Immunologic Methods for the Detection of Benzo[a]pyrene Metabolites in Urine

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Two monoclonal antibodies (10C10 and 4D5) have been developed from the spleen cells of Balb/c mice immunized with 6-aminobenzo[a]pyrene covalently coupled to bovine serum albumin. These antibodies have been used in an immunoassay for the detection of benzo[a]pyrene and its metabolites in mouse urine. The antibodies were characterized in terms of sensitivity and specificity by competitive enzyme-linked immunosorbent assay (ELISA). With both antibodies, 50% inhibition of antibody binding is at 4 pmol of BP. The antibodies also cross-react with a number of BP metabolites as well as with several other polycyclic aromatic hydrocarbons (PAHs) including pyrene, 1-aminopyrene, and 7,12-dimethylbenz[a]anthracene but with different sensitivities. These results suggest that this assay will detect multiple PAH metabolites in urine. To test the assay on biological samples, mice were treated with [^{3}H]BP, and urine was collected and digested with β-glucuronidase and aryl sulfatase. Several methods were used to isolate BP and its metabolites from the urine, including ethyl acetate extraction, Sep-pak C18 cartridge chromatography, XAD2 resin chromatography, and immunoaffinity chromatography with antibody 4D5. Analysis of the urine extracts with antibody 4D5 gave 50% inhibition at 12–15 pmol of metabolites. Thus, quantitation of metabolites in this sample by competitive ELISA against a standard curve of BP would have underestimated actual metabolite levels by about 70%. This assay will be applied to the analysis of urines from individuals with environmental or occupational exposure. Since humans are usually exposed to BP in complex mixtures of PAHs, multiple metabolites may be present in the urine, making absolute quantitation difficult. This assay should thus serve as a general indicator of exposure to this class of chemicals.

Introduction

A number of techniques have been developed for monitoring human exposure to environmental or occupational carcinogens. Among these methods are the measurement of the chemical itself or its metabolites in body fluids (e.g., serum or urine) or the chemical bound to cellular macromolecules including DNA and protein, measurement of mutagens in urine, and measurement of the early biological effects, such as sister chromatid exchange and chromosomal aberrations (*1*). Although the analysis of biological samples can provide extremely valuable information on carcinogen exposure, human biomonitoring studies are limited by the availability of test material. While blood collection is feasible and has been utilized in a number of studies (reviewed in ref 2), urine is the most readily and repeatedly available material.

Studies monitoring exposure to polycyclic aromatic hydrocarbons (PAHs)[1] have utilized mutation assays as well as chemical analysis. The *Salmonella typhimurium* mutation assay has demonstrated elevated levels of urinary mutagens in populations with environmental, dietary, medical or workplace exposures (reviewed in ref 3). These have included coal tar treated psoriatic patients, cancer chemotherapy patients, and individuals ingesting fried meats. While measurement of chemical levels in urine has had wide application in the clinical setting, few assays are available for the detection of human exposure to PAHs. Pyrene is one of the major compounds always present in environmental mixtures of PAHs and is metabolized primarily to 1-hydroxypyrene (*4*). Urinary excretion of 1-hydroxypyrene has been measured by HPLC with fluorescence detection in psoriasis patients treated with coal tar and workers handling petroleum coke (*5–8*). 3-Hydroxybenzo[a]pyrene has also been quantitated by HPLC with fluorescence detection in psoriasis patients, but levels were about 2500-fold lower than those of 1-hydroxypyrene (*9*). An alternate method quantitated PAHs after extraction of metabolites from urine, reduction to the parent PAHs by hydriodic acid, and analysis by HPLC with fluorescence detection (*10*). However, this method was recently compared to measurement of urinary mutagenicity and 1-hydroxypyrene and found to underestimate PAH excretion, probably due to the inefficiency of the reduction procedure (*7*).

Chemicals can also be quantitated by immunological techniques. Since immunoassays can easily be carried out on large numbers of samples, they are ideal for application to epidemiologic studies. Polyclonal and monoclonal antibodies have been previously developed against BP, but these antibodies have not been well characterized in terms of cross-reactivity with BP metabolites or other PAHs (*11–14*), nor have they been used to monitor biological samples for BP levels. In order to develop immunologic methods for monitoring internal exposure to BP, we have isolated and characterized two monoclonal antibodies recognizing BP and its metabolites. They have been used

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[1] Abbreviations: BP, benzo[a]pyrene; BPDE-I, (±)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 7,8,9,10-BP, tetrol produced by the hydrolysis of BPDE-I; ELISA, enzyme-linked immunosorbent assay; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate-buffered saline.

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to quantitate metabolites extracted from the urine of mice treated with [^{3}H]BP and should be applicable to monitoring human exposures to PAHs.

Materials and Methods

Chemicals. [^{3}H]BP (34 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL. Goat anti-mouse IgG–alkaline phosphatase, *p*-nitrophenyl phosphate (Sigma 104), β-glucuronidase type IV, aryl sulfatase, 8-methoxypsoralen, pyrene, 1-nitropyrene, 1-aminopyrene, 7,12-dimethylbenz[*a*]anthracene, and Amberlite XAD-2 were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit anti-mouse IgG1, IgG2, and IgM were purchased from Litton Bionetics, Charleston, SC. Alkaline phosphatase conjugated rabbit anti-mouse κ and λ were purchased from Southern Biotechnology Associates, and Iscove's modified Dulbecco's medium and HAMs F12 were purchased from Grand Island Chemical Co., Grand Island, NY. Fetal calf serum was purchased from Steril Systems, Logan, UT. BP metabolites were obtained from the National Center Institute Chemical Carcinogen Repository. 1-Hydroxypyrene was purchased from Molecular Probes, Eugene, OR. Cyanogen bromide activated Sepharose 4B was obtained from Pharmacia, Piscataway, NJ.

Monoclonal Antibody Development. BP covalently coupled to bovine serum albumin, prepared as described previously by coupling through an amino group at the 6-position, was obtained from Dr. T. Vo-Dinh, Oak Ridge National Laboratory (*13*). Balb/c mice (6–8 weeks old, Charles River Breeding Laboratories, Wilmington, MA) were immunized as follows: week 1, ip injection of 100 μg of BP–BSA conjugate emulsified with an equal volume of complete Freund's adjuvant; week 3, ip injection of 100 μg of BP–BSA in incomplete Freund's adjuvant; week 7, tail vein iv injection of 75 μg of BP–BSA without Freund's adjuvant. During week 6, blood samples were assayed by ELISA for antibody activity, as described below. Those animals showing the highest titer against BP–BSA were selected for fusion. Spleen cells were fused with the myeloma cell line P3x63-AG.8.653 using poly(ethylene glycol) 1000 essentially as described previously (*15*). Supernatant fluids were screened for the presence of specific antibodies by ELISA as described below. Cells from positive wells were subcloned in agarose plates containing an underlying monolayer of cloned rat embryo fibroblast (CREF) cells.

Hybridoma supernatants were screened by noncompetitive ELISA as described previously (*15*). Briefly, polystyrene U-bottomed microwell plates were coated with 50 ng of BP–BSA in phosphate-buffered saline (PBS) by drying at 37 °C overnight. After nonspecific binding was blocked with 200 μL of PBS containing 0.05% Tween 20 and 1% FCS for 1 h at 37 °C, 100 μL of supernatant was added and the plates were incubated for 1.5 h. Plates were washed with PBS–Tween and incubated with 100 μL of goat anti-mouse IgG–alkaline phosphatase (1:500 dilution) for 1.5 h. After washing, the substrate, *p*-nitrophenyl phosphate (1 mg/mL in 1 M diethanolamine, pH 8.6), was added and the color at 405 nm was measured with a Flow multiscan MC recorder (Flow Laboratories, Alexandria, VA). Positive cultures were then rescreened by adding 50 μL of supernatant to each of three wells, one coated with 50 ng of BP–BSA, one coated with BSA, and one blank uncoated well. This process eliminated clones producing nonspecific antibody. Sera from the immunized mice were also titered by this procedure using three wells. Isotype determination was carried out as described previously (*15*). For the competitive ELISA, wells were coated with 1 ng of BP–BSA. Hybridoma supernatant was diluted (1:1000 for 4D5 and 1:5000 for 10C10) and mixed with an equal volume of the competitor before adding 100 μL to the wells. Further steps were as described above. Competitors were dissolved in methanol for concentration determination by absorbance and diluted with methanol and then PBS such that the final concentration of methanol per well was <5–10%. These concentrations were found to not interfere with the immunoassay.

Preparation of Antibody 4D5 Affinity Column. An immunoaffinity column of antibody 4D5 was prepared as described previously (*16*). Briefly, ascites fluid was adjusted to 40% ammonium sulfate (w/v). The precipitate, formed overnight at 4 °C, was collected by centrifugation, redissolved, and dialyzed against PBS. The antibody was further purified by chromatography on a 1-mL bed volume protein G affinity column equilibrated with PBS. Antibody was eluted with 0.1 M glycine hydrochloride, pH 2.8.

Purified antibody (200 μg) was added to cyanogen bromide activated Sepharose 4B (15 mL) which had been swollen for 15 min in 1 mM HCl at room temperature and washed with coupling buffer (0.1 M NaHCO, 0.5 M NaCl, pH 8.3). The antibody–gel suspension (gel:buffer, 1:2) was incubated overnight at 4 °C, after which the remaining uncoupled sites were blocked by treatment with 0.1 M Tris-HCl buffer, pH 8, for 2 h at room temperature. Finally, the gel was washed alternatively for three to four cycles with low-pH buffer (0.1 M acetate, 0.4 M NaCl, pH 4) and high-pH coupling buffer to remove nonbound antibody. The antibody-bound gel was stored at 4 °C in PBS containing 0.02% NaN_3.

Treatment of Animals. Mice were treated with [^{3}H]BP (0.5 mg/animal, 20 mg/kg, 487 mCi/mmol) by ip injection, and urine was collected for 24 h in a metabolic cage. The urine was digested with β-glucuronidase (200 units/mL) and aryl sulfatase (100 units/mL) as described (*17*). Metabolites were extracted from the urine according to four different techniques: chromatography on reverse-phase Sep-pak C18 cartridges, chromatography on XAD2 resin, immunoaffinity chromatography with antibody 4D5, and extraction with ethyl acetate. A Sep-pak C18 cartridge was primed with 5 mL of methanol, followed by 10 mL of water. The urine sample (4×10^7 cpm in 1 mL of PBS) was applied and the cartridge washed with 5 mL of water. The metabolites were eluted with 5 mL of methanol. For the ethyl acetate extraction procedure, an equivalent sample of urine was extracted with 3×1 mL of acetone/ethyl acetate (1:2). For XAD2 chromatography, urine was applied to a 1-mL bed volume column and the column washed with 5 mL of water. The sample was applied, nonbound material eluted with water, and bound material eluted with 5 mL of acetone. For the immunoaffinity chromatography procedure, poly-prep columns (0.8 × 4 cm) (Bio-Rad, Richmond, CA) were filled with a 1-mL bed volume of antibody-bound gel. The columns were washed with PBS, and then the urine was applied. The column was washed with PBS (5×1 mL), and then bound material was eluted with 50% ethanol. Samples were counted on an LKB Rackbeta scintillation counter.

Reverse-phase HPLC chromatography was performed on an LKB Bromma HPLC on a 5-μm Bondapak C18 column (Waters, Milford, MA). A linear gradient from 20 to 30% methanol in water, over 10 min, followed by a linear gradient from 30 to 90% over 50 min was used for sample elution.

Results

BALB/c mice were immunized with BP coupled to BSA. Spleen cells from animals showing positive titer were fused with mouse myeloma cells (P3x63-AG.8.653) and cultured in hypoxanthine–aminopterin–thymidine selection media. Two stable clones producing antibody specific for BP-BSA were isolated from immunized animals and characterized by ELISA. Isotype classification showed that both antibodies were IgG_2, κ. Competitive ELISAs were used to determine the sensitivity and specificity of the antibodies. The competitors were BP and a number of its metabolites, including BP-phenols, -diols, and -7,8,9,10-tetrol (Table I). Both antibody 4D5 and 10C10 recognize BP with similar sensitivities (50% inhibition at 4 pmol). A number of other BP metabolites also react with the antibodies but with different sensitivities. For antibody 4D5, with BP phenols including 1-OH, 3-OH, 4-OH, and 5-OH, 50% inhibition occurs at 20, 90, 60, and 8 pmol, respectively. With BP-7,8-diol, -9,10-diol, and -7,8,9,10-tetrol, 50% inhibition is at 1.4, 4.5, and 1.0 pmol, respectively. Cross-reactivity was also seen with several other PAHs including pyrene (50% inhibition at 1.6 pmol), 1-aminopyrene (50% inhibition at 0.49 pmol), and dimethylbenz[*a*]anthracene (50% inhibition at 67 pmol). Figure 1 gives representative standard curves for inhibition of antibody 4D5 binding to BP–BSA by several of the competitors. Since the assay will be used to quantitate metabolites in urine samples, the cross-reactivity of the antibody with the potential

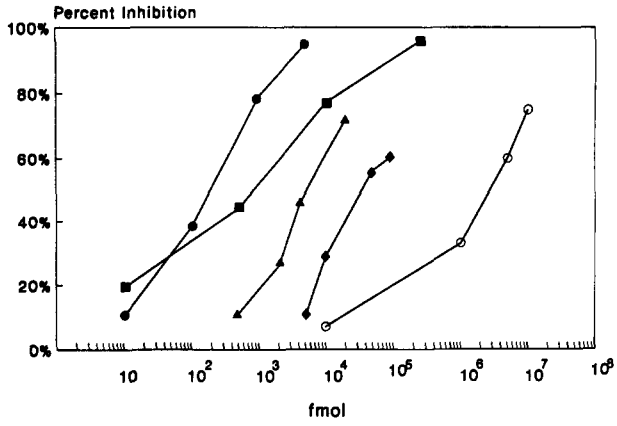

Figure 1. Competitive inhibition of antibody 4D5 binding to BP–BSA. The competitors were 1-aminopyrene (●), pyrene (■), BP (▲), 1-nitropyrene (♦), and 8-methoxypsoralen (○).

Table I. Competitive Inhibition of Antibody Binding to BP–BSA

competitor	pmol causing 50% inhibition of antibody	
	4D5	10C10
BP	4.02	4.07
BP–BSA	4.37	5.00
1-OH-BP	20.9	93.8
3-OH-BP	90.0	15.0
4-OH-BP	59.8	422.5
5-OH-BP	8.2	17.0
11-OH-BP	15.5	18.5
7,8-OH-BP	1.44	2.24
9,10-OH-BP	4.56	9.38
7,8,9,10-OH-BP	1.00	2.68
pyrene	1.65	7.00
1-hydroxypyrene	5.25	6.90
1-aminopyrene	0.49	1.4
7,12-dimethylbenz[*a*]anthracene	67.0	11.0
nitropyrene	48.5	87.5
8-methoxypsoralen	2500	460

contaminants creatinine, tryptophan, and phenylalanine was also tested. No inhibition was seen at concentrations up to 50 μg/well.

To develop an ELISA for quantitation of BP in urine, mice were treated with [^{3}H]BP, and urine was collected and digested with β-glucuronidase and aryl sulfatase to hydrolyze conjugates. Metabolites were concentrated by using four different techniques: extraction with ethyl acetate, chromatography on XAD2 resin, chromatography on reverse-phase Sep-pak C18 cartridges, and immunoaffinity chromatography with antibody 4D5. With the immunoaffinity column 52 ± 11% of the radioactivity was retained by the column and eluted with 50% ethanol; about 30% washed through in the void volume. The remainder bound to the column but could not be eluted. With the Sep-pak cartridges 51 ± 9% of the radioactivity was retained on the resin and recovered in the methanol fraction. Ethyl acetate extraction recovered only 17 ± 4% of the radioactivity while XAD2 chromatography recovered 33 ± 14%. The samples isolated by Sep-pak and ethyl acetate extraction were then analyzed by competitive ELISA (Figure 2). The concentration of BP metabolites was determined from the radioactivity levels. With the Sep-pak C18 cartridge extract 50% inhibition of antibody binding was at 15 pmol of BP metabolites, and it was at 12 pmol with the ethyl acetate extract. Extracts of urine from control animals, when tested at the highest equivalent urine concentration as the treated animals, gave no inhibition.

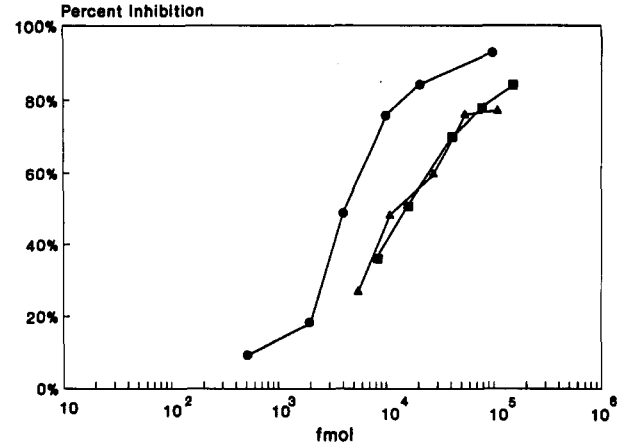

Figure 2. Competitive inhibition of antibody 4D5 binding to BP–BSA. The competitors were BP (●) and BP metabolites extracted from the urine of mice treated with [^{3}H]BP by Sep-pak C18 cartridges (■) and by ethyl acetate extraction (▲).

Urine extracted by Sep-pak C18 was also analyzed by reverse-phase HPLC with UV markers (data not shown). In agreement with previously published studies (*18*), about 10, 5, and 15% of the radioactivity eluted with the 3-OH-BP, 7,8-OH-BP, and tetrol standards, respectively. While no radioactivity eluted in the position of nonmetabolized BP, a number of unidentified peaks were present.

Discussion

Two monoclonal antibodies have been developed with broad reactivity for BP and its metabolites. Both antibody 4D5 and 10C10 have similar sensitivity for BP (50% inhibition at 4 pmol) and also recognize a broad range of BP metabolites with lower sensitivity (Table I). Several other PAHs, including pyrene and 7,12-dimethylbenz[*a*]anthracene, are also recognized, pyrene with better sensitivity than BP for antibody 4D5. The very high sensitivity for 1-aminopyrene (50% inhibition at 0.49 pmol with antibody 4D5) may be related to the antigen used for antibody development. The amino group is in the same position on the pyrene ring in 1-aminopyrene and 6-amino-BP.

Urine extracts from mice treated with [^{3}H]BP, analyzed by competitive ELISA with antibody 4D5, gave 50% inhibition values (12–15 pmol) about 3-fold higher than did BP (4 pmol). Although this analysis was carried out at only one dose of BP, studies by others on urinary excretion of BP indicated no change in the ratio of metabolites over a 5-fold dose range (*18*). Studies in hepatocytes treated in culture over a four-log range also showed no difference in metabolite ratios with dose of BP (*17*). These results suggest that the relative metabolite ratios in humans may not change with dose. Since no unmetabolized BP was seen in the HPLC analysis of the mouse urine, utilizing BP as the standard is probably not appropriate. The ideal standard curve would consists of a mixture of metabolites present in the biological samples at the appropriate ratio. However, this is not a viable method for the mouse urine since a number of unknown metabolites were present and would also be impossible with human urine. Therefore, unknown samples will be quantitated against a BP standard curve, and metabolite levels may be underestimated.

While the immunoassay was tested in animals exposed to BP as a single agent, humans are exposed to BP as part of a complex mixture of PAHs. Multiple PAH metabolites may be present in human urine and react with the antibody. This will make absolute quantitation of BP me-

tabolites difficult. Instead, this assay should serve as a general marker of exposure to this class of chemicals. A similar situation occurs with the immunoassay we are currently utilizing to monitor PAH–DNA adducts. The antisera were developed against DNA modified by BP-diol epoxide (BPDE-I) but cross-reacts with a number of other PAH-diol epoxide modified DNAs (*19*). Since the identity of the adducts present in human samples cannot be determined, absolute quantitation is not possible. However, a number of PAHs in addition to BP are carcinogenic, and thus the ELISA provides a biologically relevant general index of DNA binding by this class of compounds. The methods developed here for quantitation of urinary excretion of PAHs will complement DNA adduct measurement and be more easily applicable to routine occupational monitoring.

Determination of the sensitivity of this method is dependent upon the equivalent amount of urine assayed per microwell as well as the knowledge of what metabolites will be measured. Studies on urine of coal tar treated patients indicated the presence of 3-OH-BP but at levels 2500-fold lower than that of 1-hydroxypyrene (*9*). If environmental exposure to PAHs also gives a similar ratio of metabolites, one of the major metabolites detected by the immunoassay will probably be 1-hydroxypyrene. If the equivalent of 10 μL of urine is assayed per microwell, a urinary concentration of 90 pmol/L should be detectable. This value is well below the levels of 1-hydroxypyrene seen in coal tar treated patients and in the range of that seen in some occupational exposures (*5*, *9*, *7*). Sensitivity could also be increased by the analysis of a larger equivalent volume of urine per well or by the utilization of more sensitive ELISA methods.

Immunologic methods can also be used to complement other methods of determination. For example, immunoaffinity chromatography could be utilized to isolate metabolites from urine followed by analysis by HPLC or spectrometric methods for their quantitation or identification. Such an approach has been used successfully to monitor human exposure to aflatoxin by measurement of metabolites and excised DNA adducts in exposed populations (*20*).

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Registry No. BP, 50-32-8; 1-OH-BP, 13345-23-8; 3-OH-BP, 13345-21-6; 4-OH-BP, 37574-48-4; 5-OH-BP, 24027-84-7; 11-OH-BP, 56892-32-1; 7,8-OH-BP, 13345-25-0; 9,10-OH-BP, 24909-09-9; 7,8,9,10-OH-BP, 59957-91-4; pyrene, 129-00-0; 1-hydroxypyrene, 5315-79-7; 1-aminopyrene, 1606-67-3; 7,12-dimethylbenz[*a*]-anthracene, 57-97-6; nitropyrene, 63021-86-3; 8-methoxypsoralen, 298-81-7.

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