Quantitation of Exposure to Benzo[a]pyrene with Monoclonal Antibodies

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It is now possible to quantitate carcinogen adducts on DNA by highly sensitive immunoassays. These techniques are particularly useful for screening human populations for exposure to potential environmental carcinogens. We have developed a panel of monoclonal antibodies that react with benzo(a)pyrene (BP) modified DNA to be used in an enzyme linked immunoassay (ELISA) to quantitate adduct levels of both human and animal samples. BALBc/Cr mice were immunized with either DNA modified by 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-I-DNA) complexed electrostatically to methylated bovine serum albumin or with BPDE-I-modified guanosine conjugated with bovine serum albumin (BPDE-I-G-BSA). Four stable clones were produced from the spleen cells of animals immunized with BPDE-I-DNA and one from BPDE-I-G-BSA immunized animals. All antibodies were shown to be highly specific for BPDE-I-DNA and did not crossreact with nonmodified DNA or with N-2-acetylaminofluorene or 1-aminopyrene modified DNA. The antibodies differed in their sensitivity to BPDE-II-DNA, BPDE-I-poly G, BPDE-I-tetraols and BPDE-I-dG. In general, all the antibodies showed the greatest affinity for their original antigen. Those generated against modified DNA showed highest reactivity against modified DNA while the one antibody generated against the monoadduct showed highest reactivity with the monoadduct. These antibodies are currently being used in a highly sensitive competitive ELISA to quantitate levels of BP–DNA adducts in various animal and human tissue samples.

Benzo(a)pyrene (BP) is a carcinogenic polycyclic aromatic hydrocarbon found in heavily polluted air, cigarette smoke, and smoked foods. It is also a ubiquitous contaminant in food and water. Extensive studies from several laboratories have implicated a specific diol epoxide derivative of BP, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-I), as the major electrophilic, mutagenic, and carcinogenic metabolite of BP involved in covalent binding to DNA (*1*). The complete structure and conformation of the adduct formed between BPDE-I and DNA *in vitro* is known. It results from binding of the C-10 position of BPDE-I to the 2-amino group of guanosine and is designated BPDE-I-dG (*1,2*). This adduct has been detected as the major DNA adduct found when a variety of human, bovine, and rodent cells are exposed to BP in culture (*2–5*).

Recently it has become possible to quantify carcinogen adducts on DNA by immunologic methods (*6,7*). Since the DNA does not have to be radiolabeled, these techniques can be used on human samples to screen for exposure to potential environmental carcinogens. Polyclonal rabbit antibodies (*8–12*) as well as monoclonal antibodies(*13–16*) have been developed to several carcinogen–DNA adducts. The use of these antibodies with

highly sensitive immunoassays has led to the detection of femtomole levels of carcinogens in μg quantities of DNA (*17–20*). With this level of sensitivity (better than one adduct per 10^7 nucleotides), it is now feasible to monitor human populations for exposure to certain carcinogens. Previously a rabbit antibody to BPDE-I-dG was generated in animals immunized with BPDE-I-modified DNA (*12*). This antiserum has been used in an enzyme-linked immunosorbent assay (ELISA) to screen human samples for BP binding (*21*). Low but detectable levels of BPDE-I-DNA adducts were seen in lung DNA samples from five lung cancer patients. We decided to develop a panel of monoclonal antibodies to BPDE-I-modified DNA because of their high specificity to a single antigenic determinant and the possibility of a permanent supply of antibodies from long-term cell cultures (*22*).

Materials and Methods

Chemicals

± BPDE-I [7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, also designated "anti"] and ± BPDE II [7β,8α-dihydroxy-9β,10β-epoxy, 7,8,9,10-tetrahydrobenzo(a)pyrene, also designated "syn"] were obtained from the Cancer Research Program of the Na-

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tional Cancer Institute (Div. of Cancer Cause and Prevention, Bethesda, MD). Calf thymus DNA, goat anti-mouse IgG and goat anti-rabbit IgG-alkaline phosphatase conjugates, and p-nitrophenyl phosphate (Sigma 104) were purchased from Sigma (St. Louis, MO). Rabbit anti-mouse IgG$_1$, IgG$_2$, IgM or kappa chains were purchased from Litton Bionetics (Charleston, SC). The media F12 (HAM's) and Iscove's modified Dulbecco's Medium (IMDM) were purchased from Grand Island Chem. Co. (Grand Island, NY). Fetal calf serum (FCS) was purchased from Sterile Systems (Logan, UT).

Synthesis of Immunogens and Immunization

Calf thymus DNA was modified *in vitro* with BPDE-I to a level of 1% as described previously (*23*) and denatured before immunization. BPDE-I-G-BSA was prepared by periodate treatment of guanosine, followed by modification with BPDE-I then coupling to BSA. Details have been published (*22*).

BALBc/Cr mice (West Seneca Lab., W. Seneca, NY) were treated as follows: week 1, intraperitoneal (IP) injection of 100 µg BPDE–DNA complexed with an equal amount of methylated bovine serum albumin (mBSA) and emulsified with an equal volume of complete Freunds adjuvant (FA); week 3, 200 µg BPDE–DNA–mBSA complex with incomplete FA (IP); week 5, 75 µg BPDE–DNA–mBSA complex with incomplete FA (IP), week 7, 50 µg BPDE–DNA–mBSA complex without FA, intravenous injection in tail vein.

A similar immunization schedule was followed for BPDE-I-G-BSA except that the amounts used were: week 1, 100 µg BPDE-I-G-BSA emulsified with complete FA; week 3, 100 µg BPDE-I-G-BSA with incomplete FA; week 5, 75 µg BPDE-I-G-BSA with incomplete FA; week 7, 50 µg BPDE-I-G-BSA without adjuvant.

During week 6, blood samples were removed from the tail and assayed by ELISA for antibody activity as described below.

Cell Fusion

Spleen cells were removed 3 days after the final immunization and fused with the myeloma cell line P3X63Ag8.653 using polyethylene glycol. The hybrid cells were cultured in a HAT basal media (F12/IMDM + α-thioglycerol + progesterone + Pen-strep) with 10% FCS in 10 96-well tissue culture plates. Culture supernatants were assayed for the presence of specific antibodies by ELISA. Cells from positive wells were subcloned in argarose plates containing an underlying monolayer of CREF cells. Details of the media preparation, fusion, and subcloning procedure have been published (*22*).

Enzyme-Linked Immunosorbent Assays

Polystyrene U bottom micro plates (Corning #25855)

were coated with 20 ng heat-denatured BPDE-I-DNA (modified to an extent of 0.54%) in PBS, by drying at 37°C overnight. The plates were then washed with PBS-Tween using an automatic plate washer (Flow Multi-wash, McLean, VA.) set for five 200 µL washes. A similar wash step was done after each incubation. Nonspecific binding to the plate was then minimized by incubating the wells with 200 µL 1% FCS in PBS-Tween for 1 hr. The hybridoma supernatants were then transferred to the coated plates. After a 1.5 hr incubation 37°C, the plates were washed and goat anti mouse IgG-alkaline phosphatase (diluted 1:1000, 0.1 mL) was then added and the plate incubated for 1.5 hr. After washing the plates with PBS-Tween, 0.1 mL p-nitrophenyl phosphate in 1 M diethanolamine (pH 8.6) was added to each well. The plates were incubated for 60 min and the color development at 405 nm was read on a Flow Multiscan MC micro plate reader. Positive cultures were then rescreened by adding 50 µL of their supernatants to each of three wells, one coated with BPDE-I-DNA, one coated with unmodified DNA, and one blank, i.e., a well containing no DNA. This process eliminated those clones producing antibodies to unmodified DNA or to some unknown antigen present in the uncoated plate.

Sera from the immunized mice were also titered by this procedure. Various dilutions of the sera (100 µL) were added to sets of three wells, one blank, uncoated well, an unmodified DNA coated well, and one well coated with BPDE-I-DNA. Those animals showing the highest titer against modified DNA were selected for fusion. For the competitive assay, wells were coated with 5 ng denatured BPDE-I-DNA (0.54% modified). The hybridoma supernatant was diluted and mixed with an equal volume of the competitor before adding 0.1 mL of this mixture to the well. Further steps were as described above.

Isotype Determination

Hybridoma supernatant (0.1 mL of 1:500 dilution) was added to wells coated with 5 ng BPDE-I-DNA. After incubation, rabbit antisera specific for mouse IgG$_1$, IgG$_2$, IgM or kappa chains (1:500 dilution) were added for 1.5 hr. This step was followed by the addition of goat anti rabbit IgG-alkaline phosphatase (1:500) for 1.5 hr. The substrate was then added and the enzyme activity determined as described above.

Results

Immunization of mice with either BPDE-I-DNA complexed to methylated BSA or with BPDE-I-G-BSA led to the production of stable clones producing antibody specific for BPDE-I-DNA. A total of five clones have been isolated and characterized (Table 1). One clone designated 8E11 was obtained from the spleen cells of a BPDE-I-G-BSA immunized animal while four clones designated 5D11, 5D2, 1D7, and 4C2 were from the spleen cells of several BPDE-I-DNA immunized animals. Only antibody 5D11 is of the IgG$_2$, kappa isotype, all others are IgG$_1$, kappa.

Table 1. Immunogen and isotype analysis of antibodies.

Antibody	Immunogen	Isotype
8E11	BPDE-I-G-BSA	IgG1, kappa
5D11	BPDE-I-DNA:MBSA	IgG2, kappa
5D2	BPDE-I-DNA:MBSA	IgG1, kappa
1D7	BPDE-I-DNA:MBSA	IgG1, kappa
4C2	BPDE-I-DNA:MBSA	IgG1, kappa

Noncompetitive ELISA was utilized to reveal the specificity of the antibodies. Micro plates were coated with increasing amounts of denatured modified or non-modified DNA. For antibody 5D11, the absorbance at 405 nm, a measure of antibody binding to the plate, increases with increasing amounts of BPDE-I-DNA coating (Fig. 1). At higher levels a decrease in antibody binding is seen. This is typical of the decrease in detection seen at high antigen levels in solid-phase assays (24). There is a low, but detectable level of antibody bound to nonmodified DNA coated wells. The lowest amount of modified DNA that can be differentiated from unmodified DNA (a 3-fold difference in absorbance) is
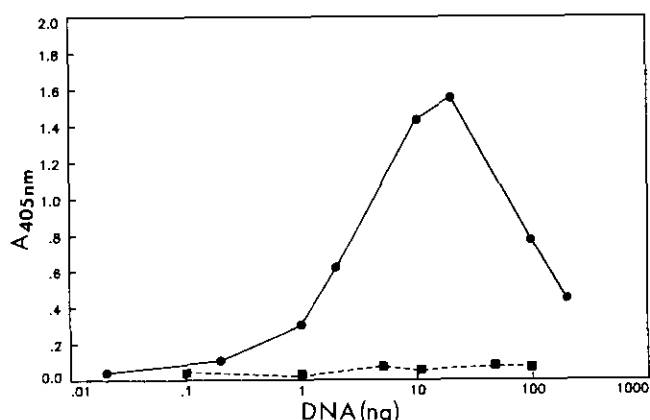

FIGURE 1.   Noncompetitive binding of monoclonal antibody 5D11 to increasing amounts of DNA. The indicated amounts of unmodified calf thymus DNA (■) or BPDE-I-modified DNA (0.54%) (●) were absorbed to each well. The antibody was used at a 1:500 dilution.
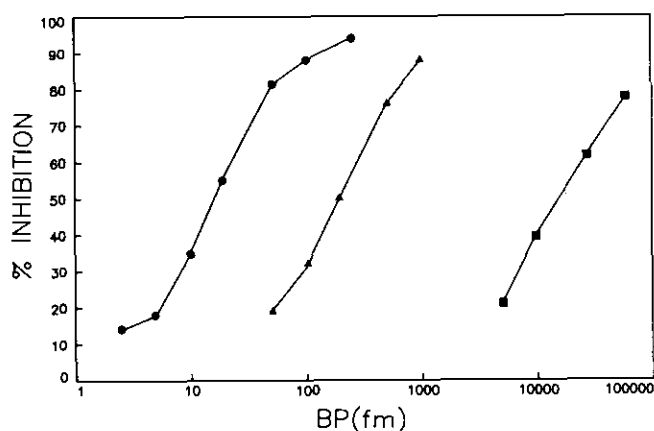

FIGURE 2.   Competitive inhibition of monoclonal antibody 5D11 binding to BPDE-I-DNA. The competitors were (▲) native BPDE-I-DNA, (●) denatured BPDE-I-DNA, and (■) BPDE-I-RNA. The antibody was used at a 1:10,000 dilution.

0.2 ng DNA or 3 fmole of bound BPDE-I.

Competitive ELISAs were used to determine the sensitivity of the antibodies and to obtain additional information about their specificity. Figure 2 shows that antibody 5D11 differs in its reactivity with modified native and denatured DNA. With native DNA, 50% inhibition is at 180 fmole BPDE-I-dG adduct, while with denatured DNA it is at 19 fmole. The antibody is even less reactive with modified RNA (50% inhibition at 18,000 fmole). The highest sensitivity of the antibody is for its original immunogen, denatured modified DNA. The specificity of 5D11 was also tested against BPDE-I-dG, the monoadduct isolated from modified DNA, BPDE-I-tetraols, the hydrolysis products of BPDE-I, and BPDE-II modified DNA. Figure 3 shows there is no cross-reactivity with free tetraols while BPDE-I-dG has a 50% inhibition at 21,000 fmole compared to 19 fmole for denatured BPDE-I-DNA. The higher reactivity with DNA compared to monoadduct suggests that the antibody recognizes antigenic determinants in the surrounding DNA structure. When tested with BPDE-II-DNA, 50% inhibition was at 4400 fmole indicating that the antibody is sensitive to the stereochemistry of the BP chromophore.

This can be contrasted with antibody 8E11 which was generated against the monomeric adduct coupled with BSA. It has a lower sensitivity to BPDE-I-DNA (50% inhibition at 350 fmole) than 5D11 (50% inhibition at 19 fmole). (Fig. 4). However, it has better reactivity against the monoadduct BPDE-1-dG (50% inhibition at 145fmole) than with DNA. In addition, it shows good cross-reactivity against BPDE-I tetraols (50% at 250 fmole). This suggests that the antibody recognizes mainly determinants present on the BP ring. It is specific for BPDE-I since it does not react with BPDE-II-DNA.

The sensitivity of antibodies 5D2, 4C2, and 1D7 for denatured BPDE-I-DNA is shown in Figure 5. Antibody 5D2 is the most sensitive with a 50% inhibition at 17 fmole while the values for 4C2 and 1D7 are 160 and 370 fmole, respectively.
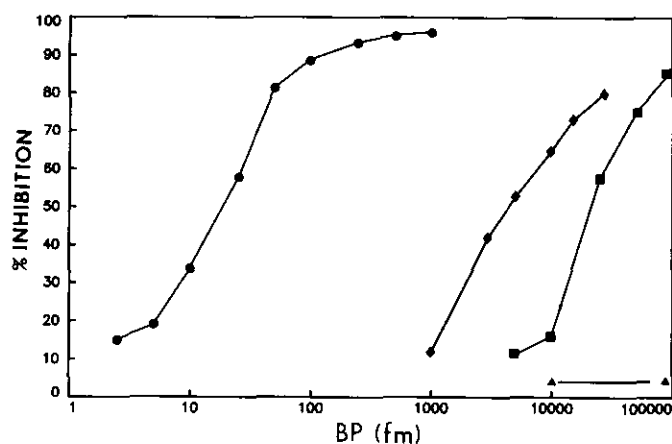

FIGURE 3.   Competitive inhibition of monoclonal antibody 5D11 binding to BPDE-I-DNA. The competitors were (●) BPDE-I-DNA, (■) BPDE-I-dG, (▲) BPDE-I tetraol, and (♦) BPDE-II-DNA. The antibody was used at a 1:5000 dilution.
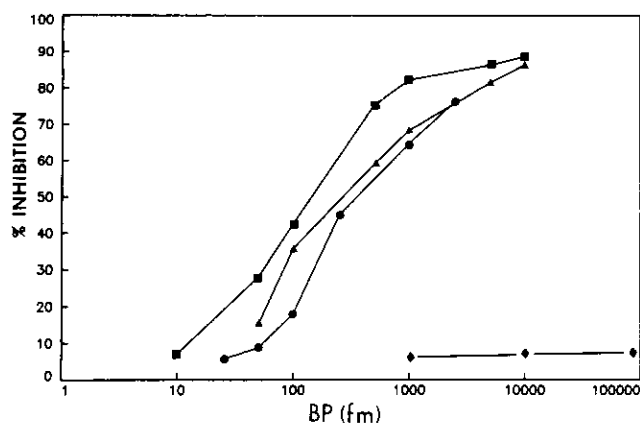
FIGURE 4.   Competitive inhibition of monoclonal antibody 8E11 binding to BPDE-I-DNA. The competitors were (●) BPDE-I-DNA, (■) BPDE-I-dG, (▲) BPDE-I-tetraol, and (◆) BPDE-II-DNA. The antibody was used at a dilution of 1:5,000.
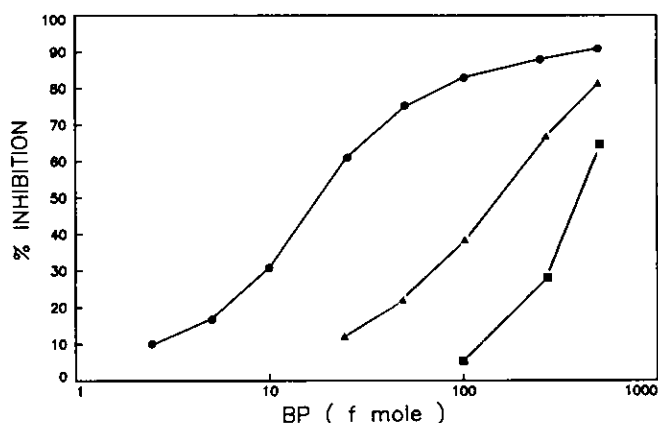
FIGURE 5.   Competitive inhibition of monoclonal antibody binding to BPDE-I-DNA. (●) antibody 5D2 was used at a 1:20,000 dilution; (▲) antibody 4C2 at 1:3,000 dilution; and (■) antibody 1D7 at 1:2,000 dilution.

Table 2. Competitive inhibition of monoclonal antibody binding.

	Level causing 50% inhibition, fmole				
	8E11	5D11	5D2	4C2	1D7
BPDE-I-DNA	350	19	17	160	370
BPDE-I-dG	145	21000	$>10^{5a}$	$>10^{5a}$	40000
BPDE-I-tetraols	250	$>10^{5a}$	90000	64000	1200
BPDE-I-RNA	580	18000	20000	2100	900
BPDE-II-DNA	$>3 \times 10^{4c}$	4400	$>10^{5a}$	$>10^{5a}$	8800
BPDE-II-dG	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$	6200
AAF-DNA	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$
AP-DNA	$>1.5 \times 10^{4b}$	$>1.5 \times 10^{4b}$	$>10^{5a}$	$>10^{5a}$	$>10^{5a}$

[a] No inhibition detected with 10^5 fmole per assay.
[b] No inhibition detected with 1.5×10^4 fmole per assay.
[c] No inhibition detected with 3×10^4 fmole per assay.

Table 2 summarizes all the data on the specificity and sensitivity of the antibodies. The four antibodies generated from animals immunized with BPDE-I-DNA show their highest reactivity to DNA with a greater than 1000-fold difference in reactivity between DNA and monoadduct. The one antibody generated to the monoadduct, however, shows a near 2-fold higher reactivity with the monoadduct than the DNA. All are fairly specific for BPDE-I adducts showing much lower cross-reactivity with BPDE-II-dG and BPDE-II-DNA. Two other carcinogen-modified DNAs were tested for antibody binding. Neither N-2-acetylaminofluorene or 1-aminopyrene modified DNA showed any cross-reactivity with any of the antibodies when tested at high concentrations.

The dependence of antibody reactivity on the secondary structure of the nucleic acids was shown by the difference in reactivity of antibody 5D11 to native and denatured BPDE-I-DNA. Studies with antibody 8E11 indicated that it reacts with a relatively similar sensitivity with the BPDE-1 chromophore whether it was free in solution (BPDE-I-tetraols), on dG or on DNA. However, when tested with BPDE-I-poly G, 50% inhibition was at 20,000 fmole. After treatment with NaOH to generate monoribonucleotides and neutralization the 50% inhibition dropped to 360 fmole. These studies indicate that the secondary structure of the nucleic acid

can interfere with antibody binding and, therefore, quantitation of modification levels.

Testing of Human Samples

In a competitive ELISA it is possible to test a maximum of 50 μg of DNA in a well. If 20% inhibition (about 5 fmole with antibody 5D11) is considered the lower limit of sensitivity in the assay, it is possible to quantitate levels of adducts in the range of 1 per 10^7 to 10^8 bases. This high level of sensitivity is especially important for quantitating DNA damage in human tissues, where the expected levels of DNA modification are likely to be extremely low. We are currently using the monoclonal antibodies described in this paper to continue studies on quantitating BP levels in human and animal samples (17,21). The use of monoclonal antibodies and further improvements in the sensitivity of the immunoassays will hopefully further facilitate human monitoring and provide a valuable tool in studies of human cancer causation.

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