection; during infection acquired later in life, circulating HBeAg would preferentially deplete inflammatory TH1 cells (which are necessary for viral clearance) via FAS mediated apoptosis. In absence of HBeAg secretion, during primary infection, HBV immunotolerance would not be induced, whereas in chronic infection the loss of HBeAg would determine the loss of immunotolerance. Activated HBcAg/HBeAg specific Th cells are directly citotoxic by infected hepatocytes and able to induce and maintain HBc/HBeAg specific CTL and down regulate HBV replication.

The fact that not only infections with pre-core mutants, but also all the conditions (mutations in the basic core promoter or HDV superinfection) where HBeAg secretion is reduced or inhibited can lead to clinical patterns characterised by exacerbations of liver damage strongly supports the hypothesis that a major pathogenetic implication of pre-core heterogeneity is mediated by the lack of HBeAg secretion.

To study the correlation between pre-core heterogeneity and the pathogenesis of liver damage, we analysed firstly the difference between crossectional and follow-up observations. We characterised multiple serum samples from 50 randomly selected patients from a cohort of 170 chronic HBV carriers (93 HBeAg and 77 anti-HBe positive) followed up in our unit for their liver disease. The characterisation of 170 baseline sera showed a mixed viral population in 36% of HBeAg and in 40% of anti-HBe positive patients. The prevalent virus was wild type and HBeAg minus in HBeAg and anti-HBe positive patients, respectively. In the follow-up study (mean 2 years, range 1-3 years) a mean of 4 serum samples (range 3-10 samples) was analysed for each patient. In the 20 HBeAg positive carriers the proportion of patients with mixed viral population remained almost unchanged during the follow-up. Overall, at the end of follow-up, 42% of HBeAg positive patients had mixed type viremia, whereas at baseline the same viral condition was present in 36%. On the contrary, 70% of the 30 anti-HBe positive patients showed, at least temporaneously, a mixed viremia, suggesting that significant changes in the virus population had occurred during the observation period (1). As further step we tried to correlate the features of the infecting virus population with the clinical patterns of chronic hepatitis B anti-HBe positive. Studying perspectively a cohort of 164 anti-HBe positive patients we showed that this form of chronic hepatitis is characterised by 2 major biochemical profiles, acute recurrent hepatitis and continuous disease. Persistent ALT elevations without flares was seen in 59 (35.9%) patients and ALT flares in 105 (64.1%), 73 of them with intervening phases of ALT normalisation (2, 3). The remaining 32 patients maintained mild-moderate ALT levels between flares. In these patients the monthly monitoring of viremia showed that HBV-DNA serum levels increased before hepatitis exacerbations and dropped abruptly thereafter. The increment in viremia levels preceding the ALT flares was always sustained by wild-type HBV, whereas HBeAg minus HBV appeared to be selected during the hepatitis exacerbations: mixed viremia was found at baseline in 81.3% of cases, during the peak in 66.7%, during the fall in 28.6% and in the remission phase in none (4). As a consequence a proportion of the anti-HBe positive patients showed an exclusive HBeAg minus HBV population in the late phase of the hepatitis exacerbations, when ALT values were within the normal range.

Taking into account the above observations and analysing all the available data, we can hypothesise some possible pathogenetic implications of HBeAg minus HBV.

The results of our dynamic studies show that in chronic anti-HBe positive patients the detection of a homogeneous HBeAg minus population occurs frequently during phases of disease remission. These findings are consistent with the reports of the literature that show the presence of pre-core mutant in HBV carriers with mild forms of liver disease. The clinical observations together with the absence of cytopathic effects of HBeAg minus HBV in «*in vitro*« experiments, *argue against* the hypothesis of the *direct pathogenicity of the mutant.* However, the close correlation between variations in the ratio of wild-type/HBeAg minus HBVs and those of disease activity during the patients' follow-up suggests a pivotal role of pre-core HBV-DNA heterogeneity in the pathogenesis of liver damage. Specifically, the *pathogenetic implications of pre-core heterogeneity appear to be mediated by the unique biological impact of HBeAg expression*, which may act either as inducer of immuno-tolerance or target of cell mediated-immune response. Two important findings are consistent with this hypothesis: firstly, the brisk attempts of HBV elimination in primary infections, sustained by HBeAg defective viruses and secondly, the close relationship between variations in wild-type/HBeAg minus HBVs ratio and liver cell necrosis episodes.

The appearance of mixed virus population in association with the disease phases in patients, in whom disease exacerbations are intervened by remissions, suggests that any increase in HBeAg expression is implicated in the triggering of a necroinflammatory event. Both in acute and chronic HBV infections, the cell-mediated attack against HBeAg expressing hepatocytes would be involved in the exacerbation of the necroinflammatory process and favour the selection of hepatocytes which lack the expression of the target antigen. This view is consistent with the inversion of the wild-type/HBeAg minus HBVs ratio (the mutant becoming the prevalent virus) during and after ALT flares in spite of an overall reduction in total viremia.

In conclusion, in *chronic HBV infection most of virologically important events and most of hepatitis exacerbations appear to be associated with detection of a mixed wild type/HBeAg minus viral population.* On the contrary wild type or HBeAg minus HBV populations are found to circulate homogeneously in the blood of patients with stable clinical conditions. Briefly, 2 major conditions can be identified in the HBeAg positive phase: the former is characterised by exclusive wild-type viremia, whit high levels of HBeAg and is associated with the absence of disease exacerbations; the latter shows frequent hepatitis flares, HBeAg minus HBV becomes detectable and HBeAg expression is fading away. A similar disease profile is observed in anti-HBe positive patients in whom the virus population is mixed and HBeAg expression is tapered. Conversely, in anti-HBe positive patients with homogeneous HBeAg minus HBV viremia, the expression of HBeAg appears completely absent and transaminases serum levels are persistently elevated in the absence of significant disease exacerbations (5).

GB C/HGV and TTV infections: pathogenetic implications in liver damage

In the last years, several research groups addressed their studies to the identification of the virus/viruses responsible for nonA-nonC hepatitis: so far 3 viruses had been hypothesised to be involved in liver damage, GB C/HGV and TTV and more recently SEN virus. We studied the prevalence of GBVC/HGV and TTV infections in patients with evidence of liver disease (cryptogenic, viral and cholestatic) and their pathogenetic implications.

GBVC/HGV. - The prevalence of GBVC/HGV infection in the general population appears to be similar to that of both HBV and HCV, but in spite of this the HGV prevalence in patients with acute or chronic non-A non-E hepatitis is low (ranging from 12-23). To study the pathogenetic implications of GBVC/HGV infection in liver damage we analysed the sera obtained from 144 patients with cryptogenic liver disease: HGV-RNA was detectable in the sera of 20 patients (13.8%). The spectrum of liver damage was extremely wide and the only commune feature was the association with the combined elevation of GGT and Aph serum levels (67% of HGV-RNA positive patients vs 25% of controls, $p<0.005$) (6). A direct implication of GBVC/HGV in the pathogenesis of the classical forms of cholestatic liver disease was ruled out by the evidence of the low prevalence of the infection in patients with Sclerosing Cholangitis or Primary Biliary Cirrhosis (3 of 48 patients, 6%). However, in spite of this evidence, we can not exclude the possibility that during a chronic liver damage the concurrent presence of GBVC/HGV could induce an elevation of GGT and Aph levels. The high degree of homology between GBVC/HGV and HCV (both viruses are classified in the flaviviridae family) could favour interferences at biological or pathogenetic levels. Thus, we studied the prevalence of GBVC/HGV infection in patients with HCV infection. HGV-RNA was detectable in 11% of 117 patients with chronic hepatitis C, in 55% of 20 patients with acute hepatitis C and 55% of 57 transplanted patients because of terminal liver disease due to HCV. The high prevalence of the infection in patients with acute hepatitis C or liver transplant was explained by the exposure to risk factors (drug addition and transfusions). An asymmetric fluctuation of GBVC/HGV and HCV viremia was observed in patients with acute hepatitis C, suggesting a possible interference at the replicative level between the two viruses, at least in the setting of acute infection (7). On the contrary, the outcome of liver disease did not appear to be influenced by GBC/HGV infection.

TTV. - Since preliminary data suggested a possible association between TTV and liver disease, world-wide epidemiological investigations took place. However, conclusive evidence on the impact of TTV in liver pathology has not been found yet. We investigated the prevalence of this novel virus and its role in liver diseases (8) testing for TTV-DNA 106 patients and 102 blood donors with the hemi-nested PCR assay reported by Okamoto. TTV DNA was found in 19 of 102 volunteer blood donors (18.6%) and in 27 of 106 patients with liver disease (25.5%): 10 of 28 chronic hepatitis B (35.7%), 9 of 28 chronic hepatitis C (32.1%) and 8 of 50 (16%) cryptogenic liver

disease patients. Previous Interferon treatment was not associated with a significant lower prevalence of TTV infection. TTV prevalence was higher in patients with blood exposure (42.8%, 6/14) than in patients without risk factors (21.4%, 18/84). Four of 5 patients (80%) with HBV familial infection and without blood exposure were also TTV positive. Therefore, we could only conclude that the prevalence of TTV infection in Italy is high in liver disease patients as in blood donors, in patients with blood exposure as in subjects without risk factors. No data from our study could support a relevant role of TTV in the aetiology of liver disease in our country. We found that G1 and G2 TTV are the most common genotypes in Italy (93.5%), however, partial nucleotide sequences from 3 isolates diverged more than 30% from the 2 prototype genotypes G1 and G2 and were 88% homologous to the recently described genotype G4.

More recently, TTV has been shown to have a very high degree of heterogeneity that greatly affected the sensitivity of TTV detection by genome amplification methods. In fact, once the complete sequence of the TTV circular genome became available, Takahashi et al., using a highly conserved domain from the 5' NCR for PCR primers design, detected TTV infection in 92% of 100 healthy subjects in Japan. By means of a quantitative «real time» PCR assay employing primers from a highly conserved region of the ORF1 such ubiquitous distribution was also found in 20 patients with chronic hepatitis co-infected with HCV and TTV who underwent interferon treatment in our center. In these patients we investigated (9) the dynamic variations of HCV-RNA, TTV-DNA and ALT serum levels before, during and after treatment. The combined quantification of HCV-RNA and TTV-DNA serum levels of our study revealed the presence of 2 patients in whom significant changes of ALT serum levels occurred with parallel variations of TTV-DNA but not of HCV-RNA serum levels. Such findings support the hypothesis that TTV and not HCV was the candidate agent responsible for liver damage in these patients, at least in the time frame investigated.

In conclusion, taking into account the high TTV genetic variability, we could speculate that the natural occurrence of pathogenic variants might occasionally cause liver damage in chronic carriers. However, a better characterisation of the TTV-DNA coding regions as well as of their protein functions are necessary to allow meaningful sequence comparison between TTV isolates with and without putative pathogenic behaviour.

The finding of a very low number of cases of liver disease possibly associated with TTV, prompt larger number of pathogenetic studies and raise the question whether liver pathogenecity of TTV is isolate or type specific. Our observations, indeed, could provide an explanation for the apparent discrepancy among the finding of a widespread distribution of the virus in the normal population and the original identification of TTV in post-transfusion hepatitis patients and several reports of a higher prevalence of TTV infection in patients with liver disease. Larger studies focused on the dynamic variations of ALT and TTV-DNA serum levels in TTV infected patients could provide an answer to the question of how frequent and under which condition such behaviour of TTV might occur.

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THE ROLE OF HCV INFECTION ON THE IMMUNE SYSTEM

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Progress Report

THE ROLE OF HCV INFECTION ON THE IMMUNE SYSTEM

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Summary

Patients with chronic HCV infection have frequently of B-cells clonal expansions. We have observed a high incidence of monoclonal B-cell expansions in Italian patients with HCV^+ chronic liver disease, without detectable cryoglobulin. An extension of this study to Japanese HCV^+ patients showed that, unlike the Italians, a polyclonal IgM fingerprint, indicating that the genetic background might determine the effects of HCV on lymphocytes. The effect of IFN-α therapy on the expanded B-cell clone was investigated in 20 patients using the Ig gene fingerprinting technique. The results showed the persistence of the monoclonal B-cell population in the PBL of all non-responders and relapsers, whereas a normal distribution was observed in the three cases that responded to treatment.

A high prevalence of chronic HCV infection has been demonstrated in patients with immunocytoma. To investigate the role of the HCV in the pathogenesis of this disorder, we have analysed the V region gene repertoire of 16 cases. V region sequences were identified in 8 of them and showed somatically mutated and clonally related V_H and V_L transcripts, indicating chronic antigen stimulation. In 9 out of 16 we found the V_H1 family gene 51p1 rearranged. And in 5 of these cases we could identify the V_L as the VKIII-kv325 gene. Expression of several combinations allowed us to rule out reactivity against HCV proteins and indicated somatic mutations as the cause of RF activity.

In addition to acute and chronic liver disease, hepatitis C virus (HCV) infection seems to be involved in the induction of lymphoproliferative disorders. Several reports have shown a strong association of Type II mixed cryoglobulinemia (MC) in HCV infected patients (1, 2). MC is characterised by the presence of serum cold-precipitable immune complexes that contain polyclonal IgG and monoclonal rheumatoid factors

(RF), usually of the IgM-kappa (3, 4). These monoclonal RF antibodies are secreted by expanded B cell clones. Type II MC frequently evolves into low grade B-cell non-Hodgkin's lymphoma (NHL), suggesting that chronic HCV infection can lead to both a bening and a malignant lymphoprolipherative disorder (5, 6). In patients with NHL, high prevalence of anti-HCV antibodies and HCV viremia has been reported, with values particularly high in Italy.

The HCV seems to infect not only hepatocytes but also B and T lymphocytes. The viral genome has been detected in PBMC from HCV^+ patients affected by MC.

In a recent study (4) we have used an immunoglobulin gene fingerprint analysis to study the IgM heavy chain mRNAs expressed by PBMC from HCV^+ patients, in particular of those with no clinical or biochemical evidence of MC. This study revealed that almost one fourth (24%) of these patients have expanded populations of clonal Ig gene rearrangements indicating that HCV infection can induce benign lymphoproliferations of IgM^+ B-cell clones. Serum RF activity in these individuals correlated with the presence of monoclonal B-cell expansions suggesting that the RF antibodies were secreted by the expanded clones.

Key words: *Hepatitis C virus, HCV infection, HCV immunology.*

Ethnic differences in the B-cell monoclonality of HCV^+ patients.

We have now extended these studies to a larger number of cases of Italian patients, and basically confirmed our previous results. The percentage of HCV^+ patients, that show a clear monoclonal B-cell expansion is of 26%. In these cases are not included those patients that have serum cryoglobulin levels corresponding to MC.

In addition, we also studied the incidence of clonal B-cell expansions in HCV^+ individuals from Japan. This represents a very interesting group since the incidence of MC in HCV infected Japanese patients is extremely low in comparison with western countries' population. We analysed 44 HCV^+ Japanese patients of different severity: 2 healthy carriers, 11 affected by liver cirrhosis and 31 by chronic hepatitis.

The expansion of monoclonal IgM^+ B-cells was studied in PBMC by the gene fingerprint technique. The results obtained in the different groups are presented in Table 1. We found that, contrary to the Italian patients, all the Japanese patients presented a complete normal distribution of bands with no indication of clonal expansions. This is a most interesting result since the Japanese population is also known to have a particularly low (almost absence) incidence of B-cell chronic lymphocytic leukaemia (B-CLL), the most common type of leukaemia in western countries, which is also characterised by expressing surface Ig of the polyreactive type with many cases of RF activity.

In selected Italian and Japanese patients we also studied the immunoglobulin fingerprint for the IgG^+ population and found no indication of clonal expansions.

Table 1. B-cell monoclonal expansions in HCV^+ Italian and Japanese patients. B cell monoclonality was established by the Ig gene fingerprint analysis performed on total RNA from PBL of HCV infected patients.

Patients	Number of cases	B cell monoclonality
Italian normal subjects	25	0
Italian patients affected by alcoholic CLD	25	0
Italian patients affected by mixed cryoglobulinemia	12	12 (100%)
Italian patients affected by HCV positive CLD	60	16 (26%)
Japanese patients affected by HCV positive CLD	44	0

CLD, chronic liver disease

α-IFN and B-cell lymphoprolipherations

It has been reported that α-IFN is effective in the treatment of HCV^+ patients with MC. The IgM^+ B-cell fingerprinting was studied in a group of 20 HCV^+ patients with MC, that were under IFN-a treatment for a period of one year before and after the treatment. This was done in collaboration with other institutions co-ordinated by Dr. Gabriele Pozzato and published in Cancer (7). The results showed the persistence of the monoclonal B-cell population in the PBMC of all non-responders and relapsers (16/20, 80%) whereas a normal distribution of bands was observed in the three responders to the treatment, thus indicating a clear correlation of the virus persistence with the abnormal fingerprint. This result also suggests that the monoclonal B-cell expansion is directly linked to the presence of HCV and probably because of a continuous antigenic stimulation.

IgM-V region repertorie in HCV associated immunocytomas

To further investigate the role of HCV infection in the pathogenesis of malignant lymphoproliferative disorders we studied at the molecular level the V_H and V_L repertoires of several HCV-associated lymphoplasmacytoid lymphomas.

The presence of an expanded IgM^+ B-cell population was initially investigated in the peripheral blood of 16 different immunocytoma patients using the Ig gene fingerprinting technique. We used 15 PBL samples and one lymph node specimen. A prominent band corresponding to the V_H gene rearrangement of the malignant clone was observed in 13 cases (PBL samples). The tumour V_H region was associated to μ sequences in all the PBL samples while it was detected in both μ and γ H chain transcripts in the lymph node specimen. This latter finding was subsequently confirmed by cloning and sequencing the amplified V_H regions, indicating that a subset of immunocytoma cells in this patient had undergone Ig heavy-chain class switching. The sequences of 8 V_H and V_L from the immunocytomas were determined. We found that

the V_H 1 family gene 51p1, which is frequently expressed in monoclonal RF antibodies from patients with Type II MC, was also overrepresented (9 out of 16, 57%) in the HCV-associated immunocytomas. Interestingly, five clones that express the 51p1 gene, also express a single V_L gene (kv325) of the V_KIII family (Table 2).

In both V_H and V_L sequences from all the immunocytomas we found nucleotide substitutions, indicating the existence of somatic mutations. This was also confirmed by repeated amplifications and sequencing, in which the same mutations were found. The distribution of replacement (R) and silent (S) substitutions seems to indicate a higher R/S ratio in the CDRs than in the framework regions, a finding consistent with antigen stimulation and selection. It was also observed, in all cases, intraclonal diversity among the tumour derived V_H sequences, indicating ongoing mutational events in the neoplastic clone. Since RF activity is expected from the antibodies expressed by the malignant clone, our findings of ongoing somatic mutations suggest a role for a chronic antigenic stimulation, most likely with immune complexes containing the HCV, in the development of the HCV-associated lymphoproliferations.

Table 2. Characteristics of HCV-associated Immunocytoma and V regions gene usage of the expressed Immunoglobulins.

Patient	Age/Sex	Involved sites	Associated conditions	cryogl.	RF	V_H	V_L
SEL	63/F	S, BM, LN	LC	1	45	III-DP51	IV-DPK24
SEG	45/M	S. BM, PB	CPH, tII- MC	3	124	I-51p1	III-kv325
SS	54/M	BM, PB	CAH, tII-MC WM, MPGM	19	6070	I-51p1	III-kv325
MEL	34/F	BM	CAH, tII-MC MPGM	3	688	I-51p1	n.d.
HAZ	68/M	S, BM, LN, PB	LC	1	57	I-51p1	III-kv325
MS	67/M	S, BM, LN, PB	WM	-	<20	III-DP47	
FAV	50/F	BM	LC, tII-MC	4	45	n.d.	n.d.
LC1	47/M	BM	tII-MC, MPGM	34	1815	I-51p1	n.d.
LC2	68/F	S, BM, PB	tII-MC	2	125	I-51p1	III-kv325
LC3	77/F	BM, PB	tII-MC, MPGM	2	824	I-51p1	III-kv325
LC4	62/M	S, BM, PB	CAH, tII-MC	1	109	IV-V_H4.21	III-Vg
LC5	57/F	S, BM, PB	tII-MC, WM	15	487	n.d.	n.d.
LC6	66/M	BM	none	-	<20	I-51p1	n.d.
LC7	51/M	BM	CAH, tII-MC	35	200	I-51p1	n.d.
LC8	58/M	BM	tII-MC	2	600	n.d.	n.d.
LC9	40/M	S, BM, PB	MPGM	-	<20	n.d.	n.d.

Lymphoproliferations in non-HCV infections

The association of monoclonal B-cell expansions in HCV infected individuals opened the possibility that this was due to the continuous presence of the immune complexes because of chronic liver disease. In order to address this problem several patients (15) affected of chronic HBV infection were analysed. We first investigated by means of the Ig gene fingerprint analysis whether there were specific V_H amplifications. We did not find any indication of B-cell expansions in these patients, thus suggesting that the results obtained within the HCV infected populations can not be interpreted in terms of the chronicity of the infection and the presence of immune complexes. Rather, they indicate that the virus itself plays a direct role in the ethiopathogenesis of the lymphoproliferative disorders. This is possible through an association with the sequences of the viral proteins themselves or, alternatively, because of specific characteristics of the liver disease caused by HCV in relation to those of HBV. These studies were confirmed by a fingerprint analysis specific for the gene V_H 51p1 (which we found associated with immunocytomas from HCV^+ infected people) that also showed no indication of expansions within the HBV^+.

Expression of selected V_L / V_H combinations

Our results on the analysis of the V_H and V_L regions from several immunocytomas have already indicated the existence of a strong bias in the repertoire of V region sequences expressed by the malignant clones. Most of them show the same association of V_H and V_L genes with indications of antigen driven somatic mutations. The gene usage of these tumours suggest that these antibodies would have RF activities.

In order to establish the reactivity of the different antibodies, we have expressed several of them in the form of a single chain Fv (scFv) in eukaryotic cells (SIPs). These miniantibody molecules are easily produced maintaining their reactivities (8). We analysed both the reactivity of these molecules against the HCV viral proteins as well as against human IgG (RF activity). As a control of polyreactivity binding to cardiolipin was also investigated. The HCV proteins were derived from Hela cells transfected with a construct containing the complete HCV cDNA genome, under the control of the T7 polymerase, and infected with a recombinant vaccinia virus expressing T7. Assays to test reactivity against these proteins were immunoprecipitations as well as western immunoblottings.

Table 3. Reactivity of SIP-antibodies expressing the Vh51p1 gene. In parenthesis, residues present in the indicated positions.

SIP	V genes	Reactivity		
		HCV	huIgG	Cardiolipin
H	VH 51p1(A33) VL (λ)	-	-	-
S	VH 51p1(G33) VL (κ)(T44)	-	+	-
H/S	VH 51p1(A33) VL (κ)(T44)	-	-	-
Sa	VH 51p1(G33) VL (κ)(A44)	-	-	-
H/Sa	VH 51p1(A33) VL (κ) (A44)	-	-	-
S/H	VH 51p1(G33) VL (λ)	-	-	-

In two different patients we have found the same 51p1 V_H gene, both with a conserved amino acid substitution in CDR1 (A33G), associated to the kv325 V_KIII gene. Also the V_K was somatically mutated though in different residues. One of these contained a substitution in the FR2 of the kv325 gene changing residue 44 from A to T.

The SIP-antibodies and expressed are shown in Table 3. None of the expressed SIPs showed any reactivity against the set of HCV proteins, thus suggesting that their expansions can not be directly associated to stimulation by viral antigens. Only one of these antibodies (SIP-S) showed rheumatoid factor activity when tested against human IgG. In several cases we analysed the effect of shuffling of L chains or reverting mutations to the germ-line sequences. For instance SIP-H contains the 51p1 gene in the germ-line configuration, associated with a λ light chain. This antibody did not react against any of the products tested and this was so, even when it was associated with the κ light chain from SIP-S. When SIP-S was mutated back in the V_H to the germline A33, reactivity was lost. The same result was obtained when T44 in the $V_κ$ of SIP-S was mutated to the germ-line A. This result indicate that both somatic mutations were necessary for the RF activity of this molecule.

The results so far obtained allow us to conclude that the immunoglobulin expressed by the malignant clones do not recognise HCV derived antigens. In addition, we could clearly demonstrate in one case RF activity which was completely dependent of two cooperative somatic mutations in both the V_H (CDR1, 33A to G) and the V_L (FR2, 44A to T), suggesting that this reactivity depends on antigen selection by immune complexes.

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PROFILE OF THE VIRAL ACTIVITY DURING THE HEPATITIS C VIRUS (HCV) INFECTION AND STUDY OF THE ANTIBODY REPERTOIRE AGAINST SPECIFIC HCV EPITOPES

Coordinator: Prof. Massimo CLEMENTI

Progress Report

MOLECULAR BIOLOGY OF HEPATITIS C VIRUS INFECTION: [A] INTRA-HOST GENETIC EVOLUTION OF HCV AND [B] RELEVANT FEATURES OF HCV ACTIVITY IN VITRO AND IN VIVO

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Summary

This research has addressed two main aspects of the hepatitis C virus (HCV) infection: firstly, the biological role of HCV evolution in infected hosts, the determinants of viral diversity, and the evolutionary characteristics of the putative HCV envelope sequence during the different phases of the infection and in different clinical conditions; secondly, the relevant features of the viral dynamics in vitro and in vivo.

The results have described some crucial features of the natural history of HCV infection and virus pathogenic potential at the molecular level, the molecular correlates of virus persistence, and the viral turnover in infected hosts. In particular, our study has described the evolution of HCV populations during acute infections of adults and newborns, documenting that the host's selective pressure is the principal determinant of HCV diversity in infected hosts and strongly suggesting that intra-host HCV diversification is compatible with a typical Darwinian model system. Moreover, the semi-quantitative evaluation of the selective constraints active on a hypervariable sequence of the putative HCV envelope region (HVR1) has described different patterns of viral evolution during persistent infection. Finally, the research has described the principal features of the viral dynamics and has addressed the role of HCV viremia in reflecting the systemic viral activity in vivo.

Key words: *Hepatitis C infection, HCV molecular virology, HCV immunology.*

Background and aims of the study

Hepatitis C virus (HCV) infection has a world-wide distribution. After primary

infection, HCV persists in a large proportions of patients, and persistent HCV infection is associated with clinical syndromes ranging from asymptomatic infection to chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma. In this context, researches aimed at understanding the pathogenesis of HCV-associated diseases and at developing effective strategies for the treatment of this important infection of humans is currently of crucial importance.

The study of the natural history of HCV infection at the molecular level and of the molecular correlates of the HCV persistent infection may have a key role in the understanding of the viral pathogenic potential. To gain more insights on the mechanisms of HCV persistence and the systemic viral activity in vivo, we have addressed in this study two main aspects of the HCV-host interaction: the relevant features of HCV activity in vitro and in vivo and the intra-host evolution of HCV populations.

Molecular strategies and results

Firstly, the study has addressed the biological and pathogenic role of HCV replication in vitro and in vivo. In this context, a highly sensitive quantitative competitive RT-PCR (cRT-PCR) method for HCV RNA molecules developed in our laboratory (A. Manzin et al., 1994) has been used for quantifying cell-free viral genomic RNA in plasma and HCV transcripts in infected cells.

In vivo, the systemic viral activity is a formal entity that consists in a sum of dynamic processes, including productive infection of target cells, release of virions outside the infected cell and eventually in the blood compartment, and *de novo* infection of permissive cells; this activity has been analysed in this study during the different phases of the HCV infection and in different clinical conditions associated with HCV persistence, including chronic hepatitis, cirrhosis, and cryoglobulinemia (A. Manzin et al., 1995; A. Gabrielli, et al., 1995; A. Gabrielli et al., 1996; F. Giostra et al., 1996; A. Manzin et al., 1996; G. Ballardini et al., 1997; R. Francesconi et al., 1997; G. Svegliati Baroni, 1999). More recently, we have described the dynamics of cell-free virus after perturbation by plasma exchange (Manzin et al., 1999). This approach has offered an alternative to studying patients treated with antivirals in order to understand the dynamics of HCV exchange among different compartments in vivo. The data have extended previous evaluations by documenting that large amounts of newly produced virions are introduced into the vascular compartment within a few hours of the drop in HCV viremia caused by plasma exchange.

In vitro, the study has addressed the dynamics of HCV infection in different cell culture systems, including human fetal liver cells and B cell lines (S. Iacovacci et al., 1997a; S. Iacovacci et al., 1997b; M.B. Valli, et al., 1997; M.B. Valli, 1998).

Secondly, the research has evaluated the relevant features of the HCV evolution during primary infection of adults and newborns (A. Manzin et al., 1997; A. Manzin et al., 1998; A. Manzin et al., 1999b). In this study, we have documented the dominant role of the selective constraints of the host on HCV envelope domains that are crucial

for HCV biology in driving the evolution of viral populations. The data have strongly suggested that intra-host HCV evolution is compatible with a typical Darwinian model system.

Finally, a collaborative study with other groups has led to the production of specific anti-HCV monoclonal Fabs using the combinatorial library technology (P. Plaisant, et al., 1997; R. Burioni et al., 1998a; R. Burioni et al., 1998b). Of note, these human monoclonal Fabs (including specific antibodies to the HCV envelope domains and non-structural antigens) may represent useful tools for further research on the immune response to this infection.

Discussion and concluding remarks

This research has employed different molecular approaches to studying HCV infection in vivo and in vitro and has addressed the evaluation of HCV activity in vivo and in vitro and the analysis of intra-host evolution of HCV populations. Overall, the data obtained in this study have principally (i) documented the viral activity during the different phases of the infection and in different clinical conditions, (ii) shown the dynamic features of the turnover of cell free HCV virions after perturbation by plasma exchange, (iii) analysed the dynamics of intra-host HCV evolution and evaluated the role of the host's selective pressure on HCV domains (iv) described the development of a combinatorial library obtained from a HCV infected subject and the features of the human monoclonal Fabs obtained after panning. These results may have implications in the understanding of the pathogenic potential of this important human pathogen and in the designing of new working hypotheses in this field.

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DETECTION OF HBV AND HCV INFECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND HEPATOCYTES OF PATIENTS WITH DUAL HBV/HCV CHRONIC INFECTION

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Progress Report

DETECTION OF HBV AND HCV INFECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND HEPATOCYTES OF PATIENTS WITH DUAL HBV/HCV CHRONIC INFECTION

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Summary

Twenty patients with chronic dual HBV/HCV infection and 20 sex- and age-matched HCV infected controls have been enrolled in the present study. Five patients were HBeAg-positive, 4 of whom were HBV-DNA-positive by hybridisation; 15 patients were anti-HBe-positive, 8 of whom were HBV-DNA-positive by PCR. The severity of liver disease in dually infected patients was inversely related to the extent of HBV replication. Suppression of both viruses was found in two patients, in whom minimal or absent histological lesions were found. In HBeAg/HBV-DNA positive patients, the PBMCs were HBV-DNA-positive, while serum, liver and PBMC HCV-RNA was undetectable. In patients with detectable liver HCV-RNA, serum HBV-DNA (hybridisation) was undetectable and liver HBcAg was not expressed, although minimal amount of HBV-DNA could be found by PCR in 64% of such patients, in whom PBMC HCV-RNA was found significantly less frequently compared to control patients with HCV infection alone. In PBMC, HBV/HCV coinfection was never found. Hepatic expression of TNF-α was significantly higher in HCV-RNA positive patients, either with coinfection, either with HCV infection alone, compared to a control group of patients with HBV infection alone. Moreover, in coinfected patients, TNF-α hepatic expression was independent from HBV replicative state.

Key words: *HCV/HBV coinfection, PBMC, TNF-α.*

Introduction

In a variable proportion of patients with chronic liver disease a dual hepatitis B (HBV) and C (HCV) virus infection can be demonstrated as shown by the simultaneous positivity of HBsAg and anti-HCV.

Both HBV and HCV can infect peripheral blood mononuclear cells (PBMC), however, data on PBMV infection in patients with dual infection are scanty and little is known about the reciprocal effects of the two viruses in liver tissue.

It is presently believed that HCV is capable of inhibiting HBV replication and that HCV is the leading virus in inducing liver damage (1). Moreover, "*in vitro*" suppression of HBV expression and replication by HCV core protein has been recently demonstrated (2). This event could occur also "*in vivo*" provided that both viruses can infect the same cells, either PBMC or hepatocytes. Alternatively, HBV suppression may occur through release of certain cytokines. In fact, HBV replication was shown to be inhibited non-cytolitically by TNF-α and IFN α/β release (3).

On the other hand, some authors have shown an inverse correlation between the replication of the two viruses in coinfected patients due to the inhibitory effect of each virus on the other (4-6).

These two conditions, namely HBV inhibition due to HCV replication and HCV inhibition due to HBV replication, probably represent two different phases during the natural history of the coinfection, each of whom could be characterised by a different pattern of interaction of the viruses within PBMC and hepatocytes and by a different hepatic cytokine profile.

The aims of our study were to examine the presence of HBV and/or HCV viral genome in hepatocytes and in PBMC obtained from HBsAg and anti-HCV positive patients with histologically proven chronic liver disease and to evaluate, in these patients, the hepatic expression of TNF-α.

Methods

HBV. - HBV-DNA detection by spot hybridisation (sensitivity threshold $\cong$ 0.2 pg/ml)and by PCR was performed as previously reported (7, 8). PCR (sensitivity threshold $\cong$ 0.2fg/ml) was performed by employing primers from the core region of HBV (sense 1732-1750; antisense 2454-2436) (9). Moreover, the detection of the e-minus mutant of HBV was performed by a selective amplification method (10).

HCV. - HCV-RNA detection was performed by nested PCR as previously reported (11) and HCV-RNA titer was measured by second generation bDNA method (Chiron Corporation). HCV genotypes were were identified by a reverse-hybridisation of biotin-labelled PCR products to oligonucleotides from the variable region of the 5' UTR immobilised on membrane strips (InnoLIpa).

All patients underwent liver biopsy and the histological changes were evaluated according to Knodell-Desmet. Concomitantly, serum and PBMC samples were

obtained. PBMC were separated by Ficoll gradient, aliquoted and stored in liquid nitrogen in 90% FCS, 10% DMSO until examined. Total DNA (12) and total RNA (13) were extracted from serum, PBMC and liver tissue samples as previously described, followed by spot hybridisation (HBV) and PCR (HBV, HCV).

Hepatic TNF-a mRNA. - Total RNA was extracted from liver tissue samples snap-frozen and stored in liquid nitrogen until tested. The extracted RNA was then treated with RNAse-free DNase (RQ1 Dnase, Promega, Madison, WI, USA) to remove any genomic DNA contamination. Hepatic TNF-α mRNA expression was evaluated, as previously described (14). To compare TNF-α mRNA expression in the different samples, it was essential to amplify equivalent amounts of substrate cDNA. Therefore, normalisation of sample β-actin cDNA content was performed by competitive PCR (Clontech, Palo Alto, CA, USA). Both competitor and target PCR products were subjected to acrylamide gel electrophoresis. The competitor cDNA generated a shorter PCR product (619 bp) compared to the target cDNA (838 bp). The density of target and competitor bands was read by densitometer (INTAS). The concentration of substrate sample cDNA was calculated by plotting the ratio of sample density to competitor density against the known amount of competitor substrate cDNA. To normalise the cDNA sample concentration, all samples were diluted until reaching the same concentration as the sample with the lowest cDNA content.

To quantify the relative levels of gene expression, the bands on the gel were scanned by transmission densitometry, and the peak areas were expressed in arbitrary units. The level of TNF-α gene expression was calculated based on the value of the internal standard (β-actin) in each test tube, and the relative value was used to compare expression across the tested samples.

Statistical analysis. - Student's t test, Fisher's exact test and χ^2 test were employed for statistical analysis, when appropriate.

Results

HBeAg-positive patients. - (5 patients, Table 1). Four out of 5 HBeAg-positive patients were HBV-DNA positive in serum by spot hybridisation with titers ranging from 135 pg/ml to 896 pg/ml (mean value 478 pg/ml). Histological examination of liver samples showed chronic active hepatitis in all cases with mean Knodell score of 9.4 (range 8-11). The 4 HBV-DNA positive patients had evidence of nuclear core antigen positivity on immunohistochemical staining. Moreover, their PMBC were consistently positive on both samples obtained 6-8 months apart. In the last patient, the serum HBV-DNA was negative by spot hybridisation, but positive by PCR. The nuclear core antigen in liver was absent and the PBMC HBV-DNA were negative, in both samples. None of these patients had evidence of e-minus mutant on selective amplification.

Serum, PBMC and liver HCV-RNA was negative in the 4 patients in whom HBV-DNA was detected by spot hybridisation, whereas the last patient was found to be HCV-

RNA positive in serum and liver, although negative in PBMC (in both samples). HCV genotype was 1a. This patient was a drug abuser who had an acute HCV hepatitis on 1993 superimposed over a chronic HBV infection. Before HCV infection, he was HBV-DNA positive and HBV-DNA titer was found to steadily decrease after acquisition of HCV infection until negativisation.

Table 1. Virological results of 5 HBeAg positive patients.

N. Pt.	HBV					HCV			
	Serum DNA Dot-blot	Serum DNA PCR	PBMC DNA	Liver DNA	Liver HBcAg	Serum RNA Meq/ml	PBMC RNA	Liver RNA	Genotype
1	+	+	+ [a]	+	+	-	- [a]	-	
2	+	+	+ [a]	+	+	-	- [a]	-	
3	+	+	+ [a]	+	+	-	- [a]	-	
4	+	+	+ [a]	+	+	-	- [a]	-	
5	-	+	- [a]	+	-	+ 0.724	- [a]	+	1a

Meq/ml: million genome equivalents/ml
a: the results were confirmed in a second PBMC sample obtained after 6-8 months.

Anti-HBe positive patients. - (15 patients, Table 2). These patients were all HBV-DNA negative in serum as evaluated by spot hybridisation, whereas 8 of them resulted HBV-DNA positive by PCR.

PCR positive patients. - In these 8 patients, liver HBV-DNA was positive, whereas PBMC HBV-DNA and liver core staining were negative. Four patients were examined for e-minus HBV mutant, none of whom resulted positive. Liver biopsy showed chronic active hepatitis in 6 patients, and cirrhosis in 2 patients. The mean Knodell score was 7.5 (range 6-9).

Six patients were HCV-RNA positive in serum with a mean titer of 5.05 million equivalents/ml (Meq/ml) (range 0.52-13.85 Meq/ml). Infecting genotypes were as follows: genotype 1b was found in 4 patients; genotype 2a/c was found in 2 patients. Only one out of these 6 patients, who had genotype 1b infection, was HCV-RNA positive in PBMC at the time of first sampling, whereas on a second examination, performed 9 months later, PBMC HCV-RNA was no more detectable. The remaining two patients were HCV-RNA negative in serum, liver and PBMC.

HBV-DNA negative patients. - (7 patients, Table 2). Liver HBV-DNA was tested by PCR in 5 patients, 3 of whom tested positive, whereas PBMC HBV-DNA was negative in all patients. Overall, 3 patients had histological evidence of chronic active hepatitis, 2 had chronic persistent hepatitis, one patient had minimal changes and one patient has normal liver. Mean Knodell score was 2.8 (range 0-7). Nuclear core staining was negative in all cases.

Serum and PBMC HCV-RNA was detectable in 2 patients (genotype 1b and 4). In one of these two patients, (genotype 1b) PBMC results were confirmed also in a second sample obtained 8 months later. The titers of serum HCV-RNA were of 0.2 Meq/ml and 3.58 Meq/ml, respectively. Liver HCV-RNA was detectable in 3 out of 5 tested patients. In the remaining 13 patients PBMC HCV-RNA was negative, and in two of them testing of further PBMC samples obtained after 7 and 8 months, respectively, showed persistently negative results.

Table 2. Virological results of 15 anti-HBe positive patients.

N. Pt.	**HBV**				**HCV**				
	Serum DNA Dot-blot	Serum DNA PCR	PBMC DNA	Liver DNA	Liver HBcAg	Serum RNA Meq./ml	PBMC RNA	Liver RNA	Genotype
1	-	+	-	+	-	+ 0.883	+	+	1b
2	-	+	-	+	-	+ 13,85	-	+	1b
3	-	+	-	+	-	+ 4,0	-	+	1b
4	-	+	-	+	-	+ 0,55	-	+	2a/c
5	-	+	-	+	-	+ 7,56	-	+	1b
6	-	+	-[a]	+	-	+ 3,50	-[a]	+	2a/c
7	-	+	-	+	-	-	-	+	
8	-	+	-	+	-	-	-	-	
9	-	-	-[b]	-	-	+ 0,2	+[c]	+	1b
10	-	-	-	-	-	+ 3,58	-	+	4
11	-	-	-[a]	+	-	-	-[a]	+	
12	-	-	-	-	-	-	-	-	
13	-	-	-	-	-	-	-	-	
14	-	-	-	NT	-	-	-	NT	
15	-	-	-	NT	-	-	-	NT	

Meq/ml: million genomic equivalents/ml
NT: not tested
a: the results were confirmed in a second PBMC sample obtained after 7 and 8 months, respectively
b: the result was confirmed in a second PBMC sample obtained after 9 months.
c: the PBMC resulted HCV-RNA negative in a second PBMC sample obtained after 9 months.

Anti-HCV positive control patients. - All these 20 patients had chronic active hepatitis on histological examination (mean knodell score 5.6, range 4-11). The HCV genotypes were as follows: subtype 1a (3 patients); subtype 1b (6 patients); subtype 2a/c

(5 patients); subtype 3 (4 patients); subtype 4 (2 patients). Mean HCV-RNA titer was 2.63 Meq/ml (range 0.2-11.76 Meq/ml). HCV-RNA was detectable in all liver samples whereas PBMC HCV-RNA was detected in 14 patients (70%), and the mean RNA serum titer was not different in patients with or without detectable PBMC HCV-RNA.

Hepatic expression of TNF-α mRNA. Hepatic expression of TNF-α mRNA was evaluated in 11 patients with detectable serum HCV-RNA without evidence of active or past HBV infection (Table 3; group A), in 8 patients with serum HCV-RNA and with serological evidence of past HBV infection (i.e., anti-HBc and anti-HBs positive; Group B); in 7 patients with detectable serum HCV-RNA and HBV-DNA (group C) and in 5 anti-HCV negative patients with replicating HBV infection (group D).

Patients with detectable HCV viremia, with or without concomitant or previous HBV infection (Table 3; groups A-D) showed a significantly higher hepatic expression of TNF-α compared to patients with HBV viremia without concomitant HCV viremia (pure HBV active infection; group C).

Table 3. Hepatic TNF-α mRNA expression in patients with HCV e/o HBV chronic infection.

Serum markers			**Hepatic TNF-α mRNA (arbitrary units)**
	Group	N°. patients	Mean ± S.D.
HCV-RNA +/anti-HBV neg	A	11	2.4 ± 1.2
HCV-RNA /anti-HBV +	B	8	3.1 ± 1.2
HCV-RNA /HBsAg +	C	7	2.7 ± 1.7
HBsAg /HBV-DNA + and HCV neg	D	5	0.3 ± 0.2

Kruskal-Wallis: D versus A p= 0.003; D versus B p= 0.0005; D versus C p= 0.012
All other comparisons were not significant.

Comment

Our results indicated that in patients with dual HBV/HCV infection, the occurrence of active HBV infection, as demonstrated by detection of serum HBeAg, serum HBV-DNA by spot hybridisation and nuclear core staining in liver tissue, was usually associated to negative serum HCV-RNA. On the other hand, in patients with replicating HCV infection, as demonstrated by detectable HCV-RNA in serum and liver tissue, liver core staining was negative and serum HBV-DNA was undetectable by spot hybridisation, although in 7 out of 11 patients (64%) with HCV-RNA detected only in liver samples a minimal amount of circulating HBV-DNA was detectable by PCR.

As far as liver histology was concerned, an inverse correlation was found between the severity of liver disease and the degree of HBV replication. In fact, in patients with circulating HBeAg and HBV-DNA (by spot hybridisation), the mean Knodell score was significantly higher compared to the mean score of anti-HBe positive patients with detectable HBV-DNA (by PCR) who, in turn, had a significantly higher score compared to anti-HBe positive patients without detectable serum HBV-DNA (9.4, 7.5 and 2.8, respectively; $p< 0.05$).

Our data showed that the proportion of coinfected patients with detectable serum and PBMC HCV-RNA is significantly lower compared to patients with HCV chronic infection alone (2/9 = 22.2% versus 5/8 = 62.5%; $p=0.04$), without any correlation, in both groups, between PBMC infection and serum HCV-RNA titer.

In our patients, the examination of the replicative status of each virus showed a reciprocal suppression that more frequently leaded to the suppression of HBV replication than *vice versa*. Interestingly, in all examined cases, the e-minus HBV mutant was never found. This could be due 1) to the inhibition of HBV replication with consequent reduction of probability of appearance of mutant particles; 2) to the presence of dual viral infection that could weaken the immune system which, thus, was less effective in exerting a selective pressure; 3) to the higher suppression activity of HCV on the e-minus mutant compared to the wild-type.

Of particular interest was the observation that in the patient who had an acute HCV superinfection during the course of chronic HBeAg/HBV-DNA positive infection, a steady suppression of HBV replication could be demonstrated over time. This finding underlines that the temporal sequence of acquisition of the two infections is not relevant in determining the final outcome of the suppression.

It is interesting to speculate about the possible mechanism(s) leading to viral suppression "*in vivo*". Since we could not demonstrate HBV core antigen by immunohistochemical staining in patients with detectable liver HCV-RNA and, on the other hand, we never found liver HCV-RNA in patients with detectable nuclear HBV core antigen, we could not demonstrate the concomitant existence of a dual infection in the same hepatocytes. The same was also true for PBM cells, in which the occurrence of both nucleic acids was never found. Therefore, it was not possible to evaluate whether "*in vivo*" could exist the possibility of HBV suppression from HCV core protein, as it was shown to occur "*in vitro*", although this mechanism seems unlikely. It seems more reasonable to speculate that the HBV suppression could be due to production and release of certain cytokines, namely TNF-α and/or IFN α/β, that have been shown to efficiently suppress HBV replication and gene expression (3). This hypothesis is also supported by the finding that in patients with chronic HCV infection hepatic TNF-α expression is high (15). Moreover, it was recently shown that in chronic HCV patients the T cells infiltrating the liver tissue are prevalently of Th1 type (16, 17), at variance to what is commonly found in patients with chronic HBV infection and liver disease, who have a type T0 prevalent T cell infiltrate (17). Our data show that patients with active HCV infection exhibit an intense TNF-α mRNA expression, significantly higher compared to patients with pure HBV infection. Moreover, this expression does not appear to be influenced by the presence of a concomitant replicating HBV infection.

This finding indicate that HCV replication seems to be the driving stimulus leading to TNF-α production that, in turn, may play a key role in the suppression of HBV gene expression and replication.

This scenario could account for the high rate of occult HBV infection detected by Cacciola et al. (18) in patients with chronic HCV infection and liver disease.

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IMMUNOGENOTYPIC ANALYSIS OF LYMPHOID AGGREGATES FROM LIVER BIOPSIES OF PATIENTS WITH HEPATITIS C VIRUS CHRONIC LIVER DISEASE AND MIXED CRYOGLOBULINEMIA

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Progress Report

CLONALITY OF B-CELLS IN PORTAL LYMPHOID INFILTRATES OF HCV INFECTED LIVERS

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Summary

Considerable evidence has been accumulating in favour of a possible role of hepatitis C virus (HCV) in the pathogenesis of human lymphoproliferative disorders. HCV infection has been documented in the vast majority of patients with essential mixed cryoglobulinemia type II (MC-II) (1, 2), that is characterised by clonal expansions of IgMk-bearing B-cells in peripheral blood and in lymphoid aggregates of bone marrow (3, 4, 5). In addition, B-cell clonal expansions have been also detected in peripheral blood and bone marrow of 24% of HCV-infected patients without cryoglobulinemia (5). Finally, a high prevalence of B-cell non-Hodgkin lymphomas (NHL) has been documented in patients with HCV-infection, associated or not with MC-II (4, 6-10), suggesting that the virus might have a role in lymphomagenesis. This hypothesis is supported by the evidence that HCV is not only hepatotropic, but also a lymphotropic virus. *In vitro*, HCV is able to replicate in a human T-cell line (11) and in normal peripheral blood mononuclear cells from healthy subjects (12). Moreover, viral genomic sequences have been found in peripheral T- and B-cell populations, as well as in monocyte-derived cells from peripheral blood and liver tissue in patients with HCV-related chronic hepatitis (13, 14). More recently, using an in situ RT-PCR technique, Muratori et al. provided evidence that peripheral blood mononuclear cells (PBMC) from patients with chronic hepatitis C contain HCV genome, suggesting that viral replication within PBMC is of common occurrence and might be responsible for the expansion of clones of B-cells, by a direct or an indirect mechanism (15).

The vast majority of studies on HCV-associated B-cell proliferations has been focused on peripheral blood and bone marrow lymphocytes. During HCV infection, liver tissue is characterised by lymphoid infiltrates, even in the form of prominent

aggregates, in portal tracts (16). These aggregates show histological and immunophenotypical features of primary and secondary B follicles (17, 18), but their nature has not been investigated in detail yet. In particular, the clonality of B cells within lymphoid infiltrates in the liver of HCV infected patients has not been analysed at the molecular level. Monteverde et al. (19) studied 12 liver biopsies from patients with MC-II and chronic liver disease and found that lymphoid infiltrates of 9 cases contained B cells expressing monotypic light chain immunoglobulins. They suggested that such monotypic B cell proliferations might be the expression of a low-grade malignant lymphoma.

In the present study, we analysed the clonality of B-cells in 35 portal lymphoid infiltrates microdissected from the paraffin-embedded liver biopsies of 16 patients with HCV infection, with and without MC-II. The B-cell clonality was tested using a polymerase chain reaction (PCR) approach designed to identify immunoglobulin heavy chain gene (IgH) rearrangements (20).

Key words: *Hepatitis C infection, HCV pathogenesis, cryoglobulinemia.*

Materials and Methods

Selection of cases. - Sixteen subjects affected by hepatitis C, 9 of which with associated MC-II were selected for this study. All of them were followed as outpatients in the Liver Disease section of the Infectious Disease Department, Spedali Civili of Brescia. Selection criteria included increase of serum transaminases, positivity for serum antibodies against HCV by ELISA and RIBA techniques, presence of HCV-RNA in serum and/or liver by reverse transcription (RT)-PCR, negativity for HBV and HDV tests, and liver biopsy containing discrete lymphoid infiltrates in at least 2 distinct portal tracts. Evaluation of cryoglobulins was performed on blood samples, and the cryocrit levels were measured as the percentage of packed cryoglobulins after cold centrifugation of the serum. Patients showing cryoglobulins even at low (<2%) percentage in at least 2 evaluations within 6 months were considered positive. The presence of a monoclonal component was investigated in all sera using agarose gel immunofixation. Liver biopsies were fixed in buffered formalin, embedded in paraffin and analysed by routine morphological techniques. None of the patients received any treatment before biopsy and in the interval between different cryoglobulin determinations. None of the patients was affected by NHL at the time of presentation, nor developed NHL during a follow-up period ranging from 24 to 60 months.

Morphological and immunohistochemical characterisation of lymphoid aggregates. - The 16 liver biopsies contained a total of 69 lymphoid aggregates in their portal tracts, ranging from 2 to 7 for each biopsy. These were morphologically distinguished into nodular, diffuse/poorly circumscribed and mixed, according to their predominant feature (16). The 69 aggregates were 40 of nodular, 13 of mixed and 16 of diffuse type. There was no significant difference in number, distribution and type of aggregates, between the biopsies of patients with or without MC-II.

The cell composition of 41 of the 69 aggregates was investigated by immunohistochemistry. In these cases, the first serial section were mounted on glass slides and immunostained using antibodies against the B-cell marker L26/CD20 (Dako, Milan, Italy) and the T-cell marker CD3 (Dako), following the indirect streptavidin-biotin-complex immunoperoxidase technique.

Microdissection of lymphoid aggregates and adequacy of samples. - Five to 20 serial sections (4 µm-thick) were cut from each biopsy. The sections were mounted on acetate strips (obtained from transparency film for plain paper copiers; 3M, Milan), deparaffinized with xylene, hydrated and lightly stained with haematoxylin. Each lymphoid aggregate was localised under a light microscope and isolated by cutting the acetate strip with a disposable sterile clade. All microdissected sections belonging to the same lymphoid aggregate were pooled in the same 0.5 ml tube. Samples were digested overnight at 37 °C in a buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCl and 200 µg/ml of proteinase K.

To test the suitability of each DNA preparation for PCR amplification we performed the amplification of the 162 bp DNA fragment of exon 1 of Ki-ras gene, as described elsewhere (21). Of the initial 69 microdissected lymphoid aggregates, 48 were successfully amplified for Ki-ras gene and were used for detection of clonality. The 21 microdissected aggregates from which no Ki-ras amplification was obtained included all 14 aggregates obtained from the biopsies of 5 patients, suggesting an overfixation of the biopsy or the presence of PCR inhibitors.

Polymerase chain reaction of IgH rearrangements. - The DNA from the 48 aggregates successfully amplified for Ki-ras gene were then subjected to clonality assessment by PCR amplification of rearranged IgH genes. After heating for 10 min at 95 °C, 2 µl from each sample were added to the PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 25 pmol of each primer, 200 µM of each dNTP, 1.25 unit of Taq polymerase and 4.5 mM $MgCl_2$ in a final volume of 50 µl.

We amplified the hypervariable complementary region (CDR-II and III), included between the second and fourth conserved framework regions (FR-II and FR-IV) of IgH genes, with a 5'-primer homologous to the FR-II region, and LJH and VLJH as 3'-primers, using a seminested protocol (20). The primers were: FR2, TGG(A/G)TCCG(C/A)CAG(G/C)C(T/C)(T/C)C(N)GG; LJH, TGAGGAGACGGTG ACC; VLJH, GTGACCAGGGT(N)CCTTGGCCCCAG. FR2 and VLJH are degenerated primers, that is composed of a mixture of primers that differ in base composition at the positions indicated in parenthesis, where N indicates the random presence of one of the four bases at that particular position. The first PCR products (30 cycles) were the template for the second PCR amplification (20 cycles), both annealing at 55 °C.

Each experiment was duplicated and accompanied by negative control containing no template DNA.

Ten µl of PCR products were analysed by electrophoresis on 3% agarose gels, stained by ethidium bromide and viewed under UV light.

Results

Sixty-nine aggregates were obtained from the 16 biopsies. Based on the results of Ki-ras amplification test, the DNA of 48 microdissected aggregates (11 biopsies) was suitable for IgH-PCR analysis. Of these, 35 showed a IgH-PCR product, whereas no IgH-PCR product was obtained from DNA of 13 aggregates, including 5 almost entirely composed of T-cells. The results of PCR amplification of IgH rearrangements obtained from each lymphoid aggregate are reported in Table 1.

Table 1. Results of PCR amplification of IgH rearrangements.

Patient	Lymphoid aggregate		Number of Bands of IgH rearrangement
	Number	Type	
Without MC-II	1	D	1
2			
	2	N	1
	3	D	2
3*	1	M	1
	2	D	2
	3	N	1
4	1	M	1
	2	M	2
5	1	N	3
	2	N	1
	3	N	2
With MC-II			
1	1	N	3
	2	N	1
	3	N	2
11	1	D	1
	2	D	3
12	1	N	2
	2	D	2
	3	D	1
13	1	N	1
	2	N	1
	3	N	1
	4	N	1
	5	N	1
15	1	M	2
	2	M	1
	3	M	1
	4	M	1
16	1	N	1
	2	N	3
	3	N	1
	4	N	1
	5	N	2
18	1	M	1
	2	N	2

Abbreviations: N, nodular; D, diffuse; M, mixed.
*: Patient with polyclonal mixed cryoglobulinemia (type III)

The 35 lymphoid infiltrates adequate for IgH-PCR analysis were 20 of nodular, 8 of mixed and 7 of diffuse type. Such distribution was consistent with that observed among the 69 lymphoid aggregates found in the 16 patients initially examined. Immunohistochemical staining showed that the proportion of B- and T-cells varied from one aggregate to another, where B-lymphocytes were usually more numerous in the aggregates of nodular and mixed type. The IgH-PCR products found in these 35 aggregates consisted in a single band in 21 cases, 2 bands in 10 and 3 bands in 4 cases. The presence and type of PCR products obtained at IgH-PCR was independent from the type of aggregates, as well as from their cell composition and proportion of B-lymphocytes. Moreover, the presence or absence of MC-II did not show any correlation with the results obtained at IgH-PCR.

In 11 biopsies, at least two different lymphoid aggregates showed IgH-PCR amplification products. The large majority were single bands suggesting that the B-cell population within each single aggregate was monoclonal. However, the comparison of the IgH-PCR amplification bands obtained from the different lymphoid aggregates of the same biopsy revealed that they all differed in size.

Discussion

In this study, we addressed the question on whether lymphoid aggregates in liver of patients with chronic HCV-positive hepatitis are clonal B-cell proliferations. Using a microdissection technique, multiple lymphoid infiltrates were isolated from portal tracts and singly analysed with a PCR approach to detect clonality of IgH rearrangements.

The appearance of one or two discrete bands (mono- or biallelic rearrangement) is the expected result of PCR amplification of a DNA from a monoclonal B-cell population, whereas the presence of multiple amplified DNA fragments, appearing as multiple bands or a smear at electrophoresis, is the expected result of the PCR amplification of DNA from polyclonal B-cell populations (20). Finally, no PCR product (neither bands nor smears) is obtained when amplifying DNAs from non-lymphoid cells or T-lymphocytes.

Thirty-five of 48 (73%) lymphoid aggregates, obtained from 11 patients, yielded from 1 to 3 rearranged IgH bands, where the number of bands corresponds to the number of proliferating B cell clones. In particular, also the ten cases with 2 rearranged IgH bands are more likely related to the presence of two distinct B-cell clones, since biallelic rearrangement in a single clone is rarely observed. The large majority of aggregates (21 of 35) showed a single band, suggesting that they were formed by a single B cell clone. In addition, we cannot exclude that at least a proportion of cases not giving IgH-PCR products was formed by single B cell clones, since not all IgH rearrangements can be amplified, due to either the incomplete specificity of primers, or the prevention of primer binding because of deletions or point mutations of target sequences (20). In human lymph nodes, B follicles take origin from more than 10 founder cells and are therefore polyclonal (22); the polyclonal nature of either lymphoid follicles and interfollicular B cells has been consistently demonstrated by

microdissection and PCR techniques (23). Clonal immunoglobulin rearrangements generally support a diagnosis of lymphoma in histologically suspicious lesions. However, these high sensitive techniques can reveal occult B-cell clones in otherwise benign lymphoproliferative disorders (24, 25). In the present study, amplification products were obtained from different lymphoid aggregates from the same biopsy, but single bands showed different size on electrophoresis and were therefore related to distinct proliferations of single B cell clones. We found similar results in patients with and without MC-II, this suggesting that the presence of B-cell clonal proliferations in liver biopsies is independent from the occurrence of B-cells producing monoclonal IgMk cryoglobulins.

The occurrence of oligoclonal B cells in liver tissue infected by HCV is intriguing. Since hepatocytes are the main reservoir for HCV infection and replication (26), selected clones of B-cells in the liver might be driven by HCV-infected cells with a phenomenon referred as to "clonal dominance" (27). This process is characterised by the development of dominant B-cell clones (monoclonal or oligoclonal) responsible for both functional and non-functional antibody production, arising in either autoimmune diseases and in response to bacterial and, especially, HIV-I infections (27, 28). Similarly to HIV-I, HCV has a high rate of mutation and the mechanism of "clonal dominance" might partially explain the low rate of immunoclearance observed in both diseases (27, 29).

Alternatively, the oligoclonal response of B-cells to HCV might be a direct consequence of infection of B-cells. Recently, using RT-PCT technique, HCV genome has been found in peripheral blood mononuclear cells (15), confirming previous hypothesis that HCV may replicate in these cell populations (13, 14). Although the HCV nonstructural protein NS3 have been shown to have oncogenic activity *in vitro* (30) and engineered HCV core protein is targeted to the nucleus (31), a direct transforming role of the virus seems unlikely, since HCV RNA sequences cannot be integrated in the host genome. However, one cannot exclude that HCV, alone or in combination with other factors, may induce a proliferative response of lymphoid cells to HCV antigens.

Oligoclonal B cell proliferations can occur in the absence of histologic evidence of lymphoid malignancy, such as in Sjögren syndrome associated myoepithelial syaloadenitis, Hashimoto's thyroiditis, and Helicobacter pylori positive chronic gastritis (24, 25, 32). In EBV-associated post-transplantation lymphoproliferative disorders, oligoclonal proliferations have been shown in both "hyperplastic B-cell hyperplasia" and "polymorphic B-cell lymphomas" (33). In all these conditions, B-cell clones are capable to evolve into flank lymphomas only following additional oncogene or tumor-suppressor gene alterations (33, 34). A multistep process has been also documented in a lymphoproliferative disorder in a HCV infected patient, in whom the bcl-2 translocation was followed by myc translocation during the clinical progression of the disease (35). However, the wide spectrum of lymphomas that have been described in patients with HCV infection, ranging from lymphoplasmacytoid (10), to MALT-type (8), to follicular-center cell lymphomas (7), seem to indicate that more heterogeneous

and complex processes are probably involved in the lymphomagenesis associated with HCV.

In conclusion, we report that each aggregate in portal tracts of HCV infected livers derives from the proliferation of one or few founder B cells, which are not related to each other. This observation rules out the possibility that such B-cell proliferations may represent a low-grade lymphoma. In addition, the fact that no difference was observed between HCV infected patients with or without MC-II supports the view that HCV may be capable to derange immune system functions (5). The long-term effects of these abnormalities and, in particular, their relationship with the documented risk of developing non-Hodgkin B-cell lymphomas is at present only speculative.

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FUNCTIONAL CHARACTERIZATION OF INTRAHEPATIC T CELL RESPONSES IN HBV AND HCV INFECTIONS
Coordinator: Dott. Carlo FERRARI

Progress Report

FUNCTIONAL CHARACTERISATION OF INTRAHEPATIC T CELLS IN CHRONIC HCV AND HBV INFECTIONS

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Summary

To further clarify the functional features of liver infiltrating T cells in HCV and HBV infections we studied the HLA class II restricted T cell response to structural and non structural HCV proteins in patients with chronic hepatitis C and the CTL response to immunodominant epitopes of the HBV envelope, core and polymerase regions in patients with chronic HBV infection. In both chronic HCV and HBV infections, intrahepatic and peripheral blood T cell responses were studied in parallel.

In HCV infected patients, HCV-specific T cells appeared to be compartmentalised into the liver and HCV core was the most powerful immunogen for HLA class II restricted T cells. Only few T cell epitopes were identified within individual HCV proteins in individual patients, showing that the intrahepatic T cell response is generally polyclonal and multispecific, but focused on a limited number of strongly immunodominant epitopes.

In HBV infected patients, the frequency of HBV-specific cytotoxic T lymphocytes (CTL) was higher in the liver compartment than in the peripheral blood in both chronic patients with active disease and high viral replication and in those without liver inflammation and low viral replication, suggesting intrahepatic compartmentalisation of virus specific CTL. Surprisingly, the frequency of HBV-specific CTL was higher in the livers of patients without liver inflammation (asymptomatic healthy carriers) than in those with active disease. Moreover, peripheral blood CD8 cells of asymptomatic healthy carriers but not CD8 cells of patients with chronic active hepatitis B were able to expand efficiently, to lyse HBV antigen expressing target cells and to produce IFN-γ after peptide stimulation in vitro. These results challenge the view that chronic healthy carriage of HBV is associated with the lack of HBV specific CTL responses and suggest

that the efficient CTL response detectable in chronic asymptomatic carriers is essential to keep infection under control. In patients with active liver disease who are unable to control HBV infection, liver damage is probably caused mainly by virus non specific cells recruited into the liver as a result of liver inflammation.

Key words: *Hepatitis B infection, hepatitis C infection, HBV immunology, HCV immunology.*

A. Intrahepatic compartmentalisation of HCV-specific, HLA class II restricted T cells in chronic hepatitis C.

It is widely accepted that the T cell response against HCV encoded antigens plays an important pathogenetic role in liver damage and viral clearance in hepatitis C. Since the peripheral blood compartment may only partially reflect the immune events taking place within the liver, at the site of cellular injury and antigen synthesis, it is crucial to develop experimental strategies to study liver infiltrating T cells. Several studies have investigated the functional features of HCV-specific intrahepatic CD8+ cells (1-5), but limited data are available about specificity and function of HLA class II restricted T cells infiltrating the liver of patients with chronic HCV infection (6, 7).

To further clarify the pathogenetic role of CD4+ T cells in HCV infection we selected 23 patients with chronic hepatitis C (CH-C) to study their intrahepatic T cell responses. The diagnosis of CH was based on the finding of elevated serum transaminase values for at least one year and was confirmed by liver histology. All patients were HCV-RNA positive by nested polymerase chain reaction and negative for HBV markers, anti-delta and anti-HIV antibodies. Patients had never been treated with corticosteroid or antiviral drugs.

Intrahepatic lymphomononuclear cells were isolated from excess liver tissue not needed for diagnostic purposes, as previously described (8), and stimulated with recombinant HCV proteins in the presence of irradiated (4,000 R) autologous PBMC and recombinant IL-2 (20 U/ml). The HCV antigens core, E1, E2, NS3, NS4 and NS5 used for T cell stimulation are C-terminal fusion proteins with human superoxide dismutase (SOD) expressed in yeast (*Streptomyces cerevisiae*) (9). Purity of the antigen preparations ranged between 85 to 95%. Restimulation of the polyclonal T cell lines was carried out every 7-10 days with antigen and irradiated PBMC and growing lines were screened for antigen specificity, phenotype and cytokine production. Some selected polyclonal T cell lines were cloned by limiting dilution.

In parallel, HCV-specific T cell lines and clones were derived from the peripheral blood of the same patients and produced by the same experimental strategies used to study intrahepatic mononuclear cells.

HCV-specific T cell lines were obtained from the liver of 14 out of the 23 chronic patients studied (Table 1). T cell lines were generated more frequently by stimulation with HCV core, followed by NS4, NS5 and NS3 that induced expansion of HCV-specific T cells in a lower number of patients. No patient showed response to E1 or E2. This finding may be due to the absence or the low frequency of T cells able to recognise these HCV proteins. In alternative, the lack of response to these highly variable proteins

may reflect a different sequence of the envelope antigens expressed by the infecting virus from that of the recombinant proteins used in our experiments.

Table 1. Significative responses of intrahepatic and peripheral blood T lymphocytes from 23 patients with chronic HCV infection. Patients who did not display significant responses are not depicted.

		Patients														Positive Responses/Patients
		1	2	3	4	5	6	7	8	9	10	19	21	22	23	
LIVER	E1	-	-	-	-	-	-	-	-	-	-	NT	-	-	NT	0/21 (0%)
	E2	-	-	-	-	-	-	-	-	-	-	NT	-	-	-	0/22 (0%)
	C22	274	32	50	3.9	4.0	-	-	7.3	4.8	-	30	6.8	46.2	87.3	11/23 (48%)
	NS3	285	-	-	-	-	-	-	-	-	-	29	39	-	15.1	4/23 (17%)
	NS4	-	-	-	-	3.2	-	22	34.5	-	-	-	4,7	4,7	35.8	6/23 (26%)
	NS5	-	-	19	-	15.3	3.3	-	-	-	15	48	-	-	34.7	6/23 (26%)
BLOOD	E1	-	-	-	-	NT	-	-	-	-	-	NT	-	-	NT	0/20 (0%)
	E2	-	-	-	-	NT	-	-	-	-	-	NT	-	-	102	1/21 (5%)
	C22	2.8	3.5	-	7.5	NT	-	-	3.7	2.5	2.9	-	27,5	-	23.7	8/22 (36%)
	NS3	-	-	-	-	NT	-	-	-	5.1	-	-	-	-	135	2/22 (9%)
	NS4	(2.3)	-	-	-	NT	-	-	-	-	-	-	16,7	-	19	2/22 (9%)
	NS5	-	-	-	-	NT	-	-	-	-	-	-	-	-	46.6	1/22 (4%)

In 8 of the 14 patients with significant T cell responses, intrahepatic T cells were able to recognise more than one HCV protein simultaneously (Table 1). However, in most patients studied T cell responses were focused on individual sequences within the stimulatory antigens. These findings suggest that intrahepatic T cell responses, though polyclonal and multispecific, are focused on a limited number of epitopes.

To further investigate this hypothesis, a chronic patient (patient n. 8) was studied more extensively and the antigen-specificity of liver-derived T cells was analysed avoiding preliminary steps of antigen stimulation before cloning, in order to draw a more representative picture of the liver environment. A preliminary step of antigen challenge can indeed facilitate the preferential expansion of cell populations that do not necessarily represent the dominant HCV-specific component of the liver infiltrates. For this purpose, T cell clones were generated not only from HCV-specific polyclonal T cell lines, but also by cloning liver infiltrating T cells directly after their isolation from liver biopsies or from polyclonal lines derived by T cell expansion of intrahepatic infiltrates with IL2 only. Interestingly, all clones, irrespective of the experimental strategy used for their production, were able to recognise only one sequence within NS4 (AA 71-90) and only one within core (AA 21-40).

All lines and clones were HLA class II restricted as shown by using allogeneic antigen presenting cells partially matched or totally mismatched with the T cell donors

(data not shown). Moreover, the prevalent cytokine profile expressed by T cell lines and clones was Th1-like (Table 2), in keeping with previous investigations of the cytokine mRNA expression in the liver tissue (7) and of the cytokine patterns secreted by intrahepatic non HCV-specific T cells of patients with chronic HCV infection (8).

Table 2. Cytokine pattern of HCV-specific T-cell lines obtained from intrahepatic T lymphocytes of 8 patients with chronic HCV infection.

Antigen Specificity	**Cytokine Pattern**		
	Th1	Th2	Th0
C22	7	1	2
C33	3	1	/
NS4	2	1	2
NS5	4	/	1
total	16	3	5

Peripheral blood T cell responses to HCV antigens were studied in parallel in the same patients analysed for their intrahepatic T cell responses. HCV-specific responses were less frequently detectable in the peripheral blood than in the liver compartment (Table 1).

Moreover, analysis of the Vβ usage by T cell clones obtained from the same patients, indicated different expression of the variable region of the T cell receptor by liver and peripheral blood derived T cells, even when T cell clones expressed the same fine specificity and the same function. Taken together, these findings suggest that the frequency of virus-specific HLA class II restricted T cells is higher in the liver than in the blood as a likely result of their preferential compartmentalisation at the site of cellular injury and viral replication. Moreover, T cell receptor analysis of HCV specific T cells derived from different body compartments raises the hypothesis that subsets of T cells with different homing capability exist in the context of functionally homogeneous populations of antiviral T cells. By this mechanism, HCV-specific T cells with antiviral function may be able to avoid exhaustive sequestration at the site of inflammation and high antigen expression, in order to recirculate throughout the body and to patrol peripheral sites preventing their infection.

In conclusion, our study of the intrahepatic HLA class II restricted T cell response in chronic hepatitis C indicates that 1) HCV core is the antigen more efficiently recognised by liver infiltrating T cells; 2) intrahepatic HCV-specific T cells are polyclonal and multispecific but are able to recognise a limited number of epitopes within the different HCV proteins; 3) HCV-specific T cells are preferentially sequestered into the inflamed liver; 4) a specific intrahepatic compartmentalisation is suggested by the analysis of the TCR of liver infiltrating and circulating T cells.

B. Cytotoxic T lymphocyte (CTL) function in patients with chronic HBV infection*

Hepatitis B virus (HBV) is a non-cytopathic virus that causes acute and chronic inflammatory liver disease. HBV-specific CD8 cells are thought to be the principal effector of both virus control and liver damage (10).

To better understand their role in the pathogenesis of HBV-related liver disease, HLA-peptide tetrameric complexes were used for the direct ex vivo study of circulating and liver infiltrating HBV-specific CD8 cells, in collaboration with Dr. Antonio Bertoletti and Dr. Mala Maini (Institute of Hepatology, Royal Free and University College Medical School, London, UK) (11). Tetramers consist of four biotinylated HLA-class I molecules, each complexed with a specific peptide. This multimeric HLA-class I/peptide complex has a high avidity for T cells displaying the appropriate T cell receptor. Binding to specific cells is detectable by FACS analysis if a flourochrome-labelled streptavidin reagent is used. This technology has facilitated the dissection of the cell mediated immunity in different viral infections (12-16) and has shown clearly that previous methods of CTL analysis have underestimated the number of virus-specific CTL (17).

HLA-A2 tetramers complexed with three different peptides representing HBV core, polymerase and envelope CTL epitopes were constructed as probes for identifying HBV-specific CTL. The peptides represented by the core 18-27, polymerase 575-583 and envelope 335-343 regions were chosen since they are recognised by HLA-A2 restricted CTL in the majority of HLA-A2 positive patients with acute HBV infection (18-20).

Two groups of HLA-A2+ patients with persistent HBV infection were studied. One group was selected for the absence of hepatic inflammation and HBV replication and was composed of 18 chronic patients who were HBeAg negative, anti-HBe positive and had normal ALT levels and less than 2 pg/ml of HBV-DNA.

The second group comprised 15 HLA-A2 positive chronic patients, positive for HBeAg, with more than 800 pg/ml and elevated ALT levels.

Tetramer-positive HBV-specific CD8 cells were preferentially sequestered in the liver in both patients groups since their frequency among liver infiltrating cells was higher than in the circulation. Surprisingly, the absence of hepatic immunopathology in anti-HBe positive patients with normal ALT levels was associated with a high frequency of intrahepatic HBV-specific CD8 cells. Frequencies of HBV core 18-27 specific CTL reached levels as high as 1 in 11 and 1 in 25 total intrahepatic CD8+ T cells in two patients of this group.

Moreover, this group of patients without liver inflammation showed a circulating reservoir of CD8+ cells able to expand in vitro after exposure to viral antigen. This is shown by experiments of PBMC stimulation with the core 18-27 synthetic peptide for 10 days, followed by staining of the short term T cell lines with the appropriate tetramer to quantify the expansion of core 18-27 specific CD8 cells. By this experimental approach, 11 out of 15 patients demonstrated a vigorous expansion of peripheral blood core 18-27 specific cells. These CD8 cells showed low expression of activation markers (HLA-DR, CD38) and were mainly L-selectin (CD62-L) and CD45RA negative, as

indicated by three colour flow cytometry. This phenotype is characteristic of antigen-experienced, resting T cells, and a similar population of 18-27 specific CD8 cells has been shown to be present in patients with self-limited acute HBV infection (11). Finally, these cells were able to produce IFN-γ and to express lytic function after peptide stimulation, suggesting that they should be able to exert antiviral function after virus-challenge in vivo.

In the second group of HBeAg positive chronic patients with elevated ALT levels and high viral replication, HBV-specific CD8 cells were generally undetectable in the peripheral blood by tetramer staining ex vivo and only 1 of 10 of these patients showed detectable expansion of core 18-27 specific CD8 cells after peptide stimulation in vitro. Moreover, HBV-specific CD8 cells were present at low frequency in the liver infiltrates that were mostly represented by virus non-specific lymphocytes.

The present results show that efficient HBV-specific CD8 responses are associated with inhibition of virus replication but not with liver damage. They challenge the view that chronic asymptomatic carriage is due to the lack of antiviral CTL responses and indicates that the CTL response is present and likely able to keep HBV infection under control. This may be the result of the antiviral effect of the cytokines secreted by CD8 cells but also a degree of direct hepatocyte lysis by HBV-specific CTL, undetectable by serum liver enzyme measurement, may be equally involved.

In the opposite situation of virus replication with liver inflammation, the frequency of intrahepatic HBV-specific CTL appears to be lower because of the massive recruitment of virus non-specific T cells. In this setting, hepatocyte damage may not primarily due to lysis by HLA class I restricted HBV-specific CD8 cells, but rather to the effect of the virus-non specific cellular infiltrate.

** This study was equally contributed by Dr. C. Ferrari's laboratory in Parma and by Dr. A. Bertoletti's laboratory in London.*

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STUDY OF HEPATITIS C VIRUS LYMPHOTROPISM AND AUTOIMMUNE-LYMPHOPROLIFERATIVE DISORDERS RELATED TO VIRAL INFECTION

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Progress Report

STUDY OF HEPATITIS C VIRUS LYMPHOTROPISM AND AUTOIMMUNE-LYMPHOPROLIFERATIVE DISORDERS RELATED TO VIRAL INFECTION

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Summary

The individuation of Hepatitis C virus (HCV) lymphotropism and the association of HCV infection not only with chronic liver disease of various severity but also with a wide series of autoimmune and/or lymphoproliferative disorders, indicated HCV's pathogenetic potential in determining diseases of the lymphatic system as well. Mixed cryoglobulinemia (MC), the extrahepatic disease most strictly associated with HCV infection, may be considered a crossroad between HCV infection, autoimmune disorders and B-cell neoplasias (non-Hodgkin's lymphoma, NHL). HCV could be the triggering factor of clonal B-cell proliferation underlying MC and in some cases, its evolution into a malignant NHL. HCV, therefore, might also be involved in the pathogenesis of "idiopathic" NHL. Indeed, a significantly higher percentage of HCV infection was first demonstrated in an unselected series of Italian B-cell NHL patients and successively confirmed, although with geographical differences. The geographical heterogeneity in the prevalence of various HCV-related clinical manifestations suggests the contribution of other genetic and/or environmental factors. For an appropriate understanding of the relationships existing between HCV infection and lymphoproliferative and/or autoimmune processes, clinico-epidemiologic studies must be completed by a deeper virological analysis of the same populations and also by molecular biology studies aimed at investigating the involvement of particular oncogenes and deregulations of lymphatic cell apoptotis and proliferation mechanisms.

Key words: *Hepatitis C virus (HCV), viral lymphotropism/lymphotropic viruses, autoimmune and/or lymphoproliferative disorders.*

Introduction

Chronic hepatitis C virus (HCV) infection is now accepted as a multifaced disease with a multitude of associated clinical manifestations (1-3). In fact, existing evidence points at an association between this viral infection and different pathologies belonging to a variety of medical fields, including rheumatology, clinical immunology, haematology, dermatology, nephrology and endocrinology (4-6). These associations, in some cases, are not supported by enough data, whereas in others evidence is abundant, in spite of ongoing controversies regarding the prevalence of these associations in different countries and surrounding etiopathogenetic mechanisms. The apparent plethora of extrahepatic manifestations of HCV infection may be re-appraised when observing that the majority of them are based on lymphoproliferative and/or autoimmune mechanisms. This suggests that the link between HCV infection and some extrahepatic manifestations may be, at least in part, related to HCV lymphotropism. HCV infection probably acts at an early stage in the pathogenetic mechanisms leading to different clinical manifestations, probably by inducing a generic tendency towards the development of lymphoproliferative and/or autoimmune phenomena through a primary action on the lymphatic system. On the basis of this virus-related predisposition, final clinical manifestations probably vary greatly in different subjects according to individual genetically and/or environmentally driven predisposing factors. In other words, on the basis of general “remote” pathogenic mechanisms, in each HCV-related extrahepatic manifestation, different pathogenic pathways, i.e. involving the production of RF, cryoglobulins, the intervention of local factors etc, may lead to fully understanding these disorders.

Analysis of an association between HCV infection and lymphoproliferative and/or autoimmune disorders.

We performed a virological analysis of different populations of patients with lymphoproliferative disorders such as MC, MC associated with chronic liver disease (CLD), MC with glomerulonephritis, MC with B-cell NHL, CLD with NHL, idiopathic B-cell NHL, chronic lymphatic leukemia and MC with hepatocellular carcinoma (HCC) (7-14). Analysis of HCV infection in these patients and in control subjects confirmed our preliminary studies, showing the existence of a significant association between HCV infection and lymphoproliferative disorders (LPDs) and in particular between HCV and MC and/or B-cell NHL. In particular, considerations suggesting that the association of HCV with B-cell NHL is not fortuitous or artifactual included the following: a- no increased risk factors for HCV exposure were recorded in HCV-seropositive NHL compared to seronegatives; b- the prevalence of HCV infection in B-cell lymphomas was statistically higher than in the general population and control groups of other haematological malignancies (4). A significant association with HCV was also observed, though at a lesser extent, for other benign and malignant B-cell neoplasias such as chronic lymphocytic leukaemia (13). The above findings were

mirrored by the increased incidence of LPDs, including B-cell NHL, in a large series of unselected patients with chronic HCV infection; for example, a monoclonal band was significantly more detected in HCV-positive than in HCV-negative chronic hepatitis patients and the prevalence of HCV genotype 2a/c was higher in patients with monoclonal gammopathies than in those without (50% compared with 18%; P = 0.009) suggesting that the prevalence of monoclonal gammopathies in patients with HCV-related chronic liver disease is striking and often associated with genotype 2a/c infection (15, 16).

Furthermore, we described for the first time the appearance of HCC, with a low percentage (3/250 cases), during the long-term follow-up of MC patients as well as the evolution towards a frank NHL in some MC patients (14/200) after an average follow up of 4 years. In patients with complicated MC, HCV infection of lymphatic cells was almost constantly found. In the same patients we also observed variations of both C_3-C_4 fractions and cryocrit levels, with potential pathogenetic and clinical interest. These data suggest that MC must be considered a pre-neoplastic disorder which should be accurately followed (17, 18). In addition, in MC patients with purpura we showed histologic aspects of leukocytoclastic vasculitis (LCV) with HCV-RNA sequences in both these lesions and in circulating leukocytes, observing furthermore, that the presence of HCV in leukocytoclastic vasculitis lesions of MC is related to CD8 lymphocytes pattern, HLA-DR and ICAM-1 expression. All these data confirm the pathogenetic role played by HCV in the pathogenesis of MC and its skin lesions, suggesting the need for assessing HCV infection in all patients with LCV of unknown cause (2, 19). Finally, the virological analysis of a large population of B-cell NHL patients at presentation, showed that no specific or more probable extranodal tumour site was identified in HCV-positive patients when compared with non-HCV associated forms of NHL, suggesting that systemic rather than local factors may trigger malignant lymphoproliferation (10). Even though confined to particular geographical areas, HCV-associated lymphomas may now be considered a new model for the study of virus-induced cancer in humans. The intimate mechanism(s) responsible for the appearance of malignancy remains still largely unknown. A variable combination of different co-factors in the development of lymphatic disorders in HCV-positive individuals should be taken into account (2, 4). The observation in the same subject of autoimmune and neoplastic diseases, concomitantly or sequentially, indicates that HCV-related disorders could be the result of a multifactorial and multistep process. HCV-related LPDs usually appear after a long-lasting period of infection. During this phase some genetic aberrations can take place. Recent data suggest that HCV infection may influence oncogenes associated with LPDs. We found a high frequency of t(14, 18) translocation, with corresponding bcl-2 protein overexpression in B cells not only in HCV-infected pts with a frank IIMC, but also in about one third of CH pts without evidence of MC syndrome. In addition, a successful antiviral treatment was shown to influence the detection of both HCV-RNA and bcl-2 recombination-positive PBMCs: among a group of HCV-positive patients who underwent antiviral therapy 8 scored t(14; 18)-positive. After treatment, t(14, 18) was negative in 7 pts. who completely or partially responded to treatment (significant reduction of viremia or ALT levels). In all cases HCV-RNA

sequences were also no longer detected in PBMCs. In contrast, t (14, 18) was still found in PBMCs from the patient that did not respond. In this patient HCV-RNA sequences (+ and – strands) were still detected in PBMCs (20-22). These data are in agreement with the hypothesis that t(14, 18) in HCV-positive patients may be involved in the complex multistep mechanisms leading from chronic HCV infection to B cell LPDs by favouring the switch from a polyclonal antigen-driven B cell expansion to monoclonality. In turn, abnormal protection against apoptosis may predispose to autoimmunity and represent one of the possible mechanisms by which HCV infection may persist in the host. The chronic stimulation of the lymphatic system by HCV, possibly amplified by the infection of lymphoid cells and/or HCV interaction with B-cell receptor/s (CD81) together with the high viral variability, could explain the high frequency of this mutation. In addition, it is tempting to hypothesise that antiviral treatment may also be effective in preventing HCV-related LPDs. Further investigations are needed to establish whether t(14, 18) and/or bcl-2 overexpression in PBMC could be used as non-invasive markers of an increased risk to develop B-cell LPDs and autoimmune phenomena in patients with HCV-related CLD.

Possible role of lymphotropic viruses in lymphomagenesis

In order to investigate whether lymphotropic viruses potentially implicated in lymphomagenesis play a role in inducing B-cell non-Hodgkin's lymphoma (NHL) either independently from HCV infection or in co-operation with this agent, we: a) evaluated lymphatic cell infection by EBV, HHV-6 and HCV in 18 patients with B-cell NHL; b) searched for infection by hepatitis G virus (HGV) and HCV in 200 consecutive patients with B-cell NHL at presentation; c) injected lymphatic cells from HCV positive NHL patients into SCID mouse.

With respect to the first study (a), it is of note that a causative role of viral agents in malignant lymphomas has been well established for Epstein-Barr virus (EBV) and has been suggested for HHV-6. EBV, whose B cell activation capacity is well known, targets CD21 on the B cell surface, which is part of the same molecular complex of the HCV putative cell receptor CD81. In agreement, HCV envelope protein E2 delivers a costimulatory signal to B cells in association with EBV. Coinfection with EBV and HCV could therefore have a synergistic effect on B-cell proliferation because of the properties shared by these 2 viruses. Recent observations obtained in the liver transplantation setting by our group and other seems to agree with this hypothesis (unpublished data). However, in a study we performed EBVDNA as well as HBV-6 DNA sequences were detected with similar frequency in PBMC from NHL patients and controls. In contrast, HCV-RNA genomic sequences were found with significantly higher prevalence ($p<O.Ol$) in PBMC from patients with NHL than in controls. Therefore, viral infection by two or more viruses in the same patients were rarely detected. These data on one hand support the hypothesis of a role played by HCV infection in lymphomagenesis, but on the other do not support the hypothesis of a co-

operative role played by the various lymphotropic viruses considered in this study in inducing NHL (23).

The second study (b) was justified by the fact that HGV is both a lymphotropic and HCV-like agent. In the NHL patients studied we detected active HGV infection (HGV viremia) in a prevalence of patients that was not elevated, but was significantly higher than those detected in healthy control matches for demographic characteristics (age, sex, geographical origin) (p0,03). HGV infection was only rarely associated with HCV infection (5% of HGV-positive patients). Flavivirus infection (HCV and/or HGV) was detected in about a third of NHL patients Anti-HGV antibody (anti-E2) detection showed that about 30% of patients studied had had contact with HGV. The majority of these patients were HCV-positive and HGV-RNA-negative. These data reinforce the hypothesis of an oncogenic potential of hepatitis-related flavivirus infection and encourage interest for further studies investigating HGV infection of lymphatic cells in NI-IL patients (24).

With respect to the third study (c), lymphatic cells from a series of HCV-positive patients including some patients with B-cell NHL were injected into SCID mice. These latter were followed for the persistence of HCV infection in PBMCs, the appearance of HCV viremia and the consequences of PBMC infection. Data obtained demonstrated that in human PBMCs, HCV infection may persist for an extended period of time and may be productive even if at very low levels. These possibilities were particularly evident in cells from patients with B-cell NHL and in these mice the appearance of ma1ignant lymphoma was particularly rapid and dramatic, with pathogenetic implications (25).

Effects of interferon alfa treatment on hepatitis virus-related lymphoproliferative disorders

We previously showed the usefulness of interferon alfa (IFN) in the treatment of patients with HCV-related MC. IFN effects on both virological and rheumatological parameters were demonstrated in these patients with evident improvement particularly regarding liver and kidney damage. However, the follow-up of a large series of IFN-treated patients showed the possibility of severe side effects. In particular we observed two cases of MC in which IFN administration was followed by severe neuropathy, prompting us to discontinue treatment. MC was related to HCV infection in one patient (47 yrs old) and to HBV in the second patient (59 years old male). The first patient had severe muscular weakness, mucocutaneous paresthesias, dyspnea, digital clubbing, and Raynaud’s phenomenon. Muscle and liver histological alterations were consistent with myositis and CAH respectively. Serum antinuclear antibody in a diffuse pattern were also present. The patient was treated with IFN (9MU/week) for 2 months. During this time an exacerbation of cranial neuropathy and a marked worsening of clinical manifestations (i.e. ALT and CPK levels), together with dyspnea due to lung fibrosis, was observed (26, 27).

In the second patient, MC was associated with HBV-related CAH and was clinically characterised by recurrent purpora and mild sensory peripheral neuropathy. This patient was treated with IFN (3MU/day). After 4 weeks of treatment, serum HBV-DNA disappeared and cryocrit, purpora and ALT levels significantly improved. However, in light of concomitant progressive worsening of parestesias and severe impairment in walking due to sensimotor neuropathy, we discontinued IFN (28).

These data suggest that in MC patients with severe immunologically-mediated symptoms, and particularly in cases of signs of neuropathy, treatment with IFN-alfa is to be considered cautiously even in case of positive effects on viral infection. In these cases, treatment with antiviral drugs lacking immunomodulatory effects or those less frequently complicated by neurologic side effects should be tested in future studies.

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HEPATITIS VIRUSES AND HEPATOCELLULAR CARCINOMA. ROLE OF THE HEPATITIS B VIRUS pX PROTEIN IN HEPATOCYTES TRANSFORMATION: EFFECTS OF GENETIC VARIABILITY ON pX ACTIVITIES ON TRANSCRIPTION, INDUCTION OF CELL PROLIFERATION AND MODULATION OF PROGRAMMED CELL DEATH AND FUNCTIONAL INTERACTIONS WITH THE HCV PROTEINS CORE AND NS3

Coordinator: Prof. Massimo LEVRERO

Progress Report

HEPATITIS VIRUSES, APOPTOSIS AND HEPATOCELLULAR CARCINOMA. THE ROLE OF THE HEPATITIS B VIRUS HBx PROTEIN IN HEPATOCYTES TRANSFORMATION: EFFECTS OF GENETIC VARIABILITY AND FUNCTIONAL INTERACTIONS WITH HCV PROTEINS

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Summary

Hepatitis B Virus (HBV) contributes to liver carcinogenesis by multiple, often synergistic, indirect as well as direct mechanisms. Several evidences indicate that the HBV encoded HBx protein might play a major role in the context of the above described scenario. HBx is a multifunctional regulatory protein that modulates transcription and affects both cell growth and cell death control. HBx sensitises rodent fibroblasts as well as human hepatoma cells to TNFa cytotoxicity and to DNA damage induced p53 dependent apoptosis. When overexpressed, HBx directly kills both fibroblasts and hepatoma cells, which can be rescued, in analogy to what has been also observed for c- and N-myc induced apoptosis, by growth factors IGF-1 and IGF-2 respectively. The functional characterisation of both genetically engineered and HCC derived HBx mutant proteins showed HBx-induced apoptosis is dependent on transactivation and strongly suggest that loss of apoptotic potential by mutated HBx proteins may contribute to the molecular pathogenesis of hepatocytes transformation. However, the relation between HBx and apoptosis is not univocous and HBx has been

shown to block apoptosis after overexpression of wild type p53 and to protect from growth factors deprivation induced apoptosis by interfering with caspase 3 activity. Thus, the fate of infected cells expressing HBx is likely to be determined by the balance between apoptotic and antiapoptotic signals of viral, cellular and environmental origin.
Key words: *HBx, transcriptional activation, apoptosis.*

Hepatocellular carcinoma (HCC) is one of the most common malignancies affecting humans. More of 250.000 individuals per year die from this type of cancer. Persistent HBV and HCV infection are major risk factors inducing the development of HCC. However, since liver cancer occur several years after the infection, the precise role played by these virus is yet unclear. Indeed, most of human hepatomas are late complications of chronic viral hepatitis and cirrhosis. As in the case of many other viruses, HBV and HCV genes products may interfere with the ability of the host immune system to eliminate infected cells, thus rendering liver cells susceptible to accumulate mutations that either activate oncogenes or inactivate tumour suppressor genes conferring to the cells an uncontrolled proliferative advantage.

Several evidences indicate that the HBV encoded pX protein might play a major role in the context of the above described scenario by either modulating the susceptibility of infected hepatocytes to apoptosis or by inducing uncontrolled cell growth. The available evidences indicate that pX is a multifunctional regulatory protein that affects transcription, cell growth and cell viability. The molecular mechanisms underlying these different pX activities are only partially defined. Indeed, pX has been shown to have pleiotropic and potentially conflicting effects on a variety of molecular targets and both nuclear and cytoplasmic sites of action of pX have been described. Several groups reported that pX modulates the intracellular signalling apparatus including the PKC and the Ras/Raf signalling pathways. Moreover, pX has been shown to physically interact in vivo and/or in vitro with DNA-binding proteins involved in transcriptional regulation, with a putative nuclear DNA repair protein, with cellular proteases and components of the proteasome complex, and, finally, with p53. Transgenic animals have been used to study the effect of wild type pX protein in different organs. The results obtained, in agreement with many evidences in the literature, support the assumption that expression of the wild type pX does not reproducibly results in cellular transformation. It is also important to note that, although hepatocellular carcinoma is a frequent disease, only a minority of patients among the many suffering from chronic viral hepatitis and cirrhosis undergo neoplastic transformation in a given time lap. These observations strongly suggest that other events are likely involved in complementing chronic inflammation and hepatitis viruses infection in the multistep process of hepatocytes transformation in vivo. One possibility is that pX (and other viral proteins) may require either the activation of oncogenes or the inactivation of genes involved in the regulation of apoptotic cell death of hepatocytes. In addition, HBV, as most RNA viruses and retroviruses which use RNA polymerases and RNA dependent-DNA polymerases for replication, undergo an high rate of spontaneous mutations and there is uncreasing evidence for the existence of X

gene mutations in HBV chronically infected individuals. Thus the possibility exists that transformation may be linked to the emergence of mutant pX proteins more than to the expression of a wild type pX protein.

Objectives of the two years project funded by the ***"ISS 1st Viral Hepatitis Research Program Project"*** were: a) to further characterise HBx activities (signalling, transcription, proliferation and apoptosis); b) to study the effects of core and NS3 proteins of different HCV genotypes on cell proliferation on cell susceptibility to apoptosis and to characterise the molecular basis of these activities and c) to evaluate the genetic variability of the X region of the HBV genome in chronically infected and HCC patients and its effects on HBx activities on transcription, cell proliferation and apoptosis.

Characterisation of pX effects on cell proliferation and cell viability

HBV pX induces quiescent rat embryo fibroblasts (REF-52 cells) to progress from the G0/G1 phase into the S phase of the cell cycle and that its prolonged expression provokes apoptosis in cells expressing wild type p53 (Chirillo et al., 1997). pX is also able to to prompt cells to programmed cell death after exposure to DNA damaging drugs. These effects of pX are not shared either by the other HBV products or by the truncated preS/S transactivating proteins, frequently expressed in human liver cancers. Using a dominant negative deletion mutant of c-myc we found that pX induced apoptosis is c-myc mediated. Moreover, taking advantage of a stable transfectant carrying the p53val135 temperature sensitive allele, we directly demonstrated that induction of apoptosis by pX is, at least in fibroblasts, dependent on the presence of a proficient p53 protein. In p53 null fibroblasts, pX activates transcription and confers an evident growth advantage without evident loss of cell viability.

pX dissects the apoptotic and transactivating/cell cycle regulatory functions of p53

It has been reported that pX binds and inactivates the transactivating function of p53. The ability of p53 to activate and/or repress gene transcription is strictly linked to its function as a cell-cycle regulator. Less clear is the relationship between the transactivating properties of p53 and its role in the execution of the apoptotic program induced by DNA damage, aberrant cell cycle induction and growth factors deprivation. The recent demonstration that the p53-related family of p73 proteins are also recruited in the cell response to DNA damage renders the scenario even more complex. We have shown that pX, unique among viral transactivators, is able to dissect the apoptotic and transactivating/cell-cycle regulatory functions of p53. First, in cotransfection experiments pX expression significantly impairs the transcriptional activation of the cdk inhibitor p21 promoter by p53. We generated stable clones from BalbC-3T3 cells expressing a termosensitive p53 dominant mutant (BalbC-p53TS) and, subsequently,

BalbC-p53TS stable transfectants expressing pX under different promoters. pX expression forces BalbC-p53TS cells to enter into S phase at 32°C (permissive temperature) and inhibits p53 transactivating function. The same pX expressing cells start the p53 dependent apoptotic program upon treatment with DNA damaging agents. At the non permissive temperature of 37°C, pX confers a growth advantage to cells lacking a functional p53. Our data indicate that pX dissects the p53 functions, thus reconciliating virus replication requirements and host liver growth homeostasis.

HBx activities and HBV mediated apoptosis

Since HBx ability to coactivate transcription of cellular genes in trans has been implicated in the development of HBV-related liver cancer. To dissect the transformation and the transcription activation properties of HBx we generated REV2 rat fibroblast derived stable cell lines expressing the wild type and truncated versions of the protein.

REV2 cells are SV40 T-antigen positive tumour revertant cells, isolated from the SV40 transformed Ref-52 cells (SV-52). Although these cells synthesise the wt SV40 T/t-antigens with the same efficiency as the parent SV-52 cells, they are unable to grow in soft agar or to form tumors in nude mice. These cells can be re-transformed by Polyoma or Ad2 with a high efficiency but not by SV40 DNA. To test whether HBx also has the capability to re-transform REV2 cells, 2-4 pCMV-X/F molecules, a CMV promoter based plasmid that encodes a FLAG tagged version of HBx, were microinjected into the nuclei of REV2 cells grown on small glass slides, as described elsewhere. About 25-30% of the injected cells were converted into HBx positive cell lines and 10 randomly selected cell clones were further analysed. Transgene expression was demonstrable in all lines by Northern and by Western blot analysis. Staining experiments revealed that the HBx protein was localised in the cytoplasm of stable cell lines. Interestingly, intranuclear accumulation was not demonstrable as well 48 hours after microinjection of the pCMV-X/F DNA when the HBx concentration is higher than in cells from the stable lines.

Cells of all pCMV-X/F expressing lines display an altered morphology, grow to a higher density than the REV2 cells and acquired the capability to form large colonies in soft agar (Gottlob et al., 1998a). To exclude that the HBx functions might have been altered by the FLAG sequence, we also established REV-2 derived cell lines expressing the wild type HBx and these cells behave as the CMV-X/F lines. These results demonstrate that full-length HBx efficiently re-transforms the REV-2 cells and we analysed the possible mechanisms involved. HBx has been described to transactivate the SV40 enhancer and to affect the expression rate of cellular genes involved in growth control in a cell type specific manner and this has been linked to its transforming activity. HBx did not significantly affected the expression levels of neither SV40 T-antigen nor of cellular proto-oncogenes such as c-myc or c-jun. An alternative strategy by which HBx may cause cell transformation involves the disruption of p53 function as a tumor suppressor. Therefore, REV2 and REV-X/F cells were lysed and protein

extracts were treated with either anti-T-antigen, anti-p53 or anti-HBx-antibodies and immunoprecipitates were subjected to Western blot analysis. As expected, p53 and SV40 T-antigen were readily co-precipitable but complex formation between HBx and p53 or with SV40 T-antigen was not demonstrable in any of our REV2-derived cell lines. To exclude that this negative result might be due to clonal selection we repeated similar experiments with cells extracts obtained from REV2 cells 48 hour after pCMV-X/F transfection. In addition we could not demonstrate cytoplasmic trapping of the p53 by co-immunofluorescence staining. To test whether the high transformation efficiency of HBx is restricted to the REV-2 cells the grand-parental Ref-52 cells were microinjected with the full-length HBx/F DNA and six independent HBx expressing stable cell lines were established. None of these cell lines displayed morphological alterations or acquired anchorage-independent growth properties, even after repeated passages (more than 50 passages).

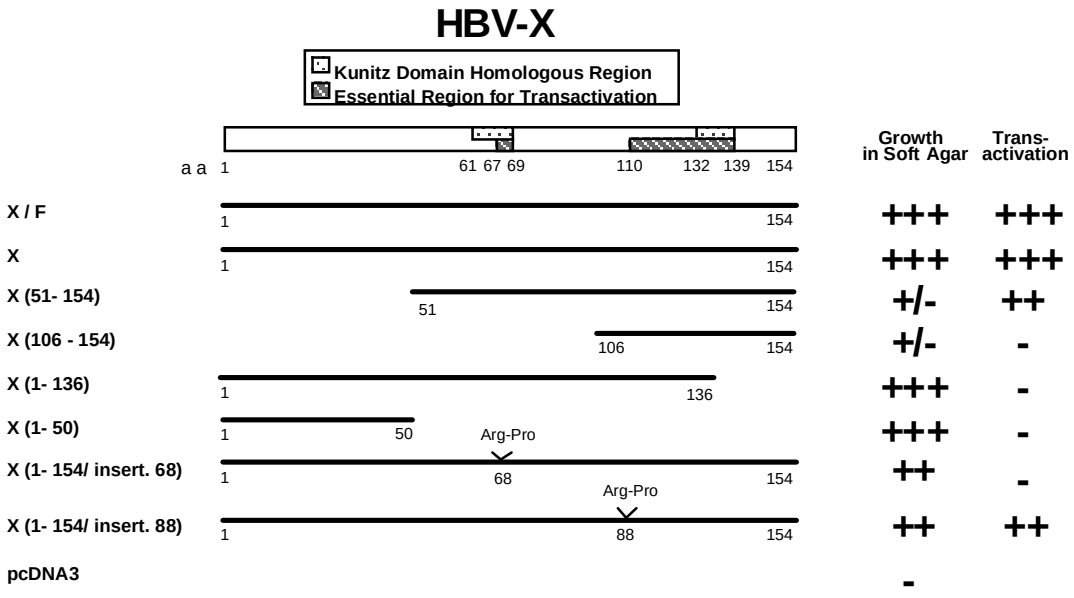

Figure 1. *The ability of HBx to transform Rev2 cells is independent from its transactivating potential.*

Finally, we analysed whether HBx transactivation function is essential for tumour promotion. Two transcription activation domains have been identified so far are, one located in the central region (aa 67-69) and the second in the COOH terminus (aa 110-139) of the protein, that are both required for efficient transactivation. To address the role of these sequences in cell transformation, we established REV-2 derived stable cell lines using a number of HBx deletion mutants and two amino acid insertion (Arg-Pro) mutants with different transactivating activities. The expression of the different mutants was confirmed by Northern blotting and by RT-PCR analysis and the growth properties of the corresponding cell lines were analysed as described above. These experiments revealed that the two transactivation domains play no role in the tumour

promoter function. We observed that the first fifty N-terminal amino acids of HBx (HBx/1-50) (see Figure 1) are sufficient to transform the REV2 cells with the same rate as the full length HBx does. A truncated HBx lacking the 50 N-terminal amino acids (HBx/51-154), but retaining an intact transactivating potential, has a very low transforming capability and cells of all the corresponding lines formed only small colonies in soft agar (2-8 cells). The mutant HBx/106-154, which lacks the first transactivation domain and contains the putative p53 binding domain, has no transactivating potential and a severely impaired transforming capability. The dispensability of the transactivation function for cell transformation is further illustrated by the behaviour of the HBx/1-136 deletion mutant, which does not activate transcription but transforms efficiently, and of the two insertion mutants.

In conclusion, HBx is an efficient tumour promoter and abrogation of HBx transcriptional activation by either deletion of the transactivation domains or by two amino acids insertions whereas it does not affect cell transformation greatly impaires the induction of apoptosis (Gottlob et al., 1998a). So far this N-terminal region has only been implicated in HBx dimerisation and in the negative regulation of transcriptional activation but no definitive role has been assigned to this region in any of HBx functions nor in the interactions between HBx and cellular protein.

HBV genetic variability and liver cancer: identification of pX naturally occurring mutants. Although hepatocellular carcinoma is a frequent disease, only a minority of patients among the many suffering from chronic viral hepatitis and cirrhosis undergo neoplastic transformation in a given time lap. This observation and the low transforming potential of the wild type pX protein observed both in *in vitro* assays as well as in transgenic mice, strongly suggest that other events are likely involved in complementing chronic inflammation and hepatitis viruses infection in the multistep process of hepatocytes transformation in vivo. HBV, as most RNA viruses and retroviruses which use RNA polymerases and RNA dependent-DNA polymerases for replication, undergo an high rate of spontaneous mutations and there is uncreasing evidence for the existence of X gene mutations in HBV chronically infected individuals. Thus, the possibility exists that transformation may be linked to the emergence of mutant pX proteins more than to the expression of a wild type pX protein. To identify and isolate mutant pX we performed PCR and RT-PCR amplification of the relevant regions in the viral genomes from a number of selected patients with HBV chronic infection or hepatocellular carcinoma. After direct sequencing and sequence analysis PCR products have been cloned into expression vectors. Four clones displaying potentially relevant mutation have been retained and used for both transient and stable expression in transfection and microinjection experiments in liver derived cell lines and in immortalised fibroblasts. None of these selected clones have shown changes in the subcellular localisation of the expressed pX polypeptide, as compared to the wild type, while their transactivating properties have been differentially affected by the mutations. The functional characterisation of HBx mutant proteins from four carcinomas (and the adjacent non tumorous tissue from the same patients) showed that defined naturally occurring mutations differentially affect HBx functions (transactivation vs apoptosis) (Pagano et

al., manuscript in preparation) (Figure 2). These results strongly suggest that loss of apoptotic potential by mutated HBx proteins may contribute to the molecular pathogenesis of HCC development.

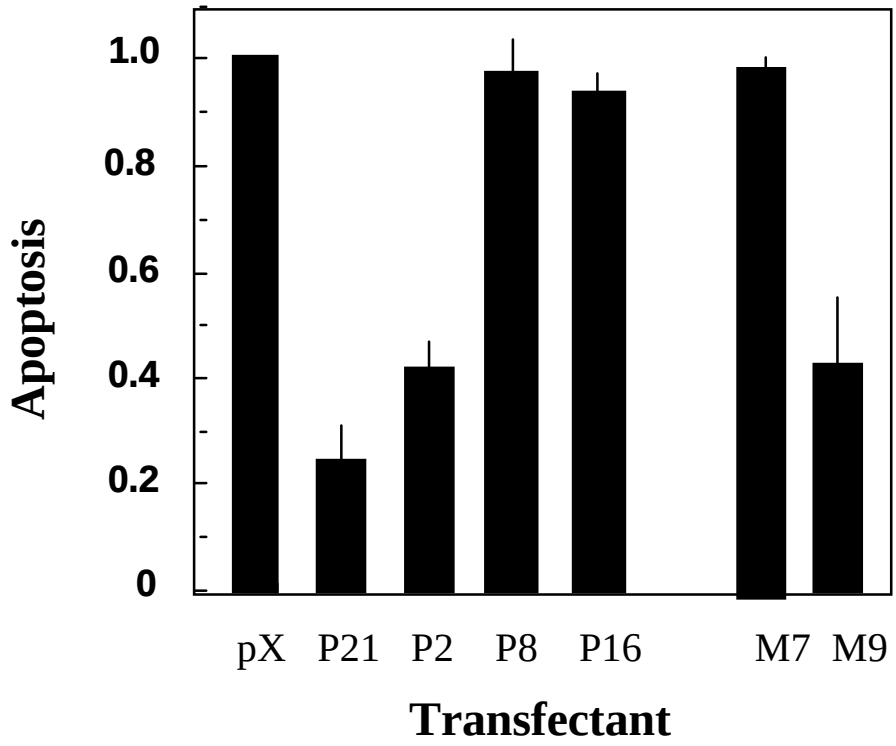

Figure 2. *Loss of transactivation correlates with an impaired apoptotic potential in both HCC-derived (p21 and P2) and genetically engineered (M9) HBx mutant proteins*

Dual effects of HBx on apotosis

Further, we studied the effects of HBx and HBx mutants on REV2 cells apoptotis in response to growth factor depletion. In these cells, HBx prevents DNA fragmentation and cell death in the absence of de-novo protein synthesis, with a similar efficiency as the competitive caspase 3 substrates inhibitors VAD-FMK and DEVD-FMK. Protein extracts obtained from the HBx positive cells contained a very low caspase activity and addition of anti-HBx antibody restored the endogenous caspase activity (Gottlob et al., 1998b). To obtain a functional map of the anti-caspase activity of HBx various cell lines were established which synthesised either N-terminal- or C-terminal-truncated HBx molecules. These gene dissection experiments revealed that the regions required for the anti-caspase activity overlap with the two known transactivation domains of HBx (Gottlob et al., 1998b).

HCV core protein and HBx share common cellular targets

HCV core protein has been shown to have other functions in addition to its role in viral morphogenesis. In fact, HCV core has been shown to affect cell lipid metabolism and to modulate gene transcription, cell proliferation and cell death. In addition, evidence from the transgenic mice model indicate that HCV core is likely to play an important role in the development of both hepatocyte steatosis and HCV-related hepatocellular carcinoma. Although it is not established which of the known activities of HCV core is relevant in either the induction or the maintenance of the hepatocyte transformed phenotype, we have evidence indicating that HCV core activates the ERK1/2 intracellular signaling pathway. The extracellular signal-regulated (ERK) kinases are part of one out of several evolutionarily conserved mitogen-activated protein (MAP) kinase cascades which play an important role in the regulation of both cell growth and differentiation. Activation of Erk-1 and Erk-2 by a wide variety of both extracellular and intracellular factors occurs through the phosphorilation of specific threonine and tyrosine residues (Thr202 and Tyr204) by a single upstream MAP kinase kinase, MEK1. We have found that wild type HCV core sharply increases both p44 and p42 MAP kinase (Erk1 and Erk 2) phosphorilation as detected by a specific anti-phospho-p42/p44 MAP Kinase polyclonal antibody without affecting the total p42/p44 levels in the cell. Maximal activation was already observed at very low levels of HCV core expression and reached a plateau at higher core levels. Interestingly, a truncated HCV core protein which lacks the two carboxiterminal hydrophobic domains and diplays a preferential nuclear localisation, also increases p42/44 phosphrilation and activation. Erk-1 and Erk-2 activation by HCV core may be pathogenetically relevant in view of the ability of these kinases to favour cell proliferation without inducing concurrent cell death by apoptosis in several epithelial cell systems.

Conclusions

In conclusion, HBx has the potential to alter the coordinated balance between proliferation and programmed cell death, being able to either induce or block apoptosis. Prevention of hepatocyte death and selective loss of apoptosis induction due to HBx heterogeneity may represent in vivo critical steps toward the development of hepatocellular carcinoma.

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IMMUNE RESPONSE TO THE HCV HYPERVARIABLE REGION 1 (HVR1) AND VIRAL QUASISPECIES EVOLUTION.

Coordinator: Prof. Mario MONDELLI

Progress Report

CHARACTERISATION OF THE HUMORAL IMMUNE RESPONSE TO THE HYPERVARIABLE REGION 1 (HVR1) OF HCV: EVIDENCE FOR ANTIBODY DRIVEN VARIANT SALECTION AND BIOLOGICAL IMPLICATIONS

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Summary

The hypervariable region 1 (HVR1) of the E2 protein of HCV is highly heterogeneous and is responsible for significant inter-and intra-individual variation of the infecting virus, which may represent an important pathogenetic mechanism leading to escape and persistent infection. We prospectively studied serological responses to synthetic oligopeptides derived from HVR1 sequences of patients with acute and chronic HCV infection obtained at baseline and after a defined of follow-up period. Extensive serological cross-reactivity for unrelated HVR1 peptides was observed in the majority of the patients. Murine monoclonal antibodies generated following immunisation with peptides derived from natural HVR1 sequences also showed cross-reactivity for several HVR1 sequences attesting to the existence of conserved amino acid motifs within different variants. A statistically significant correlation was found between HVR1 sequence variation, and intensity, and cross-reactivity of humoral immune responses providing strong evidence in support of the contention that HCV variant selection is driven by the host's immune pressure.

Key words: *HCV, HVR1, immune response*.

Similarly to human immunodeficiency virus (HIV), HCV is able to persist for a virtually indefinite period of time in the host, despite the coexistence of virus-specific immune responses (1). How HCV induces chronic infection in the face of detectable cellular and humoral immune responses is currently unknown, but the ability of the virus to undergo rapid and substantial sequence modifications is thought to be a major factor in this process (1-10).

Sequence variation is unevenly distributed along the HCV genome with maximal nucleotide and aminoacid replacements being localised in a stretch of 31 residues at the N-terminus of the second envelope glycoprotein (E2) region named hypervariable region 1 (HVR1) (11). Sequential studies of sequence changes in acute and chronic HCV infection have shown that viral variants are continuously being selected in this region (12, 13) and, therefore HVR1 variation may be a mechanism by which HCV evades neutralising responses, leading to persistent infection.

Most studies point to HVR1 as the major immunogenic domain of E2, although the presence of additional B-cell sites outside HVR1 has been documented (14), and a conserved B-cell epitope, only partially overlapping with HVR1, has been recently described which competes for E2 protein binding to its receptor (15, 16). Although an early anti-HVR1 response has been associated with self-limiting acute infection (17), anti-HVR1 antibodies are frequently produced in the majority of chronically infected individuals (6,7,9,10,18-20) and only rarely display unique variant specificity (21-23). Furthermore, even though anti-HVR1 antibodies have been shown to block viral attachment to tissue culture cells (18) and a hyperimmune serum raised against a HVR1 peptide was able to prevent HCV infection *in vitro* (21) and *in vivo* (22), in most instances such antibodies appear to coexist with the HVR1 variants they recognise and are frequently cross-reactive with unrelated HVR1 sequences (6-10,18-20,23). Thus, the significance of HVR1-specific humoral immune responses and their relationship to HVR1 sequence variation are largely undefined.

Available evidence would suggest that HVR1 variation has an adaptive significance and results from a continuous selection process which is likely controlled by humoral immune responses, as suggested by a minimal or absent sequence mutation rate in subjects with congenital immunoglobulin defects (24,25). Moreover, appearance of circulating anti-HVR1 antibodies in chimpanzees inoculated with an identical HCV strain was associated with HVR1 sequence variation, whereas no sequence mutations were observed in the absence of detectable HVR1-specific humoral immune responses (26). However, formal evidence in support of antibody-driven HVR1 variant selection is lacking and, in principles, other mechanisms of variation, such as random drift or adaptive changes in cell tropism and penetration into host cells, as described for the HIV-1 V3 loop (27-29), could be envisioned. In contrast with HIV infection, in which well standardised cell culture techniques can be used to investigate a direct effect of antibodies on the generation of viral variant selection, studies in patients with acute or chronic hepatitis C can only be based on correlative evidence between serological responses and sequence mutations in variable regions.

We prospectively studied serological responses to synthetic oligopeptides derived from HVR1 sequences of patients with acute and chronic HCV infection obtained at baseline and after a defined of follow-up period. Extensive serological cross-reactivity for unrelated HVR1 peptides was observed in the majority of the patients (Figure 1). Antibody response was restricted to the IgG1 isotype and was focused on the carboxyterminal end of the HVR1 region. Cross-reactive antibodies could be readily elicited following immunisation of mice with multiple antigenic peptides carrying HVR1 sequences derived from our patients. The finding of a statistically significant

correlation between HVR1 sequence variation, and intensity, and cross-reactivity of humoral immune responses provided evidence in support of the contention that HCV variant selection is driven by the host's immune pressure (30).

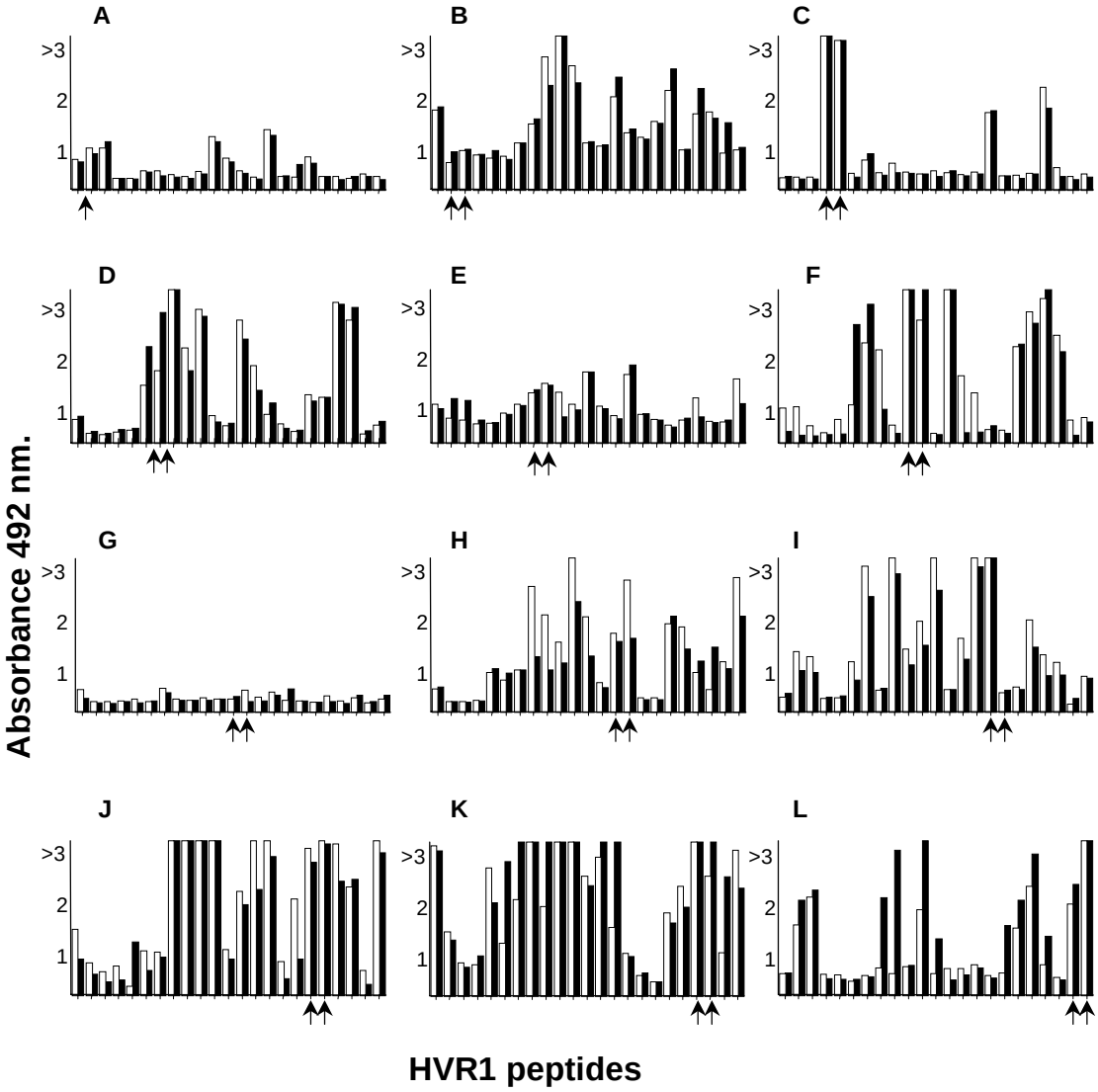

Figure 1. *Serological responses to twenty-three 16-mer synthetic peptides derived from dominant HVR1 sequences isolates from 12 patients with chronic HCV infection (see also Table 1). Empty bars indicate values obtained with baseline sera (t0); solid bars indicate values obtained with sera drawn after 12 months of follow-up (t12). Upper case letters represent individual patients: A-G were infected with HCV type 1b, whereas H-L were infected with type 2a/c. Arrows indicate peptides derived from sequences obtained from the patients reported in each graph.*

Another interesting finding which may be relevant to the pathogenesis of chronic HCV infection was that the extent and the quality of cross-reactive responses to this region may influence the outcome of HCV-induced liver disease. It is noteworthy that in

a recent report we documented a significantly higher HVR1 sequence diversification as a function of time in patients infected with type 2a/c compared with those infected with other types (predominantly type 1b) (31). As HVR1 variation has an adaptive significance, it is conceivable that higher variant selection would result from stronger immune pressure targeted on HVR1 in this patient subgroup, as also suggested by others (20). Since maximal time-related HVR1 sequence diversification is observed in type 2-infected patients (31), our findings provide additional corroborative evidence that HVR1 sequence variation is driven by antibody. It is of interest that type 2-infected patients generally show milder histological lesions and lower serum levels of enzymes associated with hepatocellular necrosis (29, 32), and it is therefore tempting to postulate that more vigorous and heterogeneous cross-reactive responses to HVR1 are associated with a more benign prognosis and possibly slower disease progression in this setting. This situation is analogous to that described for HIV-1 infection in which the rate of viral diversification is more rapid in slow progressors compared to fast progressors (33-34) as a result of a more efficient host immune response (35). Consistent with a central role for humoral immune responses in the control of HCV infection is also the observation that HCV RNA positive individuals with congenital immunoglobulin defects are characterised by a more severe liver disease, compared with those negative for HCV RNA (36). Whether these findings reflect specific characteristics of the HCV type 2 E2 glycoprotein or an overall higher immunogenicity of this virus genotype is unknown. The recent demonstration of stronger Th-cell responses in type 2-infected patients upon interferon treatment compared with other genotypes (37) would favour the latter hypothesis.

To investigate further the molecular basis for antibody cross-reactivity we generated a panel of murine monoclonal antibodies (mAb) from mice immunised with HVR1 surrogate peptides (mimotopes), affinity-selected with sera from HCV-infected patients from a phage-display library. A significantly higher number of antigen-specific clones was obtained after immunisation with a pool of 9 mimotopes compared with immunisation with only one mimotope (21% of specific hybridomas vs. 0.7%, respectively), and in the latter conditions only IgM mAb were generated, whereas also IgA- and IgG1-secreting mAb were obtained using the former experimental approach (38). HVR1 mimotope-specific mAb were also shown to recognise a number of 16-mer peptides derived from natural HVR1 sequences isolated from patients with acute and chronic HCV infection and for some of them a minimal binding site could be mapped at position 398-405, akin to our previous findings using human sera. HVR1 mimotope-specific mAb were also able to efficiently compete with sera from HCV-infected patients for binding to peptides derived from natural HVR1 sequences thus confirming previous data obtained with polyclonal antibodies showing that HVR1 peptide mimotopes are efficient antigenic and immunogenic mimics of naturally occurring HCV variants. Moreover, cross-absorption inhibition experiments confirmed that HVR1 mimotope-specific mAb were cross-reactive for a large number of natural HVR1 variants. These findings demonstrate that it is possible to induce a broadly cross-reactive antibody response at the clonal level and that the efficiency of this process can be greatly enhanced following immunisation with multiple HVR1 surrogate peptides.

Such mechanism could be exploited for the development of an effective HCV vaccine inducing broad specificity for a large repertoire of viral variants.

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ROLE OF CD4+T LYMPHOCYTES, IMPROPERLY ACTIVATED BY NATURALLY MUTATED EPITOPES OF HEPATITIS C VIRUS, IN IMMUNOPATHOGENESIS OF CHRONIC INFECTION: ANALYSIS OF MOLECULAR MECHANISMS RESPONSIBLE OF THE SUPPRESSIVE AND CYTOLITIC PHENOMENA ACTIVATION

Coordinator: Prof. Enza PICCOLELLA

Progress Report

HYPERVARIABLE REGION 1 VARIANTS MEDIATE BOTH AMPLIFICATION AND SUPPRESSION OF HCV-SPECIFIC CD4$^+$T CELLS: IMPLICATIONS FOR THE OUTCOME OF HCV INFECTION

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Summary

The evidence that the hypervariable region 1 (HVR1) of the putative Envelope 2 protein of the hepatitis C virus (HCV), the most variable part of the whole HCV polyprotein, contains both an important neutralization determinant and TCR antagonists for HVR1-specific CD8$^+$T cells, prompted us to clarify the role of HVR1-specific immune responses during HCV infection. At this aim we performed a detailed analysis of the immunogenic potential of HVR1 with particular emphasis to the research of MHC class II restricted agonistic and antagonistic epitopes and to the correlation between anti-HVR1 humoral and lymphoproliferative responses and to the outcome of the disease. By generating HVR1 specific T cell lines and clones we identified HLA-DR4 restricted T cell epitopes in the carboxy-terminus of HVR1 and we demonstrated that broadly cross-reactive HVR1 T cells are elicited by HVR1. We also evidenced that variants of HVR1 can act as powerful TCR antagonists for HVR1-specific CD4$^+$ T cells isolated from HCV-infected individuals. Moreover we observed that the frequency of anti-HVR1 T cell responses was significantly higher in patients who recovered after IFN-α therapy than in those who did not, while no differences in the anti-HVR1 antibody reactivities were detected. In conclusion our data underline the ability of HVR1 to regulate either in positive or in negative the immune response during HCV infection.

Key words: *HCV Immunity, epitopes, cellular activation.*

HCV is a major cause of chronic hepatitis worldwide (1) in that it establishes persistent infection in more than 50% of the cases. Treatment with α–interferon (IFN-α) is the only available therapy, but long-term benefit is obtained in about 20% of treated cases (2). For this reason the development of an anti-HCV vaccine represents an urgent need although a major obstacle to this goal is the high variability of the virus (3). Indeed, the high mutation rate of HCV produces escape variants rendering ineffective both humoral and cellular responses (4, 5). Some of these variants can function as TCR antagonists resulting in the loss of activatory signals and when co-presented with the agonist, abolish agonist-induced T cell activation (6). However, sequence variation in the surface proteins of infectious agents often contain or are part of neutralising epitopes (7). This means that variable portions of viral proteins can mediate both agonistic and antagonistic functions. Within the HCV genome, a hypervariable region (HVR1), consisting of the N-terminal 27aa of the putative Envelope 1 protein (E2), can represent an example of this paradigm. In fact, this region that contains both B and T cell epitopes (5), can represent an important determinant for the induction of neutralising antibodies (8). Anti-HVR1 antibodies have been shown to prevent HCV infection both *in vitro* and *in vivo*. On the contrary, variants of HVR1 can act as powerful TCR antagonists for HVR1-specific T cells favouring HCV persistence. All together these considerations suggested us to perform a detailed analysis of the immunogenic potential of HVR1 with particular emphasis to the research of MHC class II restricted agonistic and antagonistic epitopes and to the correlation between anti-HVR1 helper T cells and the outcome of the disease.

A key point of this research was the choice of a representative panel of HVR1 peptides reproducing the HVR1 sequence of natural isolates. At this aim synthetic peptides reproducing natural HVR1 variants which approximately covers the observed sequence variability was generated as described by G. Puntoriero et al. (9) and synthesised as multiple antigenic peptides. The accession numbers and sequences are behind reported:

264 Genbank:D12967 QTRTVGGQMGHGVRGLTSLFSAGSARN bp 46- bp 126
266 Genbank:D00574 HTRVTGGVQGHVTSTLTSLFRPGASQK bp1240- bp1320
267 Genbank:L19383 ETHTSGGSVARAAFGLTSIFSPGAKQN bp 46- bp 126
269 Genbank:U24616 ATYTTGGSAAKTAHRLASFFTVGPKQD bp 22- bp 102
275 PIR:PQ0835 STRITGGSMARDVYRFTGFFARGPSQN aa 6- aa 32
277 Genbank:D10934 NTYVTGGAAARGASGITSLFSRGPSQK bp1491- bp1571
284 Genbank:X79672 NTRVTGGVQSRTTGTFVGLFTPGPSQR bp 1- bp 81
286 Genbank:D12952 STRVSGGQQGRAAHSLTSLFTLGASQN bp 6- bp 126
288 Genbank:M84754 STIVSGGTVARTTHSLASLFTQGASQK bp1491- bp1571
290 Genbank:S24080 NTYVTGGSAGRAVAGFAGLLQPGAKQN bp 46- bp 126
291 Genbank:S35631 ETHSVGGSAAHTTSRFTSLFSPGPQQN bp 580- bp 660
295 Genbank:D10687 NTHTVGGTEGFATQRLTSLFALGPSQK bp1180- bp1260
296 Genbank:D43651 NTHVTGGVVARNAYRITTFLNPGPAQN bp 39- bp 119
293 Genbank:S70291 QTRTVGGANARNTYGLTTLFTTGPKQN bp 1- bp 81
268 Genbank:M62381 ETHVTGGSAGRTTAGLVGLLTPGAKQN bp 1426- bp 1506
302 Genbank:M62382 ETHVTGGSAGRSVLGIASFLTRGPKQN bp 1426- bp 1506

299 Genbank:D30613 GTHVTGGKVAYTTQGFTSFFSRGPSQK bp 1491- bp 1571
304 (H77-1) ETHVTGGNAGRTTAGLVGLLTPGAKQN bp 1- bp 81
305 (H79) ETHVTGGSAGHTAAGIASFFAPGPKQN bp 1- bp 81
292 Genbank:S62395 ETHVTGGSAASTTSTLTKLFMPGASQN bp 43- bp 123
294 Genbank:D88472 GTTTVGSAVSSTTYRFAGMFSQGAQQN bp 1485- bp 1565
303 Genbank:D88474 ETYIIGAATGRTTAGLTSLFSSGSQQN bp 1488- bp 1568

Fifty-three patients with chronic HCV infection were enrolled in this study. Among them, twenty-four were considered long term responders (LTR) since they had normal aminotransferase (ALT) activity for a period over six months after the end of IFN-α treatment. Five responders (R; normalisation of ALT activity, having finished the IFN-α treatment within six months), four responders with relapse (RR; normalisation of ALT activity only during IFN-α treatment) and twenty non responders (NR; patients who failed to normalise ALT during IFN-α treatment). As control fifteen healthy subjects that showed no sign of past or present HCV infection (i.e., they were negative for anti-HCV in second-generation ELISA tests) were analysed. Peripheral blood mononuclear cells (PBMC), isolated from freshly heparinized blood, were used in proliferation assays and for the generation of T cell lines and clones specific for the HVR1 peptides. The analysis of the capacity of HVR1-variant peptides to function as TCR antagonists were performed evaluating the agonist and antagonist induced-T cell proliferation, -TCR down-regulation, -suppression of IL-2, IL-4 and IFN-γ production and of early tyrosine phosphorylation events. A detailed description of these methods is reported in Frasca et al (10). The results accompained with a brief discussion have been divided in three sections.

Functional characterisation of the immunogenic activity of HVR1 sequences

In order to investigate the immunogenic activity of HVR1 sequences, we analyzed the proliferative responses of PBMC from IFN-α treated HCV patients. Proliferation was evaluated using the panel of the synthetic peptides above reported. We found that PBMC from sixteen patients (30%) showed a significant response to at least one of the HVR1 variants, and that in about half of these patients reactivity was directed to multiple HVR1 peptide variants. Proliferation was specific since none of fifteen HCV sero-negative controls responded to any HVR1 peptide. Previous studies on the helper-T cell response in chronic infection have indicated the core protein as one of the most immunogenic (11, 12). Within this protein, immunodominant sequences corresponding to aa23-42, aa66-85 and aa131-150 have been identified and the frequency of recognition by PBMC of chronically infected patients estimated at around 10-20% (12). Our data indicate that a higher prevalence is displayed by epitopes in the HVR1. Moreover, since the peptides used for this analysis were derived from few, distantly related HVR1 variants, it is possible that by using a larger number of peptides representing additional HCV variants an even higher frequency of T-cell reactivity would be observed. This hypothesis is supported by a recent study in which using only four HVR1 variants it was demonstrated that PBMC from 20% of chronically infected

patients recognised HVR1 peptides (13). Thus, it appears that in spite of its large inter and intra-individual sequence heterogeneity, the HVR1 contains the most commonly recognised T epitopes in HCV infected chronic patients described to date. The evidence that PBMC from the majority of HCV infected patients recognised multiple HVR1 peptides prompted us to verify whether it was due to the activation of polyclonal T cell responses or cross-reactive T cells. At this aim we generated HVR1 specific T cell lines and clones and determined the exact restriction using homozygous partially matched, lymphoblastoid cell lines as APC. The identification of the epitope(s) within the 27 aa-long HVR1 sequence responsible for T cell activation was obtained using three overlapping peptides covering the C-, N- and central sequence of the variants 269, 291 and 266. The evidence that T cell lines always proliferated in the presence of the C-terminal peptides strongly suggested that the carboxy-terminal region of HVR1 is endowed of helper T cell epitopes. The degree of cross reactivity was evaluated by testing their reactivity to a panel of HVR1 variants. Results in Figure 1A, showing the proliferative responses of T cell lines (T2 and T20) to other two and three different HVR1 peptides respectively, suggest that truly broad cross-reactive T cells are present in the HVR1 T cell repertoire. We next investigated the HLA-restriction of cross-reactive HVR1 variants and their potency in stimulating T cell lines.

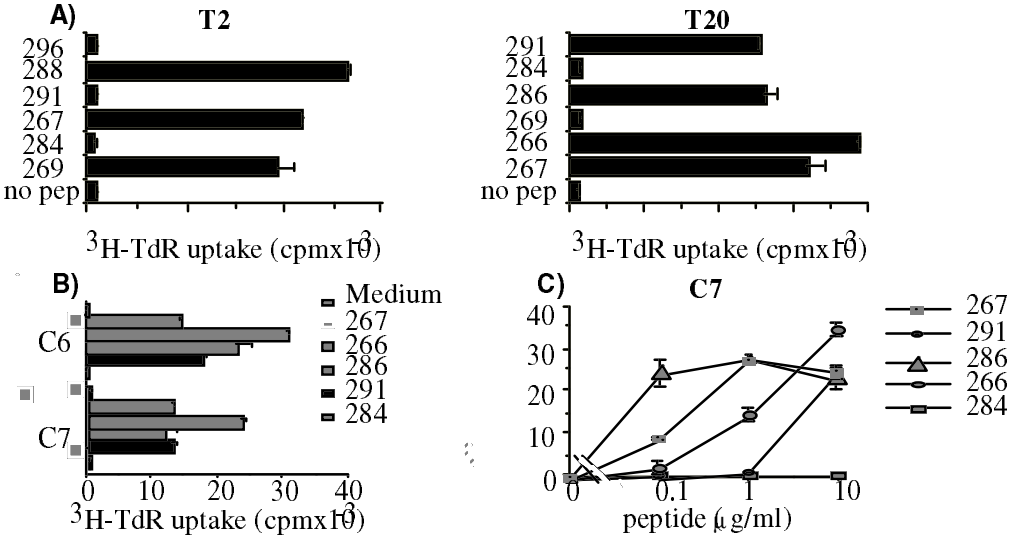

Figure 1. ***Proliferative response of HVR1 specific T cell lines and clones to different HVR1 variants.*** *T cell lines specific for variant 269 (T2), and 267 (T20) (panel A) and T cell clones C6 and C7 (panel B) were incubated with autologous B-LCL in the presence of 10 µg/ml of different HVR1 variants. Results are expressed as ^{3}H-thymidine incorporation. Dose response proliferation of clone C7 to multiple HVR1 variants (panel C).*

Restriction analysis using homozygous EBV-B expressing the HLA class II molecules pulsed with the original and the cross reactive peptides demonstrate that T cell lines use the same restriction element to recognise both the original and cross-reactive peptides. The capability of multiple HVR1 sequences to associate the same

restriction element is an interesting observation that demonstrates that the high degree of sequence variation does not modify the MHC binding capacity of many HVR1 variants. Moreover the evidence that HVR1 epitopes restricted for both HLA-DR4 and HLA-DR11 are located in the same region and the introduction of a nonconservative substitution (L to E) at residue 399 completely abolished stimulatory activity by 266 variant, strongly suggests that the carboxy-terminal sequence of HVR1 variants presents a pattern of conserved aa residues in which the hydrophobic amino acid at position 399 could represent primary anchor for peptide binding to multiple DR alleles (10). To definitively demonstrate the cross-reactive nature of HVR1 T cell recognition, clones from the line T20 were derived and their ability to recognise multiple variants was tested. The pattern of reactivity of representative clones (C6 and C7) to the original (267) and the cross-reactive (291, 266, 286) HVR1 peptide variants (Figure 1B) and the dose response curve of C7 T cell clone (Figure 1C) closely mirror those observed with the parental cell lines. The confirmation of wide cross-reactivity at clonal level indicates that a certain degree of TCR flexibility is present in the helper anti-HVR1 T cell repertoire. Our data that HVR1 specific T cells can recognise peptides which carry multiple aminoacidic substitutions are consistent with previous reports showing that several aminoacidic substitutions in immunogenic peptides are tolerated by T cells and with studies on the cross-reactivity of structurally related and unrelated antigens with the same TCR (14). However the evidence that T cell lines with different pattern of cross-recognition can recognise the same peptide–MHC complex (267 peptide-HLA-DR4 peptide for T cell lines T2 and T20 and 291 peptide-HLA-DR4 for T cell lines T20 and T16) demonstrates that the activation of each individual TCR depends on the existence of an unique spectrum of secondary T cell contacts. In conclusion our data on the characterisation of the high immunogenicity of the HVR1 suggest that this region can favour the amplification and the maintenance of protective T cells during HCV infection.

Functional characterisation of the antagonistic activity of HVR1 sequences

To identify HVR1 variants able to inhibit the T cell response to agonist ligands, six HVR1 variants out of fifteen tested with non immunogenic activity were chosen and tested as TCR antagonists by using a modified version of the method described by De Magistris et al. (6). In this way we identified four variants that powerfully inhibited the proliferation of two clones specific for variant 295 in a dose dependent manner (Figure 2, A and B). Moreover using two 18-mer peptides encompassing the C-terminal region of the agonist and antagonist variants we demonstrated that the antagonistic effect of variant 266 resided in its C-terminal region (266C). In addition we analysed the possibility that the strongest antagonist, peptide 266C, could inhibit cytokine release in both T cell clones, classified as Th0. The evidence that the inhibition of the proliferative responses by the antagonist 266C paralleled a proportional decrease of secretion of the three cytokines IL-2, IL-4 and IFN-γ supported our hypothesis. Since it has recently been reported that pure antagonists fail to induce TCR down-regulation in responding T

cells and inhibit agonist-induced TCR serial triggering (14), we also tested the ability of antagonist peptides to interfere with TCR and CD4 down-regulation in the HVR1-specific T cell clones. Our results demonstrating that peptide 266C failed to induce either TCR or CD4 down-regulation while completely blocked agonist-induced TCR internalisation confirm that this peptide acts as a pure antagonist. We next asked whether TCR antagonism mediated by peptide 266C would also occur when the agonist and the antagonist peptides were presented by different APCs.

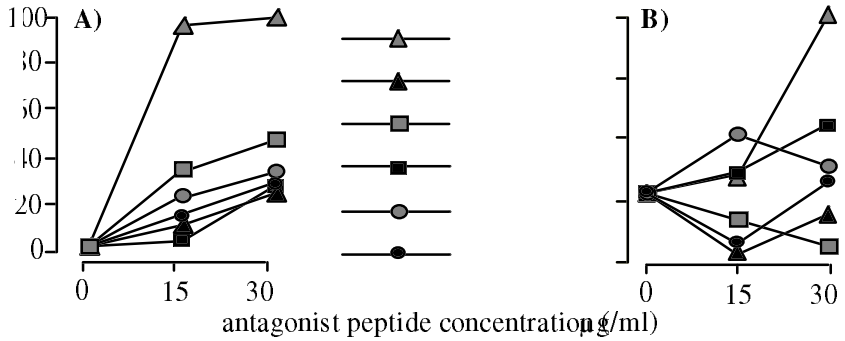

Figure 2. *Natural variants of HVR1 are able to act as TCR antagonists for HVR1-specific CD4$^+$ T cell clones. CS1 (panel A) and CS4 (panel B) T cell clones (1.5 x 10^4 cells/well) were cultured with autologous APCs prepulsed o.n. with either 15 µg/ml (panel A) or 30 µg/ml (panel B) of the agonist variant 295 and the six HVR1-variants were added directly into the wells at increasing concentrations.*

For this purpose APCs were separately pulsed with the same amount of agonist and antagonist peptides and subsequently mixed prior to incubation with HVR1-specific T cell clones. The fact that, when the agonist and the antagonist peptides were presented by different APCs T cell antagonism did not occur, suggests that an antagonist can inhibit TCR triggering only when offered to the responding T cells on the same APC as the agonist. Several reports have described the effect of TCR engagement by class II-restricted antagonist peptides on the early signalling cascade. However, most of these results were obtained following TCR stimulation by agonist and antagonist peptides separately (15). We thus investigated the effect of the co-presentation of agonist and antagonist peptides on the early TCR-induced tyrosine phosphorylation events. We obtained the evidence that TCR engagement by peptide 295C induced the dose-dependent tyrosine phosphorylation of a series of cellular substrates probably representing p95^{vav}, SLP-76, ZAP-70 and the recently cloned p36 LAT. In contrast, the maximal concentration of antagonist peptide 266C failed to increase the level of phosphorylation observed following stimulation of T cells with unpulsed APCs. We next verified whether the antagonist peptide was able to interfere with the agonist-induced tyrosine phosphorylation signals. It is interesting to underline that the antagonist peptide 266C strongly inhibited the tyrosine phosphorylation events induced by the agonist 295C while the co-presentation of the control peptide HA-306-318 did not have any significant effect. These results are the first piece of evidence in humans that class II-restricted antagonist peptides inhibit antigen-mediated early signal

transduction, as observed in class I-restricted antagonists (16). We also verified the possibility that TCR antagonism was a common phenomenon among HVR1 variants and not only a prerogative of the variants analysed and CS1 and CS4 T cell clones. Thus, we analysed the effect of different HVR1 variants by testing the response of HVR1 specific T cell lines derived from a different HCV infected patient. The use of lines instead of clones would probably reflect a more physiological system *in vitro*, since T cell responses *in vivo* are either polyclonal or oligoclonal. To this aim, two lines were obtained from a patient expressing DRB1*0404 and DRB1*1501 and DRB5*0101. These lines, T7 and T2, were specific for the variants 291 and 269 respectively, and were restricted by HLA-DRB1*0404. The results clearly show that HVR1 antagonist variants are frequently generated. In fact, both T cell lines were inhibited by at least two HVR1 variants with an efficiency ranging between 60 and 100% of the proliferative response at the agonist/antagonist ratios of 1:2.5 and 1:5. In conclusion the data reported above represent the first evidence that HVR1 variants can act as powerful TCR antagonists for HVR1-specific $CD4^+$ T cells isolated from HCV-infected individuals. We show that these variants interfere with agonist-induced T cell activation by inhibiting cellular proliferation and cytokine production, and block TCR down-regulation with the consequent suppression of early signal transduction events. Finally, we provide evidence that these effects were not due simply to competition for MHC class II-binding between the agonist and the antagonist ligand.

Analysis of the frequency of B and T cell responses to HVR1 in HCV-infected chronic patients and its correlation with long term response to IFN-α treatment

The evaluation of the frequency of the proliferative responses to HVR1 in the panel of PBMC isolated from HCV chronically infected individuals included in this study was performed according to the outcome of the disease following IFN-α treatment. HVR1-specific T cell proliferation was detected in eleven out of twenty-four LTR (45%), three out of twenty NR (15%), 2 out of five R (40%) and in none of the RR patients. The difference between LTR and NR was statistically significant ($p<0.03$), indicating an association between the activation of HVR1 specific T cells and a positive response to IFN-α. A similar trend was also observed in the anti-HVR1 responses in RR and R patients, although in these cases the limited number of patients did not allow to establish a statistically significant correlation. The observed correlation between the T cell response to HVR1 and the outcome of the IFN-α treatment led us to investigate whether any difference in the prevalence of anti-HVR1 antibodies could be detected in the LTR and NR patients. To this aim we assessed the antibody reactivity of sera from the 24 LTR and 20 NR patients to the same panel of eight HVR1 peptides. Sera from thirty-four patients (77%) recognised HVR1 sequences and the majority of them were simultaneously reactive to multiple variants. With the exception of variants 275 which was not recognised by any of the sera, and variant 277, which was scored positive only by four sera, all the other variant peptides were recognised by antibodies present in a significant fraction of the tested population, ranging from 23% to 48%. In this case,

LTR and NR patients showed similar frequencies of reactivity (75% and 80%, respectively; suggesting that no correlation between antibody response and outcome of the therapy exists. In addition we did not observe any correlation in the specificity of the B and T cell responses against HVR1 peptides in HCV chronically infected patients. In fact in only five out of fourteen patients, whose PBMC proliferated to HVR1 peptides the simultaneous recognition of the same HVR1 sequence by T cells and Abs could be demonstrated. In conclusion our results provide additional support to the role played by $CD4^+$T cells in the containment of HCV infection and underlines the importance of HVR1-specific immune responses.

Concluding Remarks

Our data on the agonistic and antagonistic activity of HVR1 variants on the activation of helper T cells represent an important analysis useful to clarify the effect of the high viral variability both on the evolution of protective immunity during HCV infection and on viral persistence. In fact, the cross-reactive nature of HVR1 recognition and the evidence of the positive responses to HVR1 peptides significantly higher in IFN-a long-term responder patients than in non-responder patients support the hypothesis that HVR1 may expand a $CD4^+$ T cell population with potential protective role against HCV infection and could represent a rationale for the development of synthetic HVR1 sequences able to activate highly multi-specific T cells. On the other side, the data showing that HVR1 variants interfere with agonist-induced T cell activation blocking TCR down-regulation with the consequent suppression of early signal transduction events strongly support the possibility that T cell antagonism could influence the priming of a $CD4^+$ T cell response toward HVR1 immunogenic variants which will be presented together during infection, favouring viral chronicity.

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SUBPROJECT

HEPATITIS VIRAL INFECTION MARKERS IN PARTICULAR CATEGORIES OF PATIENTS

FUNDS ALLOCATED ML 560

PROJECTS FINANCED N° 8

NATURAL HISTORY AND SEROLOGIC MARKERS OF HEPATITIS C IN PATIENTS WITH LEUKEMIA AND HAEMOPHILIA

Coordinator: Prof. Alfredo ALBERTI

Progress Report

HEPATITIS B AND C VIRUS INFECTION IN PATIENTS WITH LEUKEMIA AND AFTER BONE MARROW TRANSPLANTATION

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Summary

In order to assess prevalence, incidence and the clinical impact of hepatitis B virus and hepatitis C virus infections in patients with lymphoproliferative disorders and after Bone Marrow Transplantation, a series of studies in different patients cohorts are conducted.

Prevalence and natural history of HCV infection in patients undergoing chemotherapy for leukaemia were studied in 114 long-term survivors children. 49% developed HCV infection with or without detectable anti-HCV in serum.

Despite this high prevalence of HCV, none developed severe liver disease when followed for 13 to 27 years (mean: 17 years). Morbidity and mortality rates due to HBV and HCV infection after BMT were assessed in 111 consecutive children. 43% had abnormal ALT and 3.6% died of liver failure. “De novo” HBV infection occurred in 3.6% and “de novo” HCV infection in 15%. No significant association was found between HBV or HCV infection and liver disease outcome after BMT. Similar results were obtained in a prospective study of BMT recipients conducted in 15 European Centers in which “de novo” HCV infection was seen in 3.2% and “de novo” HCV infection in 7%.

Finally, the role of different HCV genotypes was assessed in relation to liver damage after BMT. In this study infection with HCV-1 was not associated with a more severe clinical outcome after BMT.

Key words: *Hepatitis C infection, HCV pathogenesis, leukaemia, transplantation.*

Prevalence and Natural History of hepatitis C infection in patients cured of childhood leukaemia (1)

In order to ascertain prevalence and natural history of hepatitis C virus (HCV) infection in patients cured of childhood leukaemia, a large cohort of patients, followed prospectively for at least 10 years since chemotherapy withdrawal, has been analysed. Among 386 consecutive children with leukaemia diagnosed and treated between 1968 and 1982, 125 were long-term survivals and 114 of them could be followed prospectively during the off-therapy period with yearly biochemical and clinical assessment. This series was made up of 59 males and 55 females, with a mean age of 4±2.6 (mean ± standard deviation) years at the time of diagnosis of the haematologic disease (acute lymphoblastic leukaemia: 109 cases; acute myeloblastic leukaemia: 5 cases). None of them received antiviral or interferon therapy during the observation period. Liver function tests and ultrasonography were used to assess presence of liver disease. Patients were also tested for antibody to HCV and for serum HCV-RNA at the end of chemotherapy and at the end of follow-up.

At chemotherapy withdrawal, 56 patients (49%) were HCV-RNA positive, often without detectable anti-HCV, and in these cases transaminase levels were more elevated during ($p=0.08$) and after ($p=0.04$) chemotherapy compared with HCV-RNA negative cases. The whole follow-up period was of 13 to 27 years (mean: 17 years) after chemotherapy withdrawal. During this period, 38 initially anti-HCV negative patients seroconverted to anti-HCV and 17 initially anti-HCV positive cases lost reactivity. Forty patients remained persistently HCV-RNA positive in serum while 16 initially viremic patients became HCV-RNA negative during follow-up. At the end of the observation period, a persistent transaminase elevation was detected only in four HCV-RNA positive and anti-HCV positive cases, while no patient developed signs or symptoms of decompensated liver disease.

Thus, hepatitis C was a frequent finding in long-term survivors after chemotherapy. It was often associated with an atypical serologic profile, as many infected patients lacked detectable anti-HCV, and was rarely associated with severe liver impairment over a prolonged follow-up period of more than one or two decades.

Morbidity and mortality due to liver disease in children undergoing allogeneic bone marrow transplantation: a 10 year prospective study (2)

In order to assess the clinical impact of HBV and HCV infection in patients undergoing allogeneic bone marrow transplantation, 111 consecutive to children who had received a BMT for different haematological disorders between 1985 and 1995 were analysed. They were followed up for a median of 5.5 years after BMT. Before transplant, 48/111 cases (43%) had abnormal ALT, none was HBsAg positive and 4/111 were anti-HCV positive. After BMT, 4/111 (3.6%) died of liver failure. No relation was found between pre-treatment HBV or HCV infection or elevated ALT levels and development of severe liver damage post-BMT. 74% of the cases had abnormal ALT

levels, either transiently (two third) or persistently (one third) after BMT but none of theses cases developed clinical signs or symptoms of end-stage liver disease or of cirrhosis during follow-up. No significant differences in prevalence of liver disease was noted between cases with or without elevated ALT at BMT (relative risk=104).

HCV was the cause of chronic liver damage in 14 out of 28 cases with persistently abnormal ALT after BMT while 2 other cases were infected by HBV and 1 by both HCV and HBV.

In the remaining 12 cases, no viral cause of the liver disease could be identified. The incidence and clinical impact of “de novo” HBV and HCV infection was also evaluated. Post-transplant hepatitis B occurred in 4/111 children (3.6%) including a recipient from a donor who had received the HBV vaccine. The rate of post-transplant hepatitis C was 15%.

Hepatitis C virus genotypes and liver disease in patients undergoing allogeneic bone marrow transplantation (3)

Hepatitis C virus (HCV) genotypes were investigated in 57 HCV infected patients undergoing allogeneic BMT at four European BMT Units where death rates for liver failure in HCV infected patients had shown a wide range of variation from < 1% to > 80%. Aim of the study was to assess whether different distribution of HCV genotypes could have influenced the outcome after BMT.

The Centers participating in the study were located in Italy (Genova and Monza), Sweden (Huddinge), Germany (Ulm). In the post BMT period under surveillance 19 patients had developed liver failure, 10 had acute hepatitis, 22 had chronic hepatitis while 6 had no signs of liver damage. 34 (60%) patients were infected by HCV-1, 15 (26%) by HCV-2, 3 (5%) by HCV-3 while mixed infection was identified in 3 cases (5%) and the genotype could not be typed in 2 (3.5%). No significant differences were noted in the distribution of HCV-1 and of other HCV types in patients with liver failure and with acute or chronic hepatitis C.

The onset of liver failure was somehow accelerated in patients with HCV-1 compared to other genotypes (45 and 68 days, respectively) but this was largely due to a different distribution of LF causes as more cases of early severe liver failure due to veno-occlusive disease occurred in the HCV-1 group. The data obtained did not support the hypothesis of different pathogenicity of HCV genotypes in this clinical setting.

European prospective study on HCV and HBV infection after allogeneic BMT (4)

On the basis of the retrospective results described above, a prospective results described above, a prospective study aimed to assess whether HCV and HBV infection could be risk factors for the development of veno-occlusive disease and severe liver impairment after BMT was initiated within the framework of the working party of the

European Blood and marrow Transplantation Group. A total of 193 consecutive patients from 15 BMT Centers were enrolled in this study.

Data on donors and recipients before and after transplant were collected and included age, gender, alanine aminotransferase (ALT), hepatitis B (HBV), and hepatitis C virus (HCV) markers, haematological disease, status and type of BMT, Conditioning regimen and graft versus host disease prophylaxis. Statistical analysis included univariate descriptive and multivariate analysis based on logistic regression on major end-points.

Forty-three of 193 patients died during the study period, and liver disease was the main cause of death (13 of 43 , 30%). Incidence of severe veno-occlusive disease was 8%, fulminant hepatic failure 0.5% and 12% of cases had ALT >500 U/L (normal ≤42 U/L). A “de novo” HBV or HCV infection occurred in 3.2 and 7% of patients respectively. Predictive risk factors for life-threatening liver disease were: unrelated donors (relative risk=5.8, confidence interval=1.7-19.8) and abnormal BMT donor ALT (relative risk=6.3, confidence interval=1.5-25.5).

This study indicates that ongoing or previous infection with HBV or HCV in donor or recipient is not an absolute contraindication for BMT.

However, abnormal ALT levels in BMT donors were a significant predictor of potentially lethal liver complications. The occurrence of “de novo” HBV or HCV infection did not correlate with severity of liver disease observed in the first 6 months post-transplant. These findings should be carefully evaluated before disregarding HBV or HCV positive siblings with normal transaminase levels in favour of unrelated donor.

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A PROSPECTIVE STUDY ON HCV INFECTION AND DISEASE IN ANTI-HCV POSITIVE PATIENTS UNDER CHRONIC HEMODIALYSIS

Coordinator: Prof. Mario ANGELICO

Progress Report

HCV INFECTION AND DISEASE IN KIDNEY TRANSPLANT RECIPIENTS AND IN PATIENTS UNDERGOING MAINTENANCE HEMODIALYSIS

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Summary

The risk for HCV infection is high in patients with kidney failure under haemodialysis as in kidney transplant recipients. Two studies were performed to better characterise the clinical and virological features of HCV infection on these patients in Central Italy.

Key words: *Hepatitis C infection, HCV pathogenesis, haemodialysis.*

Study 1. - This included 101 Italian kidney allograft recipients. Sera were assayed for, anti-HCV antibodies, HCV-RNA and HCV genotyping. Anti-HCV were found in 33%. The duration of pre-transplant dialysis was longer in anti-HCV positive than in anti-HCV negative patients (p=0.0004). Anti-HCV seropositivity was more frequent among patients grafted before than after 1990 (50% vs 27%, p=0.04) and varied depending on the country of transplant (25% in Italy; 56% in other European countries; and 40% in non-European developing countries). HCV genotype 1b was found in 48% of patients., being largely predominant in patients grafted in Europe. HCV-RNA seropositivity was associated (p= 0.03) with the use of a higher dose of prednisone (p= 0.03) and a lower dose of cyclosporine (p= 0.05) (1).

Study 2. - This included 51 HCV-infected patients undergoing chronic haemodialysis in whom HCV-RNA blood levels were measured before and after dialysis. The study showed that HCV viremia tends to be low in HCV-infected patients

with chronic renal failure under maintenance haemodialysis. Patients dialysed using filters with low or intermediate biocompatibility constantly had higher HCV-RNA levels ($p<0.05$) before dialysis compared to those dialysed with synthetic high-biocompatibility, high-permeability polymeric membranes. HCV-RNA levels decreased after dialysis in most of the patients (2).

Within the first research project on Viral Hepatitis - subproject "Hepatitis Viral Infection Markers in Particular Categories of Patients" - our research group has studied the clinical and virological features of HCV infection and disease in patients with kidney failure under maintenance haemodialysis or who had been already submitted to kidney transplantation. Two studies were completed. The first study was conducted in kidney transplant recipients and the second in patients under haemodialysis.

Key words: *Hepatitis C infection, HCV pathogenesis, haemodialysis.*

Study 1

Kidney transplant recipients are known to be at high risk of infection with HCV (3, 4). This is thought to be primarily due to pretransplant procedures performed before 1990 (5-8), especially haemodialysis and blood transfusions. However, it is often difficult to ascertaining the onset time of HCV infection in patients who do not present clinical signs of liver disease. In addition, the transplant procedure per se and the kidney graft may be other routes of HCV acquisition.

Patients and Methods. - We studied 101 kidney allograft recipients transplanted from 1975 to 1995 at various Centers in Italy or abroad. Patients included 55 males and 46 females and ranged in age from 21 to 66 years. Seventy-seven patients received the graft from a cadaveric and 24 from a living donor. Twenty-four patients were transplanted before 1990. Sera were tested for the presence of HBsAg and anti-HCV antibodies by HCV-EIA-3. All sera, including those which resulted anti-HCV negative, were analysed by reverse transcription nested PCR, using primers from the 5' UTR region. HCV-RNA positive samples were assayed for HCV genotyping using a line probe assay (INNO-LIPA 3rd generation assay). Data were collected and analysed using SOLO statistics (BMDP) and the results analysed by the Student t-test, Fisher exact test and multiple regression.

Results. - Anti-HCV antibodies were found in 32.7% of patients. Four patients were HBsAg positive. Age, gender and other demographic or occupational features did not differ between patients with or without anti-HCV antibodies. The mean duration of dialysis was significantly longer in anti-HCV positive than in anti-HCV negative patients (5.9 ± 4.3 vs 2.8 ± 1.9 years, respectively, $p= 0.0004$). No differences in relation to history of blood transfusions or other known risk factors were observed. At multiple regression analysis, a longer duration of haemodialysis and a lower dose of cyclosporine were significantly associated with anti-HCV antibodies. The prevalence of anti-HCV

positivity was 50% in the 24 patients who were grafted before 1990 and 27.3% in the 77 patients transplanted after 1990 (p= 0.04). Patients were grouped according to the country where the kidney transplantation was performed into: Group A (n= 68): in Italy; Group B (n= 18): in Western European countries other than Italy; Group C (n= 15): in non-European developing countries (mostly in India). Anti-HCV antibodies were found in 17 (25%) of patients transplanted in Italy (group A), 10 (56%) of those transplanted in other European countries (group B) and 6 (40%) of those transplanted in non-European developing countries (group C), the differences being significantly different. The mean duration of haemodialysis before grafting was not different between the three groups. Yet, the proportion of patients transplanted after 1989 was greater in group C (100%) than in groups A (75%) or B (61%) (C vs A: p= 0.02; C vs B: p= 0.007). Twenty-seven patients were found to be HCV-RNA positive. These included 22 anti-HCV positive patients and 5 anti-HCV negative patients. Forty-eight percent of HCV-RNA positive patients were infected with genotype 1b. The remaining included genotypes 1a (6 cases), 2a (1 case) 2c (2 cases), 3a (3 cases) and 4 (2 cases). HCV genotype 1b was found in 57% of patients transplanted in Italy, 83% of those transplanted in other European countries and in none of those transplanted in non-European developing countries. None of the 5 patients in whom HCV-RNA was detected in the absence of anti-HCV antibodies was infected with genotype 1b alone. None of the patients under study had evidence of decompensated chronic liver disease nor had jaundice. Five anti-HCV positive patients had ultrasonographic evidence of portal hypertension and three of these had histological cirrhosis. Abnormal ALT elevation was found in 27 patients. The frequency of ALT abnormality tended to be lower in patients infected with HCV genotype 1b. All patients received a triple immunosuppression therapy throughout the study, including cyclosporine, prednisone and azathioprine. At univariate analysis, anti-HCV positive patients received a lower (p=0.0004) dose of cyclosporine compared to anti-HCV negative patients, while there were no differences with respect to the use of prednisone and azathioprine. At multiple regression analysis, HCV-RNA positivity was independently associated, in a direct fashion, with the average maintenance dose of prednisone (p= 0.03) and inversely related to the dose of cyclosporine (p= 0.05).

Discussion. - The main findings of our study are that the prevalence of HCV infection has dropped significantly in patients grafted after 1989 (by approximately 50%) and this has been associated with a remarkable change in the pattern of HCV genotypes. Infection with genotype 1b was largely predominant in patients transplanted until 1989, while a variety of non-1b HCV genotypes have become frequent since. Overall, our results indicate that in Italy, despite the decline observed after 1989, the rate of HCV infection among kidney transplant recipients remains high. The duration of dialysis prior to transplant is the single strongest independent factor associated with anti-HCV seropositivity. This emphasises the prominent role of nosocomial infections as a risk factor for HCV infection. However, in most patients grafted in non-European developing countries HCV infection was clearly acquired at the time of transplant, as indicated by the emergence of a variety of non-1b genotypes. A complex relationship

was found between HCV infection and the level of pharmacological immunosuppression after kidney transplant. The likelihood of HCV-RNA positivity was directly related to the maintenance dose of prednisone and inversely related to that of cyclosporine. The finding has practical importance for transplant physicians.

Study 2

In this study we investigated the clinical and virological features of patients under long-term haemodialysis, a population also are known to be frequently infected with HCV, with prevalence rates as a high as 20-50% (9). However, despite such a high prevalence, the clinical outcome of HCV infection during haemodialysis is poorly understood. Clinical observation indicates that few HCV-infected patients under maintenance haemodialysis develop an overt chronic liver disease, suggesting that the clinical course of liver disease may be mild in this setting. Interestingly, we found that the serum levels of HCV-RNA tend to be low in many HCV-infected patients. Whether or not this finding may explain the apparently low pathogenicity of HCV infection remains to be established. Haemodialysis is currently performed using with filters differing in biocompatibility and permeability (10). These include filters with low (cuprofan), intermediate (cellulose acetate or diacetate) or high biocompatibility (other synthetic polymeric membranes). The use of different filters is associated not only with specific clearance rates of waste products but also with significant changes in the immunologic host responses, such as neutrophil adhesion and function, monocyte activation and the release of cytokines (11-13). A preliminary report suggested that HCV-RNA levels are reduced in blood following a single haemodialysis using a cellulose diacetate membrane (14). In the present study we further investigated also this latter point.

Patients and Methods. - Fifty-one patients, 31 males and 20 females, 51$\pm$16 years, with chronic renal failure undergoing chronic haemodialysis were studied. All patients had positive serology for anti-HCV antibodies and none of them had evidence of coinfections. Patient had been under haemodialysis for 9$\pm$7 years prior to the study and none of them had clinical and ultrasonographic evidence of progressing liver disease. HCV-RNA blood levels were measured using a quantitative PCR assay in samples obtained immediately before the start of haemodialysis and at the end of the procedure. Sera were kept frozen at -80°C until laboratory use.

Results. - Ten patients (19.6%) were HCV-RNA negative and forty-one (80.4%) were HCV-RNA positive in the blood collected before haemodialysis, in at least two separate determinations. In six infected patients in whom HCV-RNA serum levels were measured in at least four separate occasions during a period of 6-12 months of unchanged haemodialysis the percent of variation of viremia was always less than 10% of the mean values**.** In contrast, pre-haemodialysis HCV-RNA serum levels varied considerably from patient to patient. Patients dialysed using low biocompatibility filters

had significantly higher HCV-RNA levels compared both to those dialysed with intermediate biocompatibility filters and with synthetic high-biocompatibility, high-permeability polymeric membranes (757+262 vs 148+71 vs 93+19 copies/ml, respectively). HCV-RNA serum levels decreased in most patients following a single haemodialysis. Greater reductions occurred in patients with high pre-dialysis values. In two highly viremic patients dialysed with a low-biocompatibility/low-permeability filter we changed the dialyser for three weeks with a high-biocompatibility/high permeability filter. This change was associated with a marked decrease of HCV-RNA levels at the end of the third week.

*Discussion***.** - This study indicates that HCV viremia tends to be low in HCV-infected patients with chronic renal failure under maintenance haemodialysis. This could be related to the clinical observation that relatively few patients apparently progress to advanced liver disease. Low blood levels of HCV-RNA are preferentially associated with the use of high-biocompatibility/high permeability dialysers. A single haemodialysis procedure often determines a drop of circulating viral load. The mechanism of HCV-RNA reduction in blood after haemodialysis is unknown. It is unlikely that the virus may escape through the membrane. Adsorption of the virion to the haemodialysis membrane and pressure-dependent destruction could be potential mechanisms of virus elimination (14, 15). However, high-biocompatibility, high-permeability filters may also reduce HCV-RNA levels through other routes, involving the convective transport across the membrane or the modulation of immunologic host responses. Whatever are the causes, our findings suggest that HCV-infected patients with chronic renal failure should be preferably dialysed using high-biocompatibility/high-permeability filters which may increase their likelihood of cure if appropriately treated with antivirals (16).

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VIROLOGICAL AND LABORATORY CHARACTERIZATION OF UREMIC/DIALYSED

Coordinator: Prof. Arrigo BENEDETTO

Progress Report

HIGH PREVALENCE OF VIREMIA AND SIGNS OF ANTI-HCV IMMUNOLOGICAL DEFICIT IN UREMIC SUBJECTS SEROPOSITIVE FOR HCV

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Summary

In patients affected by chronic uraemia and which are dialysed, the incidence of the hepatitis-C virus (HCV) is particularly high, ranging from 2% to 59%. Nonetheless, the hepatic damage seems to be reduced in this category of patients, as indicated by normal levels of ALT. The scarce possibility to effect a biopsy in these patients, together with the normality of the ALT values renders it difficult to evaluate the hepatic damage, and it is therefore necessary to find new prognostic parameters.
Key words: *Hepatitis C infection, HCV-RNA, anti-HCV.*

Objectives

The purpose of our study was to analyse the immunological and serological parameters associated to the HCV infection in dialysed subjects. For this aim, 72 HCV seropositive patients enrolled in this study were investigated for the following parameters: 1) presence of HCV-RNA in serum and in PBMC; 2) secretion of antibodies by PBMC at first not stimulated, and then stimulated with PWM and PHA; 3) determination of IgM in serum. Finally, these parameters were correlated with the genotypes “harboured” in these subjects.

Results

The 72 subjects enrolled, 48 (66.6%) appeared to be HCV-RNA positive in the serum, 58% of these were infected with the no. 1 (1a+1b) genotype 16.6% with the no.

2 (2a+2b) genotype, 2.1% with genotype no. 4, and 2.1% with genotype no. 5; 10.4% presented coinfections, while in 5 subjects (10.4%) the species was not determinable.

In the 24 HCV-RNA negative subjects a similar presence of the various serotypes was observed: 45.8% of the patients were infected with serotype no. 2, 41% with serotype no. 3, 12.5% were infected with different species, and in the remaining 12.5% the serotypes resulted undetermined. The production of IgM appeared to be positive in 12 subjects (12.6%), and genotype no. 1 was present in 9 out of 12 (75%) of these patients. Only 16 patients (22.2%) showed in vitro secretion of antibodies when their PBMC were stimulated with PWM and PHA, while only in 3 subjects (4.1%) the production of antibodies was spontaneous.

Conclusion

The data of this research project indicate that in most uremic subjects enrolled, the state of HCV seropositivity was accompanied by the presence of HCV-RNA in serum and/or in PBMC. In spite of the high prevalence of positive cases for HCV-RNA, the PBMC of only 3 patients spontaneously secreted anti-HCV antibodies in vitro. This data suggests that uremic patients are characterised for an inactivation state of the circulating HCV specific lymphocytes. This particular immunological defect could be correlated to the reduced hepatic damage (normal levels of ALT) detected in these patients.

HEPATITIS G IN LIVER AND KIDNEY TRANSPLANT: PREVALENCE AND ASSOCIATION WITH OTHER HEPATITIS VIRUSES IN THE HEPATIC DISEASE'S NATURAL HISTORY

Coordinator: Prof. Massimo COLOMBO

Progress Report

HIGH PREVALENCE, LOW PATHOGENICITY OF HEPATITIS G VIRUS IN KIDNEY TRANSPLANT RECIPENTS

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Summary

The prevalence and the pathogenic role of hepatitis G virus (HGV) infection in kidney transplanted patients, is not fully known.

Patients. - 155 HBsAg negative kidney transplant recipients (mean age: 44+12 years) were followed up for 11+4 years post transplant. 108 (70%) received cyclosporin; 70 (45%) were anti-HCV positive and 48 (31%) had persistently elevated aminotransferase, 13 had chronic mild hepatitis, 15 had chronic active hepatitis and 5 had cirrhosis.

Methods. - Serum samples were tested for HGV-RNA by nested RT-PCR using primers derived from the NS3 region of the viral genome, and for anti-HGV by ELISA. HCV-RNA was detected by a nested RT-PCR using primers derived from the 5'NCR of the viral genome, genotyped by a LiPA and quantified by a branched DNA signal amplification assay.

Results. - HGV-RNA was detected in 37 (24%) and anti-HGV in 26 (17%) patients. HCV-RNA (median value of 5,84 MEq/ml) was found in 63 (41%). Fifteen (10%) patients were simultaneously coinfected by HGV and HCV. HGV-RNA positive and negative patients were similar in terms of age, sex, duration of dialysis, rate of transfusion, HCV-RNA, chronic liver disease and immunosuppressive therapy. HGV-

RNA positive patients showed lower levels of HCV-RNA compared to HGV-RNA negative ones (p=0.02). Chronic liver disease was found in 3 (14%) HGV infected patients, 29 (60%) HCV carriers, 10 (67%) with both HGV and HCV and 6 (15%) with neither virus.

Conclusions. - HGV prevailed among kidney transplanted patients as a consequence of multiple parenteral exposure and was associated with low prevalence of serum anti-HGV and of chronic liver disease. HGV/HCV coinfected patients circulate low levels of HCV-RNA suggesting interactions between the two viruses.
Key words: *HGV, HCV, immunosuppression.*

Introduction

10-15% of the renal transplanted recipients have chronic viral hepatitis, mostly hepatitis C virus (HCV) infection. In a residual number of these patients, chronic hepatitis is of unknown etiology (1).

Recently, hepatitis G virus (HGV), an RNA virus, was shown to be transmitted by blood transfusions, plasma products and parenteral exposures including patients on maintenance dialysis (2-4). In this patient population the prevalence of HGV-RNA varies from 3% to 19%, and is often associated with HCV infection (5,6). Chronic infection with HGV is commoner in renal transplant recipients, ranging from 14% to 36%, probably as a consequence of multiple parenteral exposure and immunosuppression (7-9).

Many data indicate that HGV infection may be self-limitated with seroconversion to anti-HGV being the marker of termination of the infection. 40% of renal transplanted patients are anti-HGV seropositive (7). One disputated point of HGV infection in the immunocompromised patient is its potential liver toxicity. Signs of cronic liver disease have been demonstrated in almost 20% of HGV-RNA patients, however in the presence of other potential hepatic viruses such as HBV and HCV (7, 10, 11).

Aim of our study was to assess the prevalence of current infection with HGV in a cohort of kidney transplanted patients and to evaluate the interaction between HGV liver disease and HCV.

Materials and methods

Patients. - Frozen serum samples were collected from 155 kidney transplant recipients followed from 1989 at the Nephrology and Dialysis Division, Ospedale Maggiore Policlinico, IRCCS, Milano, Italy. They were selected from a cohort of 445 renal transplant patients and the criteria for selection were: availability of frozen serum samples collected at -80° C from October 1995, a clinical follow-up post-transplantation of at least 6 years and the absence of serum hepatitis B surface antigen (HBsAg) and antibodies ti human immunodeficiency virus (anti-HIV). The 155 patients had similar epidemiological and clinical characteristics to the excluded patients (Table 1).

Table 1. Epidemiological and Clinical Characteristics of the 155 patients

No. of patients (M/F)	155 (88/67)
Mean age, yr	44 ± 12
Dialysis duration, mo (range)	35 ± 35 (0-192)
Post-transplant follow-up, yrs (range)	11 ± 4 (6-24)
Blood transfusions (%)	111 (72%)
Anti-HCV positive (%)	70 (45%)
Persistently elevated ALT	48 (31%)
Liver histology:	
CAH mild	13/33 (39%)
CAH moderate-severe	15/33 (46%)
Cirrhosis	5/33 (15%)

Antibody to HCV was detected in 70 (45%) patients. 48 (31%) patients showed persistently elevated alanine-aminotransferase (ALT), most (81%) with serum anti-HCV. 33 patients with elevated ALT were eligible for liver biopsy: 28 patients had chronic hepatitis with mild to severe activity and five have cirrhosis.

42 (27%) patients were treated by a combination of prednisone, azathioprine and cyclosporin, 84 (54%) by a combination of two of these drugs and 27 (17%) by cyclosporin only.

Serum assays. – 1) HGV - Hepatitis G virus RNA was detected by a nested reverse transcripted polymerase chain reaction (RT-PCR). RNA was extracted from 200 µl of serum by guanidium-thiocyanate-phenol-chloroform and was converted to complementary DNA (cDNA) using random examers. The cDNA was then subjected to nested PCR with degenerated primers derived from the NS3 region of the viral genome, as previously described (12).

IgG antibodies directed against the E2 protein of the viral envelope (anti-E2) were detected by an enzyme-linked immunosorbent assay (Anti-HGenv, Boehringer Mannheim, Mannheim, Germany). The E2 transmembrane protein was bound onto streptavidin-coated microtiter plates that were incubated with the diluted specimen, and the antibodies were detected using an anti-human IgG-peroxidase conjugate and a peroxidase substrate. Extinction was measured at 405 nm.

2) Others - Hepatitis C virus RNA was detected by a nested RT-PCR, using specific 5' UTR primers as previously described (13). HCV-RNA was quantified by a branched DNA signal amplification assay (Quantiplex HCV-RNA 2.0 assay, Chiron Corporation, Emerville CA). The assay is based upon specific hybridization of viral RNA in the sample by synthetic oligonucleotides (capture and target probes) complementary to the 5'-UTR and core regions of the HCV genome. The reaction is detected with a chemioluminescent substrate and the quantitation limit of this method is 0.2 million equivalent per ml (MEq/ml). The amount of HCV-RNA in each speciment is quantitated using a standard curve.

HCV-RNA was genotyped by a RT-PCR using biotinylated primers from the 5' non coding region. The RT-PCR products were then hybridised to oligonucleotides immobilised on membrane strips (Line Probe Assay, INNO-LIPA HCV 2, Innogenetics, Zwijndrecht, Belgium).

A second generation immunoassay was used to detect serum antibodies against HCV (anti-HCV, HCV ELISA; Ortho Diagnostic System, Raritan, NJ).

HBsAg, anti-HBs, and anti-HIV were tested in serum with immunoenzymatic assays (Abbott Laboratories, North Chicago, IL).

3) Statistical methods - Continuous variables were expressed as means values or median and standard deviations or range; categorical variables were expressed as frequency and percent values. Wilcoxon rank-sum was used to compare means and the chi-squared test to compare categorical variables. The median values were compared by the Kruskal-Wallis Test.

Results

HGV-RNA was detected in 37 (24%) and anti-HGV in 26 (17%) recipients. Three (2%) patients had both HGV-RNA and anti-HGV. Overall, 60 (39%) patients had serum HGV markers.

63 (41%) patients had serum HCV-RNA with a median concentration of 5.84 million equivalent/ml (MEq/ml). Most patients circulated either 1b or 2a/2c HCV (41% and 33%, respectively). 15 (10%) were simultaneously coinfected by HGV and HCV (Table 2).

Table 2. HGV and HCV prevalence

HGV-RNA		37 (24%)
Anti- HGV		26 (17%)
HCV- RNA		63 (41%)
HCV-RNA levels (MEq/ ml)		
(median, range)		5.84 (0.2-46)
Genotype	1a	5 (8%)
	1b	26 (41%)
	2a/c	21 (33%)
	others	11 (17%)

Table 3 shows the clinical and virological characteristics according to the presence or absence of HGV-RNA. The two groups of patients were similar in terms of age, sex, history of transfusion, duration of dialysis, ALT value, severity of liver disease and immunosuppression therapy. The prevalence of anti-HCV and HCV-RNA was similar in the two groups. However patients infected by both HGV and HCV had lower

levels of serum HCV-RNA compared to those infected by HCV alone (2.196 vs 10.8 MEq/ml, p=0.02).

13 (38%) HGV-RNA seropositive patients had persistently elevated ALT, including 10 coinfected with HCV. Serum ALT values were high in 29 (60%) HCV-RNA seropositive patients compared to 3 (14%) patients with serum HGV-RNA alone ($p< 0.001$).

Table 3. Clinical and virological characteristics according to the HGV status.

	HGV-RNA + (n=37)	**HGV-RNA -** (n=118)	**P value**
Age, yr	43±11	44±12	ns
Male	24(65%)	64(54%)	ns
Duration of dialysis,mo	31 (0-192)	19 (1-144)	ns
Blood transfusions	26 (70%)	85 (72%)	ns
Persistent elevated ALT	14 (38%)	34 (29%)	ns
Anti-HCV	16 (43%)	54 (46%)	ns
HCV-RNA	15 (41%)	48 (41%)	ns
HCV-RNA levels (MEq/ml)	2.196 (0.2-35)	10.8 (0.2-61)	0.02
HGV-Ab	5 (14%)	21 (18%)	ns
Knodell's score	8 (2-10)	4(1-12)	ns

Discussion

We found a high number of renal transplanted patients with serum HGV-RNA while a lower number of the patients (17%) circulated anti-HGV.

The prevalence of HGV-RNA in our study (24%) is similar to that reported in other studies including similar patients (7,10), but it is higher than that reported in Italian blood donors and immunocompetent multitransfused patients (3, 14). By converse, the percentage of HGV antibodies was lower (17%), than that observed in immunocompetent multitransfused patients (3,15), suggesting that the low rates of anti-HGV in renal transplanted recipients were due to loss of antibodies due to immunosuppression. High-dose long-lasting immunosuppression regiment in renal transplant recipients not only might cause loss of anti-HGV but also favours the persistence of HGV-RNA in the serum.

More patients infected by both HGV and HCV had sign of hepatitis following kidney transplantation than patients with HGV infection alone (60% vs 14% $p<0.001$). These data do not support that HGV may cause chronic hepatitis in kidney transplanted patients. Our data indicate also that HGV did not increase the severity of underlying hepatitis C. In fact, when renal transplanted patients with hepatitis C infection were

compared with those who had both HCV and HGV infections, no differences were found in terms of liver histology features.

An interesting finding of our study was the interaction between HCV and HGV in patients under a long-term immunosuppressive therapy. We found that HGV-RNA positive patients circulated lower amounts of HCV-RNA compared to HGV-RNA seronegative ones (2.196 MEq/ml vs 10.8 MEq/ml, p=0.02). As previously reported in liver transplant recipients, during immunosuppression HCV virus replication could be inhibited by replicating HGV (16). Interestingly, a similar interaction between HCV and HGV does not occurs in the immunocompetent patients, suggesting that immunity is priority in the context of the two viruses interaction (17, 18). However, the absence of correlation between levels of HCV and HGV viremia and the underlying liver disease in renal transplanted patients, further indicate that load of hepatotropic viruses is not a key factor for determing severity of liver damage.

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DIAGNOSTIC VALUE OF HCV MARKERS IN HAEMODIALYLIS PATIENTS

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Progress Report

THE DIAGNOSTIC SIGNIFICANCE OF HEPATITIS C VIRUS (HCV) MARKERS IN HAEMODIALYSED SUBJECTS

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Summary

The monitoring of dialysis in Apulia confirmed the high prevalence of HCV infection in subjects undergoing periodic dialysis, often correlated to the number of transfusion received and length of time on dialysis. Between 1992 and 1999 a decrease in HCV positive patients was observed due to a reduction in the number of transfusions, the use of heritropoietin and blood products previously tested for HCV. Furthermore HCV-RNA and IgM anti-HCV, useful markers of viremia present in 40% and 30.4% of HCV positive patients respectively, showed a close correlation with the increase in transaminase levels and number of transfusions.

The spread of HCV serotypes in 114 HCV positive dialysis patients from the same geographical area was also evaluated. Serotypes were detected in 102 subjects (89.5%) with type 1 being the most frequent (37.7%), followed by types 2 (19.3%), 4 (8.8%) and 3 (7.9%). Types 5 and 6 were the least prevalent (3.5%). Ten samples (8.8%) revealed mixed infections: type 1 was detectable in all and the coinfecting HCV types were types 2, 3 and 4 in 3, 4 and 3 cases, respectively. These results suggest the serotyping assay as an alternative method of distinguishing the major types of HCV, also for particular risk groups and especially in laboratories which lack the specific expertise to perform genotyping methods. Age related differences in patients with type 5 compared to those with types 3 and 6 may provide evidence of a more recent spread of these latter types.

Genotyping test provided similar information obtained with genotyping test. The agreement between serotyping and genotyping tests was 85.7%.

Key words: *HCV, serotypes, genotypes, HCV-RNA, IgM-antiHCV.*

Introduction

Several epidemiological studies have confirmed the high prevalence of anti-HCV antibodies in subjects at high risk of acquiring hepatitis C virus infection. Among these groups the high risk in patients undergoing periodic dialysis (1, 2, 3) is mainly related to the number of blood transfusions received and length of time on dialysis.

In anti-HCV positive asymptomatic subjects with no signs of liver damage non-invasive procedures may be preferable to evaluate liver status. HCV-RNA and IgM anti-HCV may be useful predictors of liver damage if correlated with liver function and may differentiate active from resolved HCV infection in patients considered at risk.
A high prevalence of HCV-RNA positivity observed in anti-HCV positive blood donors involved in post-transfusion hepatitis suggests a possible correlation between anti-HCV positivity and viremia (4, 5, 6).

The presence of IgM reactivity towards HCV core epitopes has been demonstrated both in acute and chronic HCV infections, and titres correlate with liver disease necro-inflammatory activity, increasing earlier in patients with post-transfusion acute hepatitis C (7, 8, 9, 10). This may have implications in the pathogenesis of chronic hepatitis C. Moreover, the presence of IgM anti-HCV may influence the outcome of antiviral therapy (11, 12). In fact, a progressive disappearance of anti-HCV IgM in patients responsive to interferon therapy in the long term has been observed (13, 14).

Studies on IgM anti-HCV correlated with the clinical stage in the diagnosis of acute HCV infection or evaluation of liver damage in haemodialysis patients, but involved only small patient groups (15, 16).

The first aim of our study was to determine HCV-RNA and IgM anti-HCV in haemodialysis patients and evaluate the correlation with duration of dialysis, increase in transaminase levels and the number of transfusions received (17).

Recently, molecular studies have established that nucleotide variations within the HCV genomic sequence may occur (18). HCV-RNAs from different geographic areas have, in fact, been completely sequenced and the comparison of their nucleotide sequences has provided evidence of a similarity within HCV isolates, subtypes and types of 99% to 87%, 86% to 73%, and less than 72%, respectively (19). The classification proposed most widely applied is currently that of Simmonds which takes into account six main genotypes (20, 21).

The introduction of commercially available assays for genotyping and, more recently, of immunometric methods for serotyping (22) has allowed epidemiological studies of the world-wide distribution of HCV types and subtypes. Moreover, a high correlation between genotyping and serotyping methods has been demonstrated (23).

In Europe, the distribution of HCV type appears to vary according to patient age and also to the different sources of infection (24). In Italy 95% of HCV infections, in blood donors and patients with HCV related chronic liver disease, are sustained by genotypes 1 and 2 and less frequently by genotype 3 (25). However, variations of the genotype prevalence in restricted geographic regions of Italy and groups of patients with different sources of infection and severity of liver disease have been reported (26). Patients undergoing chronic dialysis treatment can sometimes be put at risk of infection

if hygienic precautions are not strictly adhered to. In fact, HCV infection is highly prevalent in haemodialysis patients who are also at higher risk due to their necessary multiple transfusions (27, 28). Thus, the characterisation of HCV infection in this patient population might help in understanding some aspects of the spread of HCV in dialysis units. To date, only a few studies have characterised HCV infected dialysis patients by serotyping and these involved a small number of subjects.

For these reasons, the second aim of our study was to evaluate the HCV serotype and genotype distribution in chronic dialysis patients from Bari in South-East Italy, with seropositivity for HCV markers.

Patients and Methods

A cohort of 315 chronic dialysis patients (178 males, 137 females, mean age 43 yrs. range 19-85 yrs; mean length on dialysis 106 $\pm$ 63 months) from Bari, Italy, who attended the Santa Rita dialysis unit, was first studied. From this cohort, 135 anti-HCV positive chronic dialysis patients were enrolled in the study. Epidemiological data were collected by staff of the dialysis unit by personal interview. For each patient a record was kept, noting physical statistics, past history of liver disease, duration of dialysis, number of transfusions and increase in transaminase levels (ALT). Subjects were systematically asked about documented risk factors, such as previous blood transfusions, intravenous drug use, at risk occupations, any history of chronic hepatitis and trips abroad by family members. Other possible sources of infection, such as acupuncture or tattoos, were also considered.

All samples were tested for antibodies to HCV using Ortho Third generation HCV Elisa Test System (Ortho Diagnostic System, Raritan, New Jersey). All reactive specimens were retested with Chiron Ortho RIBA Third generation supplemental assay to determine the specific reactivity of the repeatedly reactive screening results. The supplemental test increases antibody specificity by providing different test conditions and presenting the antigen in a slightly modified form.

IgM anti-HCV was determined by a solid phase enzyme immunoassay for the detection of IgM class antibodies to a protein expressed by the putative structural region of the HCV genome (Abbott HCV IgM EIA). Specimens with an absorbance value greater than or equal to the cut-off value were considered reactive. All reactive specimens were retested in duplicate and any reactive ones were then interpreted as positive.

HCV-RNA in serum was determined by Amplicor HCV test (Roche Molecular Systems, Branchburg). This test is based on specimen preparation, reverse transcription of target RNA to generate complementary DNA (cDNA), PCR amplification of target cDNA using HCV specific complementary primers, hybridisation of amplified products to oligonucleotide probes specific to the target(s) and detection of the probe-bound amplified products by colorimetric determination (29, 30).

The specificity of the RIBA, HCV-RNA and IgM HCV tests used was evaluated in 45/180 (25%) of randomly selected HCV negative patients.

Serotyping was performed on all sera using Murex HCV Serotyping 1-6 Assay, (Murex Diagnostics, Rome, Italy) a second generation, enzyme immunoassay for the detection of antibodies to HCV serotypes 1,2,3,4,5 and 6. This method uses synthetic peptides, representing the variable antigenic region of NS4 of HCV types 1 to 6. As there is a degree of cross reactivity between antibodies to one type of HCV and peptides from heterologous types, competition from peptides in a free solution is then required to yield type specific binding of antibody to the solid phase.

Genotyping was performed using the INNO-Line Probe Assay (Innogenetics, Nuclear Laser Medicine, Milan, Italy). After RT-nested PCR amplification of 5'NCR using biotinylated primers, amplicons are hybridised with specific oligonucleotide probes and then labelled with streptavidin and immobilised as parallel lines on membrane strips. Detection is obtained by staining of the hybrid with alkaline phosphatase. The reactivity of the amplicons with one or more probes on the strips allows the recognition of the HCV genotypes.

For serum samples resulting non serotypeable, we performed a Western blot test using Wellcozyme HCV Western Blot to qualitatively confirm antibody reactivity to both structural and non structural HCV proteins. This assay is a composite Western Blot containing recombinant antigens representative of the core, NS3, NS4, and NS5 regions of HCV. Partially purified recombinant antigens from each individual region were co-electrophoresed on polyacrylamide gels and the separated proteins transferred to a nitrocellulose membrane by electroblotting.

All tests were performed according to the manufacturer's instructions on sera aliquoted immediately after blood collection and stored at – 80 C° until use.

The results were statistically analysed using Fisher's exact test, Mantel-Haenszel chi-square for stratified analysis and Student's t-test, as necessary. In all cases, a p value < 0.05 was considered statistically significant.

Results

Of 315 patients on regular haemodialysis at our institution, 135 (42.9%) were anti-HCV positive and 180 (57.1%) were negative by EIA-3 screening. Of those resulting positive 126 (93.3%) were positive to RIBA-3 and 9 (6.7%) were classified as indeterminate.

In 45/180 (25%) randomly selected HCV negative serum samples, RIBA-3, HCV-RNA and IgM anti-HCV resulted negative thus confirming the specificity of these tests.

Table 1shows HCV-RNA, IgM anti-HCV results in 135 anti-HCV positive patients, stratified by RIBA. Fifty-four/135 (40%) were positive for HCV-RNA and IgM anti-HCV was present in 41/135 (30.4%).

Table 1. HCV-RNA, IgM anti-HCV and RIBA tests in anti-HCV+ patients

Anti-HCV POS			HCV-RNA +	HCV-RNA -	IgM anti-HCV +	IgM anti-HCV -
	126	POS	54(42.9%)	72 (57.1%)	41(32.5%)	85(67.5%)
RIBA	9	IND	0 -	9 (100%)	0 -	9(100%)
	0	NEG	0 -	0 -	0 -	0 -
	135		54(40.0%)	81 (60.0%)	41(30.4%)	94 (69.6%)

The correlation between IgM anti-HCV and HCV-RNA is summarised in Table 2. The agreement between the two tests employed was 77%. In fact, 32 specimens were positive and 72 negative, respectively, with both tests used. HCV-RNA was present in 22 IgM anti-HCV negative sera, the latter was detectable in 9 HCV-RNA negative sera.

Table 2. IgM anti-HCV and HCV-RNA in anti-HCV+ patients

	IgM anti-HCV POS	IgM anti-HCV NEG	Total
HCV-RNA POS	32	22	54
HCV-RNA NEG	9	72	81
	41	94	135

P<0.0001

Table 3 summarises the characteristics (sex and age) of HCV-RNA or IgM anti-HCV positive patients and the risk factors (time on dialysis, number of blood transfusions and ALT behaviour).

Table 3. Characteristics and risk factors in HCV-RNA and IgM anti-HCV positive and controls

	HCV-RNA POS	HCV-RNA NEG	IgM anti-HCV POS	IgM anti-HCV NEG	Controls
SEX M	30	48	19	59	20
	n.s.		n.s.		
SEX F	24	33	16	41	16
AGE Mean ±SD years	48±12	35±9	34±13	46±11	42±10
Mean ±SD months dialysis	53±30	37±31	35±36	51±29	42±32
TRANSFUSIONS <10	16	48	23	52	17
			p<0.001		n.s.
TRANSFUSIONS >10	38	33	18	48	19
ALT NORMAL	21	54	14	68	24
	p<0.003		p<0.001		
ALT INCREASED	33	27	27	32	21

No statistical significance was found in terms of HCV-RNA and IgM anti-HCV patterns when patients were subdivided according to sex, age and dialysis time. However, HCV-RNA was more frequently found in older patients with a longer history on dialysis while IgM-HCV was restricted to younger subjects with a shorter dialysis history.

In order to evaluate the correlation between transfusions and HCV-RNA or IgM anti-HCV status, patients were subdivided into two groups according to the number of transfusions received (more or less than 10). HCV-RNA viremia was present in 38 patients in the first group compared to 16 in the second, with a statistically significant difference ($p < 0.001$). Eighteen/41 IgM anti-HCV positive patients had more than 10 transfusions, but the difference between the two groups was not statistically significant.

A higher rate of both HCV-RNA and IgM anti-HCV positivity was observed in patients with increased ALT compared to those with normal ALT, with statistically significant differences ($P < 0.003$ and $P < 0.001$ respectively).

Serotyping was performed in 114/135 anti-HCV positive patients using an assay based on serological discrimination between the major HCV-types 1 to 6, by measuring type-specific NS-4 antibodies. The results are summarised in Table 4.

Table 4. HCV Serotype prevalence in relation to age of patients and months of dialysis

HCV serotypes No.(%)	**Age of patients ± = sd**	**Months on dialysis**
HCV 1 no. 43 (37.7%)	60.9 ± 11.6	118.4 ± 59.3
HCV 2 no. 22 (19.3%)	59.7 ± 14.3	94 ± 63.3
HCV 3 no. 9 (7.9%)*	54.0 ± 15.3 *	96 ± 58.5
HCV 4 no. 10 (8.8%)	58.7 ± 11.1	132 ± 68.4
HCV 5 no. 4 (3.5%)* ^	73.7 ± 10.3 * ^	114.8 ± 69.8
HCV 6 no. 4 (3.5%)^	54.5 ± 8.5 ^	92.8 ± 57.4
Mixed Infections no. 10 (8.8%)	58.8 ± 11.6	97 ± 57.7
Non Serotypeables no.12 (10.5%)	61.1 ± 10.8	98.5 ± 62.9

* $p < 0.02$ ^ $p < 0.03$

Type-specific antibodies were detected in 102 (89.5%) of 114 sera. HCV serotype 1 proved to be the most frequent among subjects enrolled in the study (37.7%) followed by HCV serotype 2 (19.3%). HCV serotypes 3 and 4 showed a prevalence of 7.9% and 8.8%, respectively, serotypes 5 and 6 had the same prevalence (3.5%). Ten samples (8.8%) revealed mixed infections: HCV type 1 was detectable in all and the coinfecting HCV type was type 2, type 3 and type 4 in 3, 4 and 3 cases, respectively. Of the thirteen RIBA indeterminates, 7 were HCV type 1, 4 HCV type 2 and 2 were HCV type 3.

Twelve of the 114 sera (10.5%) could not be serotyped and repetition of serotyping yielded identical results. Therefore, a Western Blot test to verify antibody reactivity to both structural and non-structural HCV proteins was performed. All 12

samples resulting positive contained anti-NS4 antibodies and were also C100 RIBA positive.

When we analysed HCV serotype distribution in relation to patient age, a statistically significant difference was found between patients with HCV serotype 5, those with HCV serotype 3 (mean age 73.7 versus 54.0 years, respectively, $p < 0.02$) and those with HCV serotype 6 (mean age 73.7 versus 54.5 years, respectively, $p < 0.03$).

No statistically significant difference was noted when analysing HCV serotype distribution in relation to the duration of dialysis treatment. (Table 4).

Genotyping was performed in 63 patients resulted HCV-RNA positive (Table 5). Type specific antibodies were detected in 43 sera, HCV genotype 1 proved to be the most frequent among subjects enrolled in the study (38.1%) followed by HCV genotype 2 (19.0%). HCV genotype 4 showed a prevalence of 7.9%, two samples (3.2%) revealed mixed infection (genotype 1+4). Twenty sera could not be genotyped (31.8%).

Table 5. HCV Genotype prevalence in relation to age of patients and months of dialysis

HCV serotypes No.(%)	**Age of patients ± = sd**	**Months on dialysis**
HCV 1 no. 24 (38.1%)	66615.1	128.1658.5
HCV 2 no. 12 (19.0%)	57.2618	94 .1657.9
HCV 4 no. 5 (7.9%)*	55.3614.5	127659.2
Mixed Infections no. 2 (3.2%)	56.5611.8	95.2656.4
Non Genotypeables no.20 (31.8%)	64.4612.3	95.8661.4

The age and time on dialysis in subjects with genotype 1 were higher than in those with other genotypes, without statistical significance.

The agreement between serotyping and genotyping tests was 85.7%. In fact, 43 specimens presented serotype or genotype of the same type, 11 specimens were negative for serotype and genotype. Moreover, nine specimens typed by serotyping test were negative by genotyping test.

Discussion

Some recent observations in blood donors and HCV chronic patients have suggested that HCV-RNA may represent a useful marker for evaluating liver status and monitoring HCV infected subjects. On the other hand, IgM anti-HCV is a useful serological marker for estimating relative exposure time to HCV infection or monitoring chronic patients during interferon therapy.

In this study, a prevalence of 30.4% of IgM anti-HCV positivity was noted in haemodialysis patients, which was lower compared to that of patients with chronic liver

disease. It could, therefore, be possible that this discrepancy may be the expression of an immunosuppressive state occurring in haemodialysis subjects, considering that in these patients immunological disorders, such as cellular and humoral response impairment, have been observed.

Moreover, the results of this study also confirm the significant presence of HCV-RNA and IgM anti-HCV in HCV positive haemodialysis patients, while at the same time providing further useful information, such as the strong association between HCV-RNA and number of transfusions or increase of ALT correlations. Increased ALT levels were observed in HCV positive patients with IgM anti-HCV.

Some authors have not observed any differences in disease activity in chronic HCV patients with or without anti-HCV IgM. In our data, however a greater positive association between anti-HCV IgM and disease activity (ALT levels, liver features) was found, perhaps indicating persistent HCV replication in haemodialysis patients. Moreover, IgM anti-HCV in haemodialysis patient indicates early HCV infection.

Analogous to anti-HBcIgM, in HBV infection, anti-HCV IgM positivity could be considered the effect of the host's immune response to viral replication. Therefore, the anti-HCV IgM test can be considered as providing useful information for evaluating virus induced liver damage in haemodialysis patients.

The anti-HCV IgM assay may be useful in routine clinical activity as a serological marker to indicate the presence of ongoing HCV infection.

Serotyping analysis of HCV isolates provides an indirect typing method based on the production of type specific antibodies by the infected host. In our study HCV serotype distribution was evaluated in a cohort of 114 chronic dialysis patients with seropositivity for HCV markers. A high percentage of serotypeable samples (89,5%) was observed, suggesting the applicability of the serotyping assay also in these particular patient groups. This result contrasts with the sensitivity of serotyping reported by other groups who investigated the HCV serotype distribution in haemodialysis patients. In fact, studies by Van Doorn, Brechot and colleagues, indicate that serotyping assay sensitivity in haemodialysis patients, who may have impaired immunoreactivity, is in approximately 40-60%. These studies, however, involved a very limited number of haemodialysis subjects on which to draw this kind of conclusion. Our study involved a greater number of patients, therefore the sensitivity of serotyping may be sufficiently high to consider this assay reliable also in haemodialysis patients.

Twelve non-reactive serum samples (10.5%) were found in the serotyping test: these sera did not seem to contain any antibodies to the NS4 epitopes presented in the serotyping assay. Instead, using a Western Blot test, we demonstrated that in these samples anti-NS3, anti-NS4 and anti-core antibodies were present. In this respect, we may hypothesise that these sera could have a low level of anti NS4 antibodies, detectable by confirmation assay, but not enough to characterise the HCV type.

This report shows that, though circulating in different proportions, the six major HCV types are represented in our haemodialysis patients. HCV serotype 1 was the most frequent (37.7%) followed by HCV serotype 2 (19.3%). Similar results are also reported by other groups in different geographic areas of Italy. HCV serotype 3 frequency was 7.9%, which is consistent with the prevalence of HCV type 3 in other risk groups, such

as drug addicts and haemophiliacs. In our group we found a relatively high prevalence of mixed infection (8,8%), possibly due to multiple blood transfusions. Also, HCV serotype 4 frequency was 8.8%. An interesting fact was that HCV serotype 4, which is more frequent in countries such as Egypt, Saudi Arabia and Central Africa, and has a low frequency in Italy in non risk groups, is nevertheless often encountered in haemodialysis patients in Italy. In fact, an outbreak entirely attributable to HCV type 4 has been reported in a haemodialysis unit in Northern Italy. Frequency of HCV serotypes 5 and 6 was 3.5%. These HCV types, rarely encountered in Europe, are more frequent in Africa and South-East Asia. None of the subjects in the study were African or Asian nor, to our knowledge, had recently visited these continents. This observation is confirmed by data obtained in our previous study in which HCV serotypes 5 and 6 were found in our area (31).

The age of patients with HCV type 5 was significantly higher compared to those with HCV serotypes 3 ($p < 0.02$) and 6 ($p < 0.03$), respectively. These data may suggest that HCV serotype 5 persists for a longer time compared to HCV serotypes 3 and 6, thus confirming a more recent spread of these latter HCV types.

Genotyping test provided similar information obtained with genotyping test.
The present study indicates that the serotyping assay is a useful epidemiological method for characterising HCV types also in particular risk groups, that may often have modified immunoreactivity. The serotyping assay offers an alternative method of distinguishing the major types of HCV, especially in laboratories which lack the specific expertise to perform genotyping methods. In our group HCV serotype 1 occurred frequently. Age related differences in patients with HCV serotype 5 compared to those with HCV serotypes 3 and 6, provide evidence of a more recent spread of these latter types.

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NATURAL (AND POST-THERAPEUTIC) HISTORY OF HEPATITIS VIRUS C (HCV) INFECTION AFTER LIVER TRANSPLANTATION ROLE OF JAK-STAT PATHWAYS AND ALPHA-IFN-DEPENDENT TRANSCRIPTIONAL ACTIVATION IN THE AUTCOME OF RECURRENT HCV HEPATITIS

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Progress Report

EXPRESSION OF THE JAK-STAT SIGNAL TRANSDUCTION PATHWAY IN VIRAL HEPATITIS C

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Summary

The mechanisms responsible for the persistence of HCV infection and for the limited response to interferon-α (IFN-α) treatment are not fully understood. Great importance has been conferred to the high genetic variability of HCV genome and the quasispecies distribution of the virus. However some evidence suggests that both virus escape from host immunity and interference with the endogenous IFN system may be involved. We have therefore evaluated the expression of the IFN-α dependent Jak-STAT pathway in liver biopsies from patients with 'naive' chronic hepatitis C and in patients with histological recurrence of HCV infection after OLT for HCV-related cirrhosis, to assess the possible role of an abnormality of natural immunity against viral infections in the pathogenesis of HCV-related liver damage. We found an increased activity of Stat1 in the hepatocytes of patients with HCV infection. This may lead to the overexpression of immune response genes and therefore to a condition of persistent immune activation. On the other hand, we were not able to find any nuclear localisation of Stat2 e p48. This data indicates that the ISGF3 complex formation and nuclear translocation may not succeed in HCV infection and suggests a possible mechanism by which HCV may inhibit IFN-α activity.

Key words: *HCV, Jak-STAT, IFN-α.*

Introduction

Hepatitis C virus infection persists in more than 80% of the cases, leading to chronic hepatitis and, possibly, to liver cirrhosis (1). Interferon-α in combination with

ribavirin has been shown to be effective for the treatment of HCV chronic hepatitis in approximately one third of the cases (2). Liver transplantation (OLT) has been also approved as a treatment for end-stage HCV-related cirrhosis. However, HCV reinfection after liver transplantation for HCV-related cirrhosis is almost universal and histological liver damage develops in 50-90% of patients within 24 months from OLT (3-4). The mechanisms responsible for viral persistence and limited response to interferon-α (IFN-α) treatment are not fully understood, nor the relationships between HCV infection and mechanisms of antiviral defence and hepatocellular damage are established. A key question is represented by the respective roles of viral and host factors. Great importance has been conferred to the high genetic variability of HCV genome and to the consequent quasispecies distribution of the virus (5). However, both virus escape from host immunity (6) and interference with the endogenous IFN system have been also advocated (7, 8). Useful information may derive from the analysis of the processes of natural immunity against viral infections. Virus-infected cells produce IFN-α that exerts a protective function towards the virus-free neighbouring cells, making them resistant to viral infection ("antiviral state"). IFN-α induces cell synthesis of proteins, such as 2'-5' oligoadenylate synthetase and PKR, that interfere with replication of viral RNA or DNA. Moreover it enhances the lytic potential of NK cells and the efficiency of CTL-mediated killing also by modulating expression of class I MHC antigens (9). These functions cooperate to eradicate viral infections and represent the basis for the clinical use of IFN-α in chronic viral hepatitis C. The antiviral activity of endogenous IFN-α is primarily paracrine, since virus-infected cells produce IFN to protect the virus-free cells, IFN-α induces the activation of Jak1 and Tyk2 protein kinases, that are associated with the IFN-α receptor (α-IFNr), and then the phosphorylation of the cytoplasmic proteins of the STAT family (STAT1 p91, STAT1 p84 and STAT2 p113). The STAT proteins then translocate to the nucleus and assemble into a complex, the ISGF-3 (interferon-stimulated response gene factor-3) together with the p48 protein, a sequence-specific DNA binding protein whose affinity for IFN-α response elements (ISRE) in IFN-α induced genes (ISG) is thereby increased. Following the interaction with the active ISGF3, the transcription of the IFN-α stimulated genes is activated in an IFN-α dependent manner (10-12). Some viruses have been shown to escape the antiviral activity of IFNs by specifically blocking the ISG induction. Abnormalities in the mechanisms of ISGF3 formation have been, in fact, described for adenovirus, EBV, HBV and, more recently, HCV (13-16). Expression of HCV proteins has been shown to inhibit signal transduction through the Jak-STAT pathway probably by interfering with DNA binding of STATs and by inhibiting IFN-α induced formation of ISGF3 (16). We have therefore evaluated the expression of the IFN-α dependent Jak-STAT pathway in liver biopsies from patients with ‘naive’ chronic hepatitis C and in patients with histological recurrence of HCV infection after OLT for HCV-related cirrhosis, to assess the possible role of an abnormality of natural immunity against viral infections in the pathogenesis of HCV-related liver damage.

Patients and methods

Patients. - We have analysed percutaneous liver biopsies obtained from 16 patients with chronic hepatitis C (9 mild, 5 moderate, 2 severe) and from 18 patients with histological recurrence of hepatitis C after OLT for HCV-related liver cirrhosis. We have also analysed 4 surgical liver samples with normal histology. All controls were serologically negative for markers of HBV and HCV infection.

Methods

Histological grading. - Necroinflammatory activity was graded according to Ishak et al. (17).

Immunohistochemistry

We have used a biotin-streptavidin immunohistochemical technique on paraffin sections of formalin-fixed liver biopsies with synthetic peptide rabbit polyclonal antibodies specific for the Janus and STAT families and for p48, all obtained from Santa Cruz Biotechnology, Inc. Namely, we have used Jak1 (HR-785) specific for an internal domain of Jak1, Tyk2 (C-20) specific for the carboxyterminus of Tyk2, Stat1 p84/p91 (E-23) specific for a carboxy terminal sequence common to human Stat1β p84 and Stat1α p91, Stat1α p91 (C-24) specific for the carboxy terminus of Stat1α p91, Stat2 (C-20) specific for the carboxy terminus of Stat2 p113 and ISGF-3 γ p48 (C-20) specific for the carboxy terminus of ISGF-3 γ p48.

Semiquantitative analysis of Jak-STAT expression

Semiquantitative assessment of the intensity of expression of the Jak-STAT families and of p48 was performed on a scale from 0 to 4. The mean tissue density of positive cells was assessed on the same samples and scored using a range from 0 to 4 (0, <1%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, >75%). Sections were considered negative when no clearly detectable staining above background was found in the parenchyma or when the tissue density of positive cells was below 1%.

Results

Normal liver. - In normal liver the expression of the proteins of the Janus (Jak1 and Tyk2) and STAT (1 and 2) families and of p48 was very low. Jak1 was consistently negative in hepatocytes, whereas it was clearly expressed in perisinusoidal cells. Tyk2 was negative. On the other hand, there was a weak expression of STAT1 (p91 and p84)

and STAT2 in the cytoplasm of less than 50% of the hepatocytes. There was no nuclear localisation of STAT molecules. There was no detectable expression of p48.

HCV-related chronic hepatitis

In HCV-related chronic hepatitis Jak1 was exclusively expressed on perisinusoidal cells (9 out of 16 cases) and less frequently on sinusoidal endothelial cells. Hepatocytes were consistently negative. Tyk2 showed a weak cytoplasmic reactivity in the hepatocytes of 8 out of 16 cases, that was generally diffuse in the parenchyma without any specific zonal compartmentalisation. STAT1 expression appeared to be considerably increased in the liver of patients with CAH in comparison to normal controls, as assessed by the reactivity with both antibodies either to Stat1α p91 or to the carboxy terminal sequence common to human Stat1β p84 and Stat1α p91. STAT1 was expressed in all cases examined in bile duct cells with a variable degree of intensity. There was also a variable degree of expression, both in terms of intensity and density of positive cells, in the inflammatory cells in the portal tracts and in the areas of piecemeal and spotty necrosis. Particularly strong was the reactivity in the lymphoid follicles found in some portal tracts and in occasional granulomas. In 9 out of 16 cases there was also a clear expression of STAT1 in the endothelial cells of terminal hepatic venules and portal venules. There was also a clear reactivity with perisinusoidal cells and Kuppfer cells in 8 cases. However, the most interesting finding was the marked increase of STAT1 expression in hepatocytes. In fact, STAT1 was expressed in the cytoplasm of hepatocytes in all patients. There were variations in the extent and intensity of reactivity among individual cases, with an apparent stronger expression in the periportal hepatocytes and in the areas of spotty necrosis. More importantly, STAT1 was localised also to the nucleus of hepatocytes in 15 out of 16 patients, indicating a state of STAT1 activation and STAT1-dependent gene transcription. On the other hand, STAT2 was expressed with a varying degree of intensity in the cytoplasm of the hepatocytes in 14 out of 16 cases. The intensity of reactivity appeared to be higher in zones 1 and 3. However, there was no detectable localisation of STAT2 in the nucleus of hepatocytes. Finally, p48 was weakly expressed in the cytoplasm of hepatocytes in 13 cases, but never localised to the nucleus.

Post-OLT recurrent C hepatitis

In post-OLT histological recurrence of hepatitis C, Jak1 was expressed, as observed in normal liver and chronic hepatitis, exclusively on perisinusoidal cells (14 out of 18 cases). Tyk2 showed a diffuse, even though generally weak reactivity in the cytoplasm of hepatocytes in 13 out of 18 cases. The most striking finding was represented by the very high level of expression of STAT1 in hepatocytes, as established by the reactivity with both antibodies either to Stat1α p91 or to the sequence common to human Stat1β p84 and Stat1α p91. Stat1 was expressed in all cases

examined both in the cytoplasm and in the nuclei of hepatocytes. The intensity of expression was generally high and was in some cases localised to almost all hepatocytes. There was no clear relationship with the infiltration by inflammatory cells. On the other hand, Stat2 was expressed in all cases in the cytoplasm of hepatocytes, but never localised to the nucleus. Finally, ISGF-3 p48 was expressed in the cytoplasm of hepatocytes in 16 out of 18 cases without any nuclear localisation.

Discussion

Our results provide initial evidence for an abnormality in the pathways for cytokine signal transduction in chronic hepatitis C. In particular, we show that the Stat1 transcription factor is activated in the hepatocytes of patients with chronic hepatitis C, as indicated by its nuclear localisation in the hepatocytes of all patients examined. In addition, we find that the other two members of the ISGF3 complex, STAT2 and p48, that are essential for signal transduction through IFN-α receptor and, therefore, for IFN-α antiviral and antiproliferative functions, are not translocated into the nucleus of hepatocytes. The mechanism that underlies activation of Stat1 in chronic hepatitis C and post-OLT recurrent hepatitis C is uncertain. This may be due to local production of proinflammatory cytokines, such as IFN-γ, that leads, after receptor occupation, to Stat1 phosphorylation, dimerization, nuclear translocation, and consequent binding to gamma-activated sequence (GAS) elements (18). These events drive the expression of nearby genetic targets, such as the ICAM-1 gene promoter region that enhances gene transcription and expression (19) and a condition of persistent immune activation. Other cytokines such as IL-2, IL-6, IL-10, EGF, PDGF and CSF-1 may be also involved in Stat1 activation (20) and Stat1 homodimers have been also shown to form in response to IFN-α/ß (21,22). However, since IFN-α/ß-induced Stat1 homodimers do not drive the expression of IFN-γ-responsive genes containing GAS elements, it is likely that an additional response to IFN-γ is required as shown in experimental models of inflammation (23). The meaning of the increased expression of STAT2 and, even though at a lesser degree, of p48 in the cytoplasm of hepatocytes remains to be assessed. This may reflect an effect of an increased secretion of IFN-α, as STAT2 gene appears to be transcriptionally activated by IFN-α (24). In fact much higher levels of STAT1 and STAT2 are observed in cells after IFN-α treatment (25-27). The absence of nuclear localisation of Stat2 and p48 in the hepatocytes of patients with either ‘naive’ chronic hepatitis C or post-OLT hepatitis C recurrence is consistent with the hypothesis that the ISGF3 complex formation and nuclear translocation do not succeed in HCV infection and suggests a possible mechanism by which HCV may inhibit IFN-α activity. The level of inhibition would appear to be downstream to the transcriptional level, either by the direct interference of the virus itself or through a regulatory molecule (e.g., kinase or phosphatase) that is expressed or activated or altered selectively (e.g., by the viral infection itself) in hepatocytes (28, 29). In conclusion, our data suggest the presence of a complex abnormality that may be involved in the pathogenesis of HCV-related liver

damage. First, the increased activity of Stat1 may lead to the overexpression of immune response genes and therefore to a condition of persistent immune activation. Second, the inhibition of ISGF3 formation and nuclear localisation, may hamper IFN-α antiviral function, so favouring persistent HCV infection.

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MULTIPLE INFECTIONS BY HEPATITIS VIRUSES: DIAGNOSTIC AND PROGNOSTIC VALUES OF DIFFERENT PATTERNS OF INFECTION

Coordinator: Prof. Evangelista SAGNELLI

Progress Report

MULTIPLE HEPATITIS VIRUS INFECTIONS

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Summary

We evaluated the interference between HBV, HCV and HDV in multiple hepatitis virus infections and the clinical impact of the different virological patterns in 648 HBsAg and/or anti-HCV positive subjects enrolled in 7 Italian Liver Units from 1993 to 1997. We demonstrated that the interaction between the hepatitis viruses is characterised by a reciprocal inhibition of the respective genomes. Indeed, we observed an inhibitory effect of HCV on HBV replication and confirmed the well-known depression exerted by HDV on HBV genome. In particular, HCV depresses the HBV "e-minus" strain, but it does not affect the HBV wild strain. We also demonstrated that HBV infection, alone or associated with HDV, inhibits HCV replication. The associated liver disease is more severe in patients with a multiple hepatitis virus infection than in those with a single infection. Anti-HCV positive/anti-HBc positive patients lacking HBsAg and anti-HBs may be considered a subgroup with HCV infection and "silent" HBV infection. This subgroup of patients show a more severe liver disease and a poor response to α-Interferon treatment, irrespective of the HCV genotype involved.

Key words: *Multiple hepatitis virus infection, HBV infection, HCV infection.*

Introduction

In countries with a high endemicity level of HBV, HDV and HCV infection, chronic hepatitis from multiple virus infection is a common event.

Multiple HBV and HDV infection has been extensively investigated in past years and found to be associated to a fulminant course of acute hepatitis, to the more severe forms of chronic liver disease and to a rapid progression to liver cirrhosis; an inhibitory effect exerted by HDV on HBV replication has also been demonstrated (1).

The interaction between HCV and HBV has so far been poorly investigated, since tests to detect HCV replication have only recently become extensively available. Moreover, little is known on the virological interaction, the clinical presentation, the natural history and the response to antiviral treatment of liver diseases related to HBV and HCV coinfection (2-7).

In this paper we report the data from an Italian multicentre study carried out to analyse the interference between the hepatitis viruses, using recently developed specific technology, and the clinical impact of multiple virus infections as opposed to the single HBV or HCV infections. In this study anti-HCV positive/anti-HBc positive patients lacking both HBsAg and anti-HBs were identified as a subgroup of patients with HCV infection and "silent" HBV infection who showed a severe clinical presentation. To better understand the clinical value of "silent" HBV infection in HCV-related liver diseases and its response to α-interferon treatment, two additional studies were carried out and are presented here.

The virological and clinical expression of multiple hepatitis virus infection.

Patients and Methods. - Six hundred and forty-eight consecutive patients with chronic hepatitis from a multiple hepatitis virus infection or a single virus infection first observed at one of the seven participating Liver Units from January 1993 to December 1997 were selected for the study. The patients were divided into two case types: subjects who were HBsAg and anti-HCV positive were defined as Case group B-C, and subjects who were anti-HCV positive/ anti-HBc positive and lacked HBsAg and anti-HBs were defined as Case group b-C. It was planned that for each B-C case two controls should be selected: an HBsAg positive, anti HCV negative control (Control group B); an HBsAg negative, anti HBs negative, anti HBc negative, anti-HCV positive control (Control group C). The control group C was used as the control for Case group b-C. In all Liver Units the number of controls only slightly exceeded the number established.

In HBsAg positive subjects serum HBV-DNA was quantified by a sandwich capture hybridisation assay (Diagene Diagnostics). For Case group b-C patients and HBsAg positive/HBV-DNA negative patients, HBV viremia was also determined by the polymerase chain reaction (PCR) assay. In viremic HBsAg/anti-HBe positive chronic carriers the presence of the pre-core stop codon mutation was identified by a point mutation-specific PCR. This procedure uses primers which have a match or mismatch at

the 3^1-nucleotide for either the wild type or a mutant virus at position 1896 of the HBV genome and allows the specific amplification for both virus types.

HCV-RNA was determined by RT-PCR in the 5^1-noncoding region of the viral genome using a commercial kit (HEPA-Check-C, Nuclear Laser Medicine, Settala, Mi, Italy). Transcutaneous liver biopsy was performed for 356 of the 648 patients, applying a modification of the Menghini technique. Patients who did not undergo liver biopsy were asymptomatic carriers or patients with clinical evidence of cirrhosis or those who refused this procedure.

Results. - A higher prevalence of subjects with serum HBV-DNA was observed in Control Group B (54% of 161 cases) compared to Case Group B-C (35.7% of 84 cases, $p<0.01$), Case sub-group B-C+HDV (12% of 33 cases, $p<0.0001$) and Control sub-group B+HDV (4.5% of 22 cases, $p<0.0001$), indicating an inhibition exerted by HCV and HDV on the replication of HBV. The prevalence of patients showing circulating wild type HBV was similar in Case Group B-C (14.7%) and in Control Group B (17.4%), but the prevalence of patients with a detectable serum HBV e-minus strain was lower in Case Group B-C (10.7 vs. 30.4%; $p<0.0001$). This may suggest that HCV selectively inhibits the replication of the HBV e-minus strain, but exerts no influence on the wild strain.

The patients in Control group C showed a higher prevalence of HCV-RNA positive cases (90.7% of 130 cases) than those in Case Group B-C (65.2% of 69 cases, $p<0.0001$) and those in Case sub-group B-C+HDV (29.6% of 27 cases, $p<0.0001$); instead, no difference was observed between Case group b-C (92% of 110 cases) and Control group C.

The clinical presentation of the liver disease was more severe for patients with a multiple hepatitis virus infection than for those with a single infection, since they showed higher serum aminotransferase values and a more severe liver histology. Indeed, the analysis of the biochemical data obtained at the time of the first observation shows higher serum AST values for Case Group B-C compared to Control groups B ($p<0.05$) and C ($p<0.01$); moreover, patients in Case Group B-C showed higher serum ALT values than those observed for Control group C ($p<0.005$), but similar to those for Control group B. More severe histological liver damage (moderate or severe CAH, or cirrhosis) was more frequent in Case Group B-C (62.9% of 65 cases) than in Control group B (46.7% of 90 cases, $p<0.05$) and Control group C (40.8% of 98 cases, $p<0.005$). In addition, Case group B-C+HDV more frequently than Control group B, Control group C and Control group B+HDV showed severe liver disease (84% of 19 cases vs. 46.7%, 40.8% and 37.5% of 8 cases, respectively; $p<0.005$, $p<0.0005$ and $p<0.05$, respectively).

In this study we tested the hypothesis that anti-HCV positive, anti-HBc positive patients lacking both HBsAg and anti-HBs, namely Case group b-C, might be a group of patients with a “silent” HBV + HCV coinfection. Although there was no inhibition exerted on the HCV genome in Case group b-C, serum HBV-DNA was detected in 40.8% of cases by PCR and the clinical presentation was as severe as in patients with dual HBV-HCV infection. Indeed, compared to patients in Control group C, patients in

Case group b-C showed higher serum aminotransferase values ($p<0.001$) and more frequently had severe histological liver damage (71.1% of 76 cases vs. 40.8% of 98 cases, $p<0.005$).

"Silent" HBV infection and HCV genotypes as predictive factors of a more severe liver disease

Patients and Methods. - In this work we studied the clinical presentation of liver disease in 205 anti-HCV/HCV-RNA positive, HBsAg and anti-HBs negative patients at the time of their first visit to our Liver Unit during the period from January 1993 to December 1997, in relation to the HCV genotype, and to the presence of anti-HBc, as the only marker of a possible "silent" HBV infection. We evaluated the clinical presentation both in patients who had undergone liver biopsy (134 cases) and patients without liver biopsy for whom a "clinical diagnosis" was possible: namely, subjects with no sign or symptom of active liver disease or cirrhosis (16 "asymptomatic carriers"), or patients with unequivocal clinical signs of liver cirrhosis (35 patients with "clinical cirrhosis"). The 20 anti-HCV positive patients with high serum aminotransferase levels who did not undergo liver biopsy and did not show any sign of "clinical cirrhosis" were not considered for clinical evaluation because their clinical diagnosis was uncertain. Two main diagnosis groups were thus established: a group we named "mild liver disease group", composed of 16 "asymptomatic carriers" and 60 patients with histological evidence of minimal CH or mild CH, and a group we named "severe liver disease group", composed of 35 patients with "clinical cirrhosis" and 74 patients with histological evidence of moderate-CH, severe-CH or cirrhosis.

HBV-DNA and HCV-RNA were detected by PCR, as previously described. HCV genotyping was done using Line-Probe-Assay (INNO-LIPA HCV II, Innogenetics, Zwigndrecht, Belgium).

Results. - The prevalence of patients with severe liver disease was only slightly higher for patients with HCV genotype 1 than those with a non-1 HCV genotype (61.3% of 98 cases vs. 52.9% of 70 cases, respectively); this difference is not significant to the statistical analysis and apparently rules out the fact that HCV genotype 1 is more aggressive than the other genotypes. We, instead, observed that patients with a silent HBV infection (33% of whom showed serum HBV-DNA by PCR) more frequently had a severe liver disease than those with no marker of HBV infection (72.7% of 88 cases vs. 46.4% of 97 cases, $p<0.0005$), indicating that a silent HBV infection may unfavourably influence the course of HCV-related chronic hepatitis. As regards the anti-HBc positive group, the prevalences of patients with severe liver disease were similar for the subgroup with HCV genotype 1 and that with a non-1 HCV genotype (68.1% of 47 cases and 73.5% of 34 cases, respectively). Instead, as regards the anti-HBc negative group, the prevalence of patients with severe liver disease was higher in the subgroup with HCV genotype 1 (55% of 51 cases) than those with a non-1 HCV genotype (33.3% of 36 cases, $p<0.05$).

"Silent" HBV infection in chronic hepatitis C predicts a poor response to interferon treatment

Patients and Methods. - We retrospectively assayed for other HBV markers besides HBsAg and for the HCV genotype 132 patients with anti-HCV/HCV-RNA positive, HBsAg negative chronic hepatitis, recruited from January 1995 to December 1996 to a randomised controlled trial on the safety, tolerability and efficacy of three types of alfa Interferon (IFN). The patients had been treated with 3 mega units (MU) IFN three times weekly for one year and subsequently followed-up for at least another year. No patients had been treated with α-IFN or Ribavirin before entering the trial. Forty-nine patients received α-2a-IFN, 42 patients α-2b-IFN and 41 patients lymphoblastoid IFN at a dose of 3 MU subcutaneously three times a week for 48 weeks. Ninety-two patients were infected by HCV genotype 1 and 40 by a non-1 HCV genotype. There was no difference in age, percentage of males, IFN schedule and severity of liver disease between patients with HCV genotype 1 and those with a non-1 genotype.

Forty-one patients were anti-HBs and anti-HBc negative (Group A), 47 anti-HBs and anti-HBc positive (Group B) and 44 anti-HBs negative and anti-HBc positive (Group C). The three groups were similar for age, distribution of HCV genotype and type of IFN administered. Severe liver damage was more frequently observed in patients in Group C than in those in Groups A or B (75% vs. 56% and 61.7%, respectively); this difference, however, is not significant to the statistical analysis.

HBV-DNA, HCV-RNA and HCV genotypes were sought as previously described.

Results. - A sustained response to IFN treatment was observed in 28 patients (21.2%), 23 (17.4%) showed a response to treatment but they relapsed once IFN was discontinued and 81 (61.4%) showed no response. Patients with a non-1 HCV genotype more frequently showed a sustained response than those with HCV genotype 1 (30% vs. 17.4%); this difference, however, was not significant to the statistical analysis.

Patients in group C, 25.5% of whom showed serum HBV-DNA by PCR, had a lower percentage of sustained response than those in Groups A and B, who were all negative for HBV-DNA by PCR (6.8% vs. 26.8%, $p<0.02$ and 29.8%, $p<0.005$ respectively). In particular, the sustained response rate to α-IFN was high in patients with a non-1 HCV genotype both in Group A (42.8%) and Group B (38.4%), intermediate in patients with HCV genotype 1 both in group A (21.4%) and B (24.2%), and very low in patients in Group C, irrespective of the HCV genotype (6.4% for HCV genotype 1 and 7.6% for a non-1 HCV genotype).

Conclusions

Our studies make a contribution to the knowledge on multiple hepatitis virus infection and on the clinical impact of "silent" HBV infection in HCV-related chronic hepatitis.

Our data demonstrate that the interaction between HBV, HDV and HCV is characterised by a reciprocal inhibition of the respective viral genome. Namely, we observed an inhibitory effect of HCV on HBV replication and confirmed the well-known depression exerted by HDV on HBV genome. The inhibitory activity of HCV on HBV replication has been previously hypothesised on the bases of few studies on small numbers of patients or studies performed several years ago when specific technology was not available. In a follow-up study of 6 years Sheen demonstrated a rate of HBsAg clearance of 2.5 times higher in HBsAg/anti-HCV positive cases than in those with HBV infection alone (4); Pontisso (2) observed a reciprocal inhibitory effect between HBV and HCV in small groups of patients, and Shih (3) described a reduced expression of HBV transcripts in the presence of HCV structural genes in HuH-7 cells.

Our data demonstrate that HCV depresses the HBV "e-minus" strain, but does not affect the HBV wild strain. As far as we know no other data on this have been published to date, except for the study of Yeh (8), who reported the absence of the pre-core stop mutant HBV in all 7 chronic hepatitis patients with HBV and HCV infection he studied. We also demonstrated that HBV infection inhibits HCV replication; this phenomenon is more evident when HDV infection is associated. Previous studies have investigated this phenomenon but the results are discordant: Pontisso (2) found a low prevalence of serum HCV-RNA (56%) in 25 chronic hepatitis patients with HBV-HCV coinfection, but Zarski (6) observed detectable HCV-RNA in 82.6% of the 23 patients with HBV and HCV coinfection and in 88.4% of the 69 patients with HCV infection alone.

Our data also indicate that the clinical presentation of the liver disease is more severe in patients with a multiple hepatitis virus infection than in those with a single infection. This observation confirms the data previously published by us and by other authors (2, 5, 6).

We also demonstrated that anti-HCV positive/anti-HBc positive patients lacking both HBsAg and anti-HBs may be a subgroup with HCV infection and "silent" HBV coinfection, who frequently show severe liver damage and a poor response to IFN treatment (see below).

Our findings suggest that, at least in our geographical area, the severity of HCV-related liver disease may depend more on the presence of a "silent" HBV infection than on the HCV genotype. Whether genotype 1 may be responsible for a more severe course of liver disease is still controversial. In some studies carried out in Italy, Japan and France, HCV genotype 1b was found to be associated to a more advanced stage of liver disease (9, 10), whereas more recent studies carried out in the same countries and in USA and Benelux did not confirm this association (11-13). As regards our anti-HBc negative patients, those with genotype 1 more frequently than those with a non-1 genotype showed a severe liver disease, the difference being significant to the statistical analysis ($p<0.05$). This may indicate that genotype 1 may be moderately more aggressive in the pathogenesis of liver diseases than the other genotypes, but the presence of a silent HBV infection or other unknown factors may obscure this.

Consistent with our data are the observations of Koike (10) who found anti-HBc more frequently in HCV infected patients with hepatocellular carcinoma (HCC) (69.6%) than in those with chronic hepatitis without HCC (38.2%). Other authors

reported that HBsAg sero-negative patients with chronic hepatitis with or without HCC had clonally integrated HBV-DNA sequences in the liver tissues, regardless of the anti-HBc status (11-12). Recently, Cacciola et al. reported that among anti-HCV positive/HBsAg negative patients, HBV sequences were found in the liver or serum in 46% of 100 anti-HBc patients and in 20% of 100 patients with no serological markers of a past HBV infection ($p<0.001$). He also found liver cirrhosis in 33% of 66 cases with HBV-DNA sequences and in 19.4% of 134 cases without ($p=0.04$) (17).

Our data also demonstrate that patients with a "silent" HBV infection, whether of HCV genotype 1 or non-1, very rarely show a sustained response to alpha-IFN given at a dose of 3 MU three times weekly. This IFN schedule, however, is effective in about 22% of patients with genotype 1 and with no silent HBV infection. Instead, the 3 MU schedule is effective in about 40% of patients with a non-1 HCV genotype and with no silent HBV infection.

These data are supported by Cacciola who in a study on 83 patients with HCV-related chronic hepatitis treated with α-IFN found HBV sequences in 47.2% of the 55 patients for whom IFN was ineffective, and in 25% of the 28 for whom IFN was effective (17). The data were also supported by Mason who found an ongoing low-level HBV replication in the hepatocytes of seven patients who had cleared HBsAg from serum, had evidence of either HBV transcription or an intact direct repeat region of HBV-DNA in the liver, but only one of whom had HBV-DNA in serum by PCR (18).

In conclusion, the presence of HCV genotype 1 or a silent HBV infection seem to be prognostic factors of an unfavourable response to alpha-IFN treatment and the presence of both factors seems to nullify the efficacy of alpha-IFN treatment.

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NEUTRALIZING ANTIBODIES AND CELLULAR IMMUNITY AGAINST HCV: THE MODEL OF LIVER TRANSPLANT PATIENT
Coordinator: Prof. Erica VILLA

Progress Report

NEUTRALIZING ANTIBODIES AND CELLULAR IMMUNITY AGAINST HCV: AN INVESTIGATION IN THE LIVER TRANSPLANT PATIENT MODEL

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Summary

Hepatitis C virus infection (HCV) is a common event in patients undergoing Orthotopic Liver Transplantation (OLT). Recurrence of infection is very high in patients who were anti-HCV-positive before OLT while variable percentage are reported for patients who were anti-HCV-negative. We have previously reported that some pre-OLT anti-HCV-negative patients became HCV RNA positive after OLT but, they did not develop antibodies against HCV but were able to clear HCV infection, becoming persistently HCV RNA negative. In the attempt to understand how it is possible to clear the virus despite heavy immunosuppression, we investigated using the Phage Display Technology whether it was possible to evidence antibodies (which could therefore be considered as neutralising) other than those evidenced with routine tests. As additional support to the hypothesis of immunising contact with HCV, also cellular immunity to HCV has been investigated.

A random peptide library displaying on phage all the epitopes (phagotopes) has been prepared. Affinity selected phagotopes with human sera were immunoscreened using anti-HCV-positive sera. Immunopositive phagotopes were assayed using large panels of anti-HCV-negative and positive sera, then selected as HCV-specific on basis of common reactivities shown with anti-HCV-positive sera. Pre- and post-transplant sera of our patients have been then assayed with characterised and mapped phagotopes HCV-specific in the attempt to discover unknown antibody reactivities. On the whole we have tested 36 of 70 sera pre- and postOLT of 6 patients, 4 of whom presented with the above described phenomenon, one served as comparison, being a pre-OLT anti-HCV-positive subject with recurrent infection and one pre-OLT anti-HCV-negative became persistently HCV infected. Our results show that before OLT there are no

relevant serological reactivities; after OLT it has been possible to evidence multiple heterogeneous reactivities (especially against NS4b) whose specificity against HCV has been confirmed. Preliminary investigation of CD4 and CD8 reactivities have shown that two of the four patients having antibody reactivities with mimotopes have also demonstrable specific sensitisation to HCV.
Key words: *HCV infection, liver transplant, Phage Display Technology.*

Background

HCV infection occur more often after liver transplant (LT) especially in patients who were already HCV-positive before LT (1, 2). In those who were anti-HCV negative before LT, HCV infection occur in variable percentage (1, 3). Published data refer usually to short periods of follow-up post-transplant while long follow-up studies are lacking.

Twenty-two patients were followed-up in our Unit for more than 3 years after LT with routine serological tests (Matrix HCV EIA -Abbott), ELISA II e III generation (Ortho), RIBA (Ortho) and molecular-biological tests (RT/PCR of 5' untranslated region) (4), to investigate HCV infection both before and after LT to evaluate the role of HCV infection and of different HCV genotypes (determined by the technique set up by Okamoto et al.) (5) in determining the recurrence rate and the severity of hepatic disease in the post-transplant period. It was clear from our study that HCV infection, in patients who were HCV-positive before LT, recurred in the totality of patients and was followed by development of severe hepatic disease, especially in patients characterised by genotype 1b. Hepatic disease was much less severe in those patients in whom genotypes 1a and 2b were present (3).

Some of the patients who were anti-HCV negative before LT developed HCV infection in the absence of any antibody marker and with the onset of HCV RNA as the only marker of infection. The surprising data was that, despite the lack of any demonstrable antibody (at least of those who are routinely tested) and despite the heavy immunosuppression, some of these patients were able to clear HCV infection. The hypothesis under investigation has been therefore that the subjects could efficiently react against HCV infection despite the unfavourable immunological conditions of the immediate post-transplant period. This could occur either through a cell-mediated or a humoral response which however were not identifiable through routine tests. The study in the liver transplant patient has been therefore continued and expanded using a new approach for the study of immunological epitopes which are not recognised by standard methods.

Methods

To study the long term effects of HCV infection in liver transplant patients, different molecular biological and immunological techniques have been employed.

Molecular-biological methods. – HCV RNA testing on pre- and post-transplant sera, on lymphocytes and post-transplant hepatic biopsies. HCV RNA has been evaluated by one step RT/PCR using primers localised in the 5' UTR region (3). PCR products have been then elecrophoresed in a 1.8% agarose gel, hybridised with a 5'-labelled ^{32}P probe as already described (4). HCV genotypes have been studied by nested RT/PCR with the method by Okamoto (6) using primers localised in the core region in the first PCR and in the second PCR a universal primer and a mix of type-specific different antisense primers. Genotype are identified on the basis of the fragment length after electrophoresis and classified as 1a, 1b, 2a 2b etc. accordingly.

As the main pitfall of this technique, for its high level of sensitivity and possible occurrence of carry over, can be the high rate of false positive results, it has been necessary to introduce a substantial number of precautions: high number of positive and negative controls; decontamination measures for lab surfaces; physical separation of rooms where the different phases of the procedure occur. This has been a constant worry, as the starting point of the study was the demonstration of a labile viremia in a series of immunosuppressed patients. For this reason, nested PCR has not been used (for its high rate of possible false positive results) and instead one step PCR plus hybridisation has bee utilised, which gives both high sensitivity and preserved specificity.

Non routine methods to evidence antibody reactivities. – 1) Set up of a Phage library. - This is composed by random nonamers expressed on the surface of filamenous phages as fusion proteins at the N-terminal expremity of the major surface protein pVIII (pVIII9aa). This technology permits to manipulate the genome of the phage to obtain phages each expressing a different epitope on its surface (phagotope). Phagotopes can be recognised by corresponding antibodies. Recently, a specific technique has been set up to select phagotopes able to mimic abnormal antigens randomly expressed on phages as sources of ligand and human sera as ligates (5,6). Phagotopes have been selected on the basis of affinity for human sera. Selected phagotopes have undergone immunological screening using HCV-positive sea.

2) ELISA on pre- and post-transplant sera of antiHCV-positive subjects with HCV-specific epitopexpressed on phagi (Phage Display Technology). - This technique requires the coating of multiwell plates with monoclonal antibodies against the protein III of phagus fl-11,1 then followed by anti-HCV specific phagotopes so that the phagotope can be bound to the well. In the meantime, the sera under test are pre-incubated with phagic supernatant to eliminate aspecific reactivities. Subsequently, the reaction follows the rule of a classical ELISA.

3) Lymphomononuclear cells culture. - These techniques consist in the separation of lymphomononuclear cells (PBMCs) from peripheral blood and the setting up in

cultures for at least 7 days in a medium enriched with Pokeweed Mitogen (PWM); after 7 days the presence of antibodies against HCV will be tested in the supernatant in ELISA and Recombinant Immuno Blotting (RIBA). It has already been reported that other viruses (i.e. HIV) PWM stimulation may render evident an "in vitro" production otherwise non evidenced as they may be related with an latent infection. In those subjects in whom it will impossible to evidence antibodies with the routine technique, sera of patients have been assayed by the method of random peptide expressed in phagus (Phage Display Technology)(5).

4) Study of the proliferative response of T cells. - Both CD4 and CD8 response has been studied.

CD4 response: after purification of PBMCs from heparinised blood on Ficoll-Hypaque gradient, cells were put in culture and pre-incubated for 4-6 hours with HCV-specific recombinant proteins expressed in yeast as fusion proteins with human SOD. As control for unspecific proliferation, the yeast extract and the SOD protein alone were used. After washing, PBMCs were cultured for 7 days; 15 hours before terminating growth, ^{3}H-tymidine was added: the proliferation index is obtained by the rate of cpms in presence and in absence of antigen. Together with the stimulation with recombinant protein, also overlapping HCV-specific peptides have been used: these stimulate the activation of CD4+ specific clones.

CD8 response: after purification of PBMCs are put in culture in presence of HCV-specific peptides, selected on the basis of HLA-I of the patient (usually, HLA-I A2 has two anchors to which peptide aminoacids bind: leucin in position 2 and valine in position 9 or 10). Stimulation continues for 72 hours in presence of IL2. After wasing, PBMCs are put again in cultures for 7 days, thereafter cultures are split in two. EBV+ HLA-I A2 cell lines, labelled with ^{51}Cr, are then added, half of these cells being prepulsed with the same HCV-specific peptides and half not. The recognition by patients' CD8+ PBMCs of the EBV cells presenting the same antigen determines a significant higher release of ^{51}Cr.

Results and comment

Two of the six OLT patients, who were anti-HCV-negative before OLT, had persistently a viremia below the level of sensitivity of the method. Four of six liver transplant patients became HCV RNA positive after OLT, three transiently and one persistently. None of the patients had antibody demonstrable by routine methods neither before nor after OLT. However, it was possible to reveal, with the mimotopes technique, antibody reactivities against NS4b. This demonstrates the significant sensitivity of the technique in comparison with the routine technique: it has been not only possible to evidence unknown reactivities but also to identify against which viral subregions these reactivities are directed. This can be of notable importance for understanding the mechanisms of viral elimination. A screening on a larger number of OLT patients, conducted with a larger number of mimotopes, has confirmed the involvement of NS4b region. This involvement is somewhat surprising as NS4b region

is not hypothetically involved in viral clearance; the involvement of epitopes of the envelope region would be more logical. A deeper insight in the function of the NS4b region would help explaining the role in these patients.

In the attempt to elucidate the meaning of the results, we have investigated with the same test a larger number of sera from other 10 transplanted patients, collected before and after 1, 3, 6, 8, 12, 24 e 36 months. HCV RNA was negative in all patients at all time points studied: these data suggest that the blood screening has certainly improved and confirm that the somewhat "vanishing" viremia observed in the previous four patients was not a false positive result.

To have major support to the fact that these patients had actually had a transient HCV infection, we have also investigated the role of cellular immunity. Preliminary investigation of CD4 and CD8 reactivities have shown that two of the four patients having antibody reactivities with mimotopes have also demonstrable specific sensitisation to HCV.

Taking all these data together, it can be concluded that:

1. these patients have in fact experience a HCV infection which has been cleared despite heavy immunosuppression.
2. Phage Display Technology has been able to evidence antibodies reactivities not otherwise demonstrable with routine techniques and
3. the specificity of these reactivities for HCV is supported by the demonstration of specific sensitisation of T cells for HCV.

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PUBLICATIONS

Books, publications and abstracts of congress proceedings of the First research project on Viral Hepatitis: Etiopathogenesis and diagnosis (1997-1999)

SUBPROJECT
Structural and biological characterisation of recently identified viruses and viral variants

Books

ICARDI, G.C., BONANNI, P., WICKS, R., FARALDI, L., ROCCATAGLIATA, A., SANTO, M., ORIONE, L., DE CONCA, V., CROVARI, P. Characterization of hepatitis C virus genotypes in Italian subjects at different risk of infection. In: *Viral Hepatitis and Liver Disease*, Rizzetto M., Purcell R.H., Gerin J.L., Verme G. (Eds.), Edizioni Minerva Medica, Turin (Italy) 1997, p. 598-600.

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SUBPROJECT
Hepatitis viral infection markers in particular categories of patient

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