

# Carcinogen-modified DNA and specific humoral immunity toward carcinogen-DNA adducts. A review

Alessandra Verdina

*Dipartimento Sviluppo e Programmi Terapeutici, Centro Ricerche Sperimentali, Istituto Nazionale Tumori Regina Elena, Rome, Italy*

**Summary.** Carcinogenesis is a complex and multistep process starting from initiation to tumor progression. Damage to DNA, induced by the covalent binding of chemical carcinogens on critical DNA segments, reflects exposure and is directly related to tumor formation. For this reason it's very important detect and quantify DNA-adducts by using highly sensitive methods. During the last 30 years sophisticated methods have been developed, in particular immunoassays that have a widespread application in monitoring animal models and human tissues for evidence of carcinogen exposure. In this paper we describe the work done in our laboratory, from the production of antibodies specific for two different carcinogens, 2-Acetylaminofluorene and Benzo[a]pyrene, to their application in chemical carcinogenesis studies. Moreover, we describe as immunological methods can be used for detecting the presence of specific antibodies in sera of exposed individuals.

**Key words:** DNA adducts, carcinogen, humoral immunity, exposure marker, host factor.

**Riassunto** (*DNA modificato da cancerogeni e specifica immunità umorale verso gli addotti DNA-cancerogeno. Una review*). La cancerogenesi è un complesso processo a più stadi che vanno dalla iniziazione alla progressione del tumore. Il danno indotto dal legame covalente di cancerogeni chimici su segmenti critici del DNA riflette l'esposizione ed è direttamente relazionato alla formazione del tumore. Per questa ragione è molto importante determinare e quantificare gli addotti al DNA usando metodi altamente sensibili. Durante gli ultimi 30 anni sono stati sviluppati metodi sofisticati, in particolare metodi immunologici che hanno un vasto spettro di applicazione per il monitoraggio di modelli animali e tessuti umani al fine di evidenziare una esposizione a sostanze cancerogene. In questo articolo viene descritto il lavoro svolto nel mio laboratorio nell'ambito della cancerogenesi chimica, dalla produzione di anticorpi specifici per due differenti cancerogeni, 2-Acetilaminofluorene e Benzo[a]pirene, alla loro applicazione in studi di cancerogenesi. Inoltre viene descritto l'uso dei metodi immunologici per la determinazione della presenza di anticorpi specifici in sieri di individui esposti.

**Parole chiave:** addotti al DNA, cancerogeno, immunità umorale, marker di esposizione, fattori dell'ospite.

## INTRODUCTION

The first event of chemical carcinogenesis is the covalent binding of electrophilic molecules (direct-acting carcinogens or metabolically generated reactive intermediates) on critical DNA segments. The disruption of base-pairing due to the binding of an electrophile will be locked in as a point mutation or frameshift mutation if the cell replicates before DNA repair can take place. Thus, the higher the rate of cell proliferation the greater the likelihood that a molecular lesion in DNA will be locked in as a mutation. The target of electrophile-produced initiation may be a protooncogene (which thereby becomes activated to an oncogene) or a tumor-suppressor gene (which would become non-functional as a suppressor).

In the last time considerable advances have been made in the identification and detection of carcinogen DNA adducts that require highly sensitive analytical

techniques [1-6]. The most widely used methods include electrochemical detection, mass spectrometry, fluorescence and phosphorescence spectroscopy, immunoassays, immunohistochemistry and (32)P-post-labeling [7-10]. Detection limits for quantitative assays are typically in the range of 1 adduct in 107 or 109 nucleotides [11, 12]. However, accelerator mass spectrometry, which is highly sophisticated but less accessible, has a detection limit of approximately 1 adduct in 1012 nucleotides [11]. The sensitivity of radiochromatographic methods is limited by the specific radioactivity of the respective carcinogens and by the relatively large amounts of DNA required for analysis. Furthermore, their application is restricted to experiments with radiolabelled carcinogens synthesized in laboratory.

The development of immunoassays and 32P-postlabelling methods for determination of carcinogen-DNA

adducts has widespread application in investigating chronic carcinogen administration in animal models and in monitoring human tissues for evidence of carcinogen exposure [13-15]. These methods prove to be sufficiently sensitive to enable the detection and quantitation of DNA adducts without the requirement of radiolabelled carcinogens. Therefore, they could be applied directly to DNA from animals or humans exposed.

The postlabelling technique is more sensitive but does not discriminate between different types of adducted xenobiotics. The immunochemical assays offer great specificity but a lower sensitivity, even if can detect low amounts of adducts (1 adduct in 108 nucleotides) [16].

However, immunological methods have certain advantages over other techniques: they are rapid, highly reproducible, and cheap and the antibodies are specific for a particular three-dimensional structure and can be used to probe the conformation of unknown adducts on DNA. Moreover, carcinogen-DNA adduct antibodies do not cross-react with structurally dissimilar adducts of the same carcinogen, the carcinogen alone, unmodified nucleosides, or unmodified DNA.

Immunological methods for determination of adducted carcinogens rests upon eliciting and characterizing polyclonal and monoclonal antisera against these haptens. The use of such antisera has widespread application in investigating chronic carcinogen administration in animal models and in monitoring human tissues for evidence of carcinogen exposure.

Immunoassays can be used also for detection of specific antibodies to the DNA-adducts in the serum of individuals exposed [17, 18].

In this work the results of our previous studies are reviewed in order to describe the properties of the antibodies produced in our laboratory toward 2-Acetylaminofluorene (2-AAF) and Benzo[a]pyrene (BaP), the techniques applied for their development and characterization, the immunoassay optimized procedures, and the application of these methods in *in vivo* carcinogenesis studies.

## MATERIALS AND METHODS

### Preparation of carcinogen-carrier conjugates

Gelatin was chosen as the carrier molecule because of its low immunogenicity. The carcinogen-protein conjugates were prepared by reacting the carcinogen with gelatin "*in vitro*" as described in Verdina *et al.* [25] for 2-AAF and in Bucci *et al.* [24] for BaP.

Adducted DNA was obtained by reacting calf thymus DNA with carcinogens, as described in Bucci *et al.* [24] for BaP and in Verdina *et al.* [25] for 2-AAF.

### Production of specific antisera and purification of specific IgG

Two different polyclonal antisera were produced in rabbit toward 2-AAF and BaP conjugated to gelatin [24, 25]. A BaP specific polyclonal antiserum was produced and characterized also by using as antigen benzo[a]pyrene-diol-epoxide modified DNA as described in Bucci *et al.* [24].

Total IgG were isolated from immune sera by protein A Superose chromatography as described in Bucci *et al.* [24].

The specific antibodies were isolated from the immune sera or total IgG by immunoaffinity chromatography as described in Citro *et al.* [23].

### Animals

Male Swiss mice, provided by Charles River Italy (Calco, Como, Italy), were used. The animals, aged 4 weeks at the beginning of treatment, were maintained on a balanced standard chow (Mucedola, Milan, Italy) and tap water *ad libitum*, housed in polycarbonate cages at constant temperature and humidity with a light/dark period of 12/12 h. All animals were acclimatized for one week before random allocation to experimental groups.

### Immunization schedules and carcinogen treatments

Immunization schedule and carcinogens treatment are described in Verdina *et al.* [25] as regard to 2-AAF and in Galati *et al.* [26] as regard to BaP.

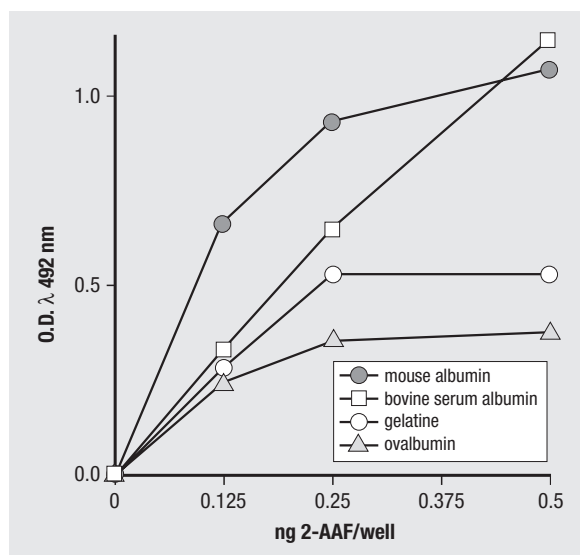
### Detection of carcinogen-DNA adducts

Carcinogen-DNA adducts were quantified by ELISA as described in previous works [23-25].

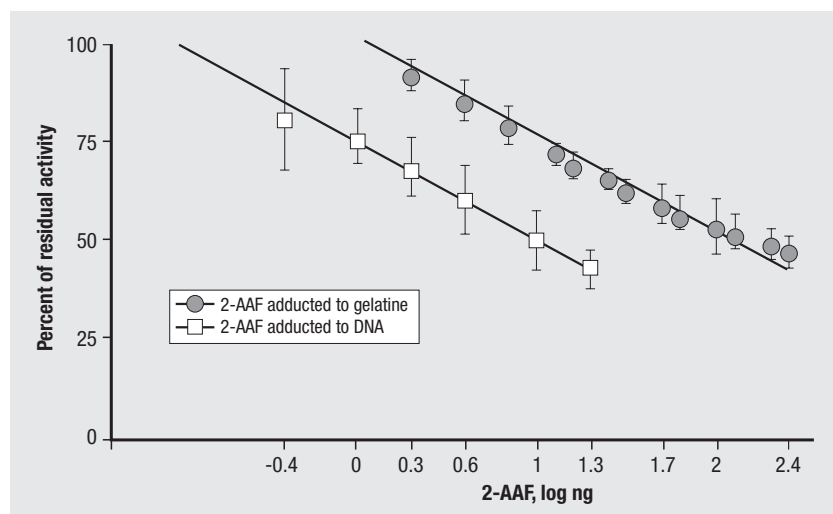
DNA was extracted by liver and spleen of mice previously treated with carcinogens as described in Verdina *et al.* and in Galati *et al.* [25, 26].

### Study population and blood sampling

194 Rome policemen (142 males and 52 females) were selected for the study. The group included 134 individuals (69%) employed in traffic control and 60 (31%) belonging to the administrative division.



**Fig. 1** | Reactivity of anti-2-AAF rabbit antiserum against 2-AAF adducted to different carrier proteins. Carrier proteins alone □—□ gave in all cases values of <0.01 O.D. The figure shows averaged values of triplicate determinations. In all cases, standard errors were <10% of the mean.



**Fig. 2** | Reactivity of rabbit polyclonal antiserum toward 2-AAF added to gelatine and DNA as determined by competitive ELISA. Plates were coated with 0.25 ng 2-AAF-gelatin conjugate/well. The level of modification of added DNA was 350 pg 2-AAF/ $\mu$ g DNA. The antiserum was diluted 1:5000.

The population of aluminium industry workers included 105 male individuals.

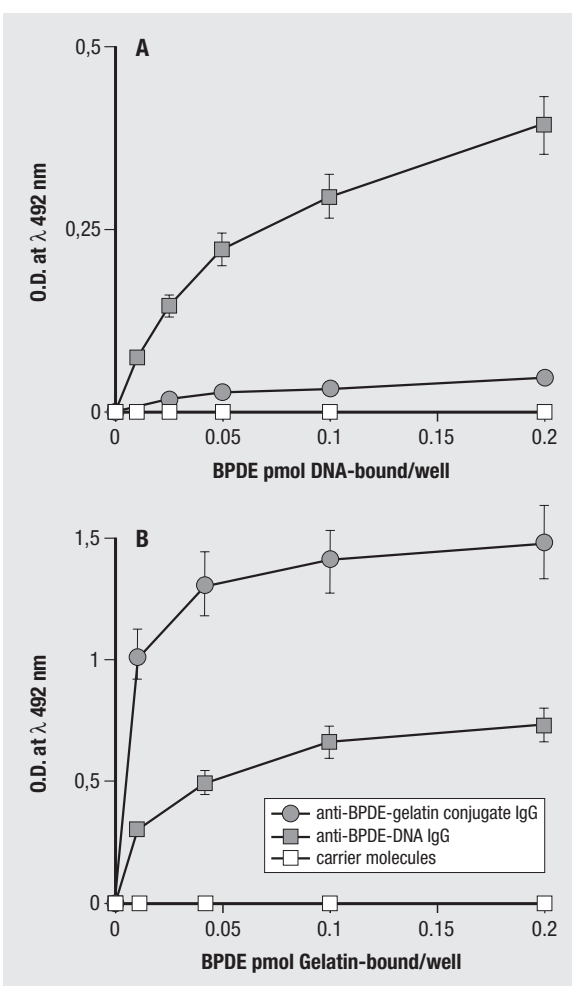
From all subjects, a sample of about 10 ml of peripheral blood was obtained by venipuncture. Total IgG were isolated from sera by precipitation with ammonium sulphate (40% of saturation).

Informed consent was obtained from all participants to the study. In compliance with the law about privacy, all samples were coded and treated in anonymous and collective manner.

## RESULTS AND DISCUSSION

In the past years, specific polyclonal rabbit antibodies toward 2-AAF and BaP were produced and characterized in our laboratory, and the optimal conditions for their detection (direct and competitive ELISA) were searched.

The anti 2-AAF antiserum was able to recognize the carcinogen adducted to different proteins (gelatin, bovine, egg and mouse albumin), and no reactivity was observed with the carrier proteins alone (*Figure 1*). In particular, no reactivity was observed against gelatin alone that was used as carrier in antiserum production since it has no antigenic activity (*Figure 1*). A comparison of the reactivity of the serum toward DNA and protein adducts, estimated by competitive ELISA, demonstrated similar activities with no reaction with carriers alone (*Figure 2*). This was widely studied also by using BaP as antigen. In this case we have produced two different antisera using BPDE covalently linked to two different carriers (DNA and gelatin). As *Figure 3* shows, the IgGs purified from both sera were able to detect adducts in modified DNA (A) and gelatin (B), even if with a different efficiency. In both cases no reaction was observed with carrier molecules alone. For both carcinogens optimal conditions for detecting adducted DNA were determined for direct and competitive ELISA and sensitivity of two methods was valued. Competitive ELISA assay was found to be more sensitive than direct method for the quantitative limitation of coating of the DNA in well in direct method.



**Fig. 3** | Reactivity of polyclonal rabbit anti-BPDE-gelatin conjugate IgG and anti-BPDE-DNA IgG toward BPDE adducted to DNA (5 pmol BPDE/ $\mu$ g DNA) (A) and gelatine (B) as determined by direct ELISA.

In both cases, carrier molecules alone gave values of  $<0.01$  OD (Optical Density). The average values of triplicate determinations are reported. In all the cases, standard errors were  $<10\%$ .

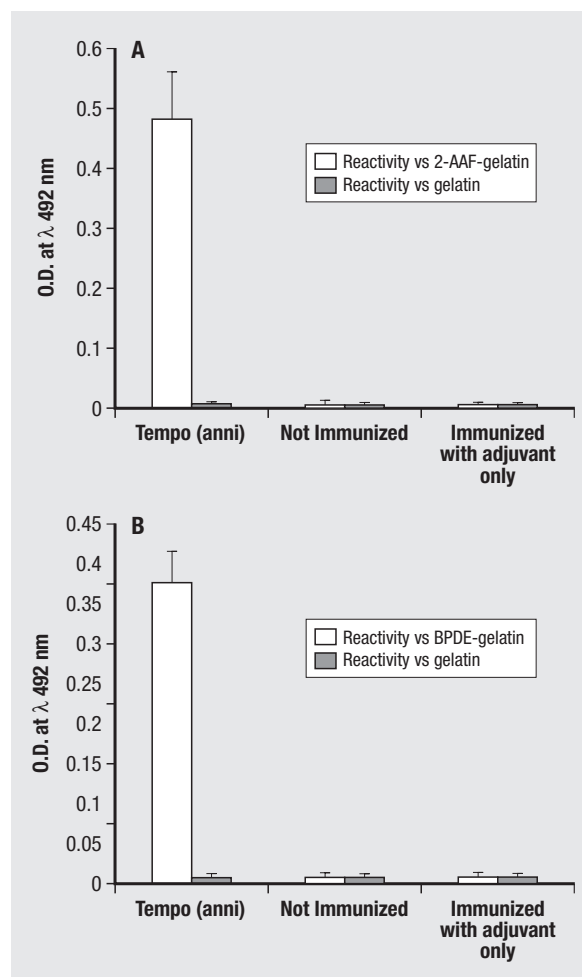
**Table 1** | 2-AAF-DNA adducts in liver and spleen of non-immunized and immunized Swiss mice after 4 weeks dietary exposure to the carcinogen

Treatment (ppm)	DNA-adducts (fmol 2-AAF/ $\mu$ gDNA) [mean $\pm$ SE (n)]	
	Non-immunized	Immunized
Liver		
50 p.p.m.	101 $\pm$ 9 (14)	47 $\pm$ 13 (9)
150 p.p.m.	245 $\pm$ 26 (14)	62 $\pm$ 13 (12)
Spleen		
50 p.p.m.	109 $\pm$ 12 (12)	51 $\pm$ 8 (16)
150 p.p.m.	128 $\pm$ 10 (12)	53 $\pm$ 10 (14)

Our specific antisera and competitive ELISA were used for studying DNA adducts formation in *in vivo* systems. In particular, anti 2-AAF antiserum was used for the analysis of liver and spleen 2-AAF-DNA adducts in mice treated with 2-AAF. The analysis of DNA adducts revealed high levels of adducts in all treated animals: liver DNA adducts were correlated with the administered dose, whereas no relationship with dose was observed for spleen DNA adducts (Table 1). The different responsiveness of liver and spleen to 2-AAF exposure can be explained by differences in cell turnover, as well as saturation of DNA repair and metabolic activities.

Similar results were obtained for BaP: in this case, by using anti BPDE-DNA IgG, the adduct levels in liver of mice treated with BaP were detected. Also in this case, high levels of adducts were demonstrated in all treated animals, with a partial quantitative relationship with respect to the administered dose (Table 2).

By direct ELISA we demonstrated the induction of a specific humoral immunity toward both 2-AAF and BaP in all mice previously immunized with 2-AAF-gelatin or BaP-gelatin conjugate respectively (Figure 3 A and B). This demonstrates that the repeated carcinogen exposure may result in the induction of a specific immune response toward the carcinogen itself: this phenomenon, which has been reported in both human population and experimental animals [27-29], is likely to be related to the persistence of macromolecular



**Fig. 4** | A: reactivity of mouse sera against 2-AAF-gelatin conjugate as determined by ELISA. Plates were coated with 0.5 ng/well conjugated 2-AAF and sera were diluted 1:1000. The mean  $\pm$  SE of the results obtained with 37 immunized mice and the response obtained with pooled sera from 20 non-immunized and 20 adjuvant-pretreated mice are shown. B: reactivity of mouse sera against BPDE-gelatin conjugate as determined by ELISA. Plates were coated with 0.6 ng/well conjugated BPDE and sera were diluted 1:100. The mean  $\pm$  SE of the results obtained with 40 immunized mice and the response obtained with pooled sera from 40 non-immunized and 10 adjuvant-pretreated mice are shown.

**Table 2** | BPDE-DNA adducts in liver of non-immunized and immunized Swiss mice 7 days after i.p. treatment with different doses of B[a]P

Treatment mg B[a]P/Kg body weight	DNA-adducts (fmol B[a]P/ $\mu$ gDNA) [mean $\pm$ SE (n)]	
	Non-immunized	Immunized
Liver		
25	1.4 $\pm$ 0.25 (11)	0.86 $\pm$ 0.20 (11)
50	1.6 $\pm$ 0.32 (11)	0.57 $\pm$ 0.32 (11)
100	1.7 $\pm$ 0.18 (11)	1.10 $\pm$ 0.21 (11)
200	4.4 $\pm$ 0.36 (11)	2.82 $\pm$ 0.46 (11)

carcinogen adducts in the bloodstream of exposed subjects. Most carcinogens, in fact, forms stable covalent adducts with blood proteins, either directly or following metabolic activation. These adducts, not processed by any specific repair system, are subject to accumulation and slow elimination. In chronically exposed individuals, this results in the continuous presence of the haptenic carcinogen linked to a macromolecular support, thus fulfilling the conditions for the induction of a specific humoral response.

The presence of specific antibodies may conceivably affect the distribution and toxicokinetics of the carcinogen in different organs and tissues, possibly exerting

**Table 3** | A) Distribution of serum samples positive for anti BPDE-DNA antibodies among Rome policemen on the basis of job category and PAH exposure. B) Distribution of serum samples positive for anti BPDE-DNA antibodies among aluminium industry workers on the basis of job category and PAH exposure

A	Anti BPDE-DNA antibodies			
	Negative	Positive	Total	%
Administrative personnel (low exposure)	59	1	60	1.7
Traffic controllers (high exposure)	124	10	134	7.5
Total	183	11	194	5.7

B	Anti BPDE-DNA antibodies			
	Negative	Positive	Total	%
Not exposed	13	2	15	13.3
Electrolysis (low exposure)	14	1	15	6.7
Ovens, Rodding (middle exposure)	45	9	54	16.7
Mixing (high exposure)	19	2	21	9.5
Total	91	14	105	13.3

a modulatory effect on the activity of the carcinogen. To provide insight on this phenomenon, we studied the modulation exerted by immune status on genotoxic effects of the carcinogens, administering 2-AAF or BaP in immunized and not immunized mice. In all cases the immunological response elicited was able to influence the extent of covalent binding of the carcinogen. In fact, adduct levels were consistently reduced in all immunized mice with respect to control groups (*Table 1* and *Table 2*). Our data suggest that the specific immune response elicited by carcinogen-conjugate exposure can modulate its genotoxicity *in vivo*. This effect could be related to the scavenging action of antibodies which could lower the availability of the carcinogen to metabolic activation and subsequent DNA interaction. On the bases of these results we can consider the specific immune response as a newly identified host factor capable of modulating the effects induced by carcinogen exposure.

The induction of antibodies toward BPDE-modified DNA has been studied also in sera from humans exposed to polycyclic aromatic hydrocarbons (PAH). The objective of the study was to assess whether low, environmental PAH concentrations could induce the formation of anti-BaP antibodies since previous investigations demonstrated the occurrence of antibodies directed against BaP-DNA adducts in sera from human occupationally exposed to high levels of PAH [19], as well as in the urban population [20, 21]. These antibodies are likely to be related to the persistence of macromolecular carcinogen adducts in the bloodstream and, for this reason are considered as a marker of a previous exposure.

In our study we have screened sera from two populations of individuals occupationally exposed to low PAH concentrations: Rome traffic policemen and workers of an aluminium production plant. In both populations different groups were identified on the bases of exposure levels (*Table 4*). In policemen, the overall

percentage of subjects with anti BPDE-DNA antibodies was 5.7%. The incidence of subjects positive for BPDE-DNA antibodies was relatively higher within high exposure group (traffic controllers). Among 105 aluminium workers screened, fourteen subjects positive for the presence of anti BPDE-DNA antibodies were identified (13.3%). Positive individuals were homogeneously distributed in different exposure and control groups with no significant difference between observed and expected values. These data did not highlight a clear association between PAH exposure and presence of anti BPDE-DNA antibodies, also if the small prevalence of positives within people exposed to traffic fumes may suggest that the exposure to environmental PAH can contribute to some extent to the formation of anti-BP adducted antibodies.

## CONCLUSIONS

In this review is described the production of specific polyclonal antibodies directed against two small carcinogenic molecules, 2-AAF and BaP and their application in chemical carcinogenesis studies. From all our data we can draw the following conclusions:

- the exposure of mice to 2-AAF and BaP resulted in the production of DNA adducts in liver and spleen (for 2-AAF) of all treated animals;
- repeated intraperitoneal injections of 2-AAF or BaP conjugated to high molecular weight carriers effectively elicited the production of specific IgG in all treated mice, demonstrating that the repeated carcinogen exposure may result in the induction of a specific immune response toward the carcinogen itself;
- the specific humoral immunological response elicited in mice by 2-AAF or BaP exposure was able to reduce consistently the adduct levels in all immunized animals respect to not immunized. For this reason, it may represent another important host factor, beyond



variations in DNA repair and metabolism, which may modulate the genotoxic effect induced by the carcinogen itself;

- a weak association has been observed between chronic exposure to PAH and formation of anti-BPDE antibodies in occupationally exposed individuals. However it

seems confirm the previous findings on the correlation between carcinogen exposure and specific immunological response.

Submitted on invitation.

Accepted on 10 April 2006.

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