Production of a Monoclonal Antibody Against Benzo[a]Pyrene Diol Epoxide DNA Adducts

Brian Peden Austin

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PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST
BENZO[α]PYRENE DIOL EPOXIDE DNA ADDUCTS

by

Brian Peden Austin
B.S. December 1998, Old Dominion University

A Thesis Submitted to the Faculty of Old Dominion University in Partial Fulfillment of
the Requirement for the Degree of

MASTER OF SCIENCE

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May 2002

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ABSTRACT

PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST BENZO[α]PYRENE DIOL EPOXIDE DNA ADDUCTS

Brian Peden Austin
Old Dominion University, 2002
Director: Dr. James H. Yuan

Benzo[α]pyrene is a ubiquitous pollutant produced from the incomplete combustion of organic material such as fossil fuels. It is found in the workplace, urban air, drinking water, and the food supply. Recently, it has been proposed that benzo[α]pyrene may be the causative agent in the formation of lung adenocarcinomas among some Taiwanese women exposed to cooking oil fumes without adequate ventilation. In this study, calf thymus DNA was modified in vitro with benzo[α]pyrene-diol epoxide (BPDE) to a level consistent with that found in biological samples. This DNA of low modification was used as an immunogen in the production of a monoclonal antibody specific for the detection of benzo[α]pyrene-DNA adducts and used in the development of a sensitive colorimetric enzyme-linked immunosorbant assay (ELISA). Following it's development, the newly developed assay will be used independently in a subsequent epidemiological study to explore the correlation between exposure to benzopyrene generated from hot cooking oils, adduct formation, and the generation of relevant carcinomas.
ACKNOWLEDGMENTS

I would like to take this opportunity to thank all those who were directly and indirectly responsible for my education.

First, I would like to personally thank my teacher, mentor, and friend, Dr. James H. Yuan, who spent a great deal of his time seeing that my development as a student was proceeding on the proper course. Additionally, I would like to thank him for going above and beyond the call of duty, for his heartfelt concern for me as an individual, and for his gentle, nurturing spirit. I owe a great debt of gratitude to him for all he has done for me and above all, for his willingness to channel God’s love and His Spirit to me from Heaven above. Thank you!

I would also like to thank my mom, dad, sister, brothers, grandparents, aunts and uncles for their support of me while I was a student. This help came in the way of a place to live and food to eat. It also came in ways that I am unable to describe. Just the mere existence of my family was enough to encourage me to continue. Late at night I would study alone and think of my family and the desire to make them proud of me drove me even in times of discouragement and despair. Thank you!

I want to conclude by thanking, above all, my God and savior, Jesus Christ, for giving me this opportunity to glorify Him, without whom, I would never have existed. Thank You!
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A. BACKGROUND

The idea that a single event is responsible for the formation of cancer has slowly waned, after many years of rigorous studies, giving way to the more reasonable concept of multistage chemical carcinogenesis. In this scheme, no single substance or action will give rise to cancer but instead will trigger or act in conjunction with a series of steps that must be in place for cell transformation to occur. There are three major steps that define the process of multistage chemical carcinogenesis. In step one, an agonist, generally thought to be an exogenous substance referred to as a xenobiotic, is responsible for compromises in the fidelity of DNA. These mutations may have the effect of altering normal signal transduction pathways within the cell including gene expression which defines the second stage of multistage chemical carcinogenesis. Lastly, as a result of modifications to normal cellular functions, there is a general loss of cell cycle controls responsible for the maintenance of cell growth. Small perturbations to any of the above steps may lead to further deviations in other stages of carcinogenesis so that the initial changes may be regarded as necessary but not sufficient for the formation of cancer.

Two hundred years ago the British surgeon Sir Percival Pott made the first association between the environmental pollutant soot and the incidence of human prostrate cancer in chimney sweeps (1). Of the environmental xenobiotics found to be carcinogenic, benzo[a]pyrene is by far the most well studied of the family of compounds known as the polycyclic aromatic hydrocarbons (PAH’s). Benzo[a]pyrene is produced
by incomplete combustion of organic materials such as fossil fuels and is a ubiquitous pollutant found in small concentrations in the workplace, urban air, drinking water, and the food supply (2). For this reason benzo[α]pyrene is generally accepted as a general indicator for the concentration of other PAH's. Approximately 1300 tons of benzo[α]pyrene are released into the air each year in the United States alone (3). Studies have measured adducts in human tissues taken from many workplace settings such as coal miners, foundry workers and coke-oven workers in addition to psoriatic patients treated with coal tar and adducts measured in the general population (2, 4-7). Benzo[α]pyrene is lipophillic and easily enters the body via lung tissue, the skin, or through absorption in the intestines. Following absorption, benzo[α]pyrene is distributed to different tissues and detoxified. Cytochrome P450 forms an epoxide on carbons 7 and 8 which is subsequently hydrolized by the enzyme epoxide hydrolase to benzo[α]pyrene 7,8-dihydrodiol. The dihydrodiol is again oxidized by the cytochrome to benzo[α]pyrene-7,8-diol-9, 10-epoxide. These oxidations make possible the conjugation of benzo[α]pyrene to polar species such as sulfates, glucuronate, or glutathione (Fig. 1). One study has reported that polymorphisms in two genes coding for class µ and π glutathione thionyl transferases may influence the level of carcinogen-DNA adducts in human tissues associated with individual susceptibility to carcinogens (8). The increased polarity of BPDE allows for it’s removal from the body in the kidneys or secreted as bile. This process has been termed metabolic activation and results in intermediates capable of binding nucleic acid in vitro and in vivo (1). It has been shown that two reactive metabolites BPDE I (anti) and BPDE II (syn) are formed during activation and that each of these compounds is optically active resulting in (+)
Fig. 1 Activation of benzo[a]pyrene to form anti-BPDE I.
and (-) enantiomers. Of the products formed, studies have indicated that BPDE I is more carcinogenic in newborn and adult mice and also forms the major product when reacted with deoxyguanosine. DNA exposed to mixtures of BPDE I and II have resulted in yields of 90-95% trans-(7R)BPDE 1-N2-deoxyguanosine (1, 9-10).

DNA, being a moderately strong nucleophile, readily forms adducts with the electron deficient nuclei of BPDE. The reaction of BPDE with native DNA results in covalent attachment to all four nucleic acid bases but by far the most prevalent and stable adduct formed is with guanine. The factors that determine the reactivity of BPDE with DNA are 1.) the species of BPDE in question, 2.) nucleophilicity of the DNA site, 3.) steric factors, and 4.) the sequence context of neighboring nucleotides (11). The variability in the nucleotide sequence of DNA gives rise to regions within the macromolecule that are more reactive and susceptible to modification. As a result, the adducts formed with DNA are not heterogeneously distributed throughout but accumulate within specific regions such as GC rich intervals. The preference of BPDE for sequences containing contiguous guanines is caused by the increased electrostatic potential of guanine when flanked by repeating guanine bases (12). BPDE preferentially binds to the exocyclic N2 of guanine as it is frequently aligned in the major groove of the DNA double helix (Fig. 2). In contrast, N3 of adenine is orientated within the less accessible minor groove and reacts at a much lower frequency. It has been reported that GC to TA, GC to AT, or GC to CG substitutions and framshift mutations are induced by benzo[α]pyrene-deoxyguanosine (BP-dG) adducts occurring at N2 and these are much more carcinogenic than N7 adducts (13-15).
The solution structure of several adducts as they occur in duplex DNA have been determined by NMR spectroscopy, energy minimizations, and molecular modeling (16-18). Each adduct can be placed into either of two categories known as Type I and Type II adducts. Type I adducts reflect those adducts in which the planar pyrene ring structure is incorporated into the DNA helix by intercalation of it’s π orbitals with those of neighboring bases. In the second type of adduct formed, Type II adducts are orientated outside of the helix and exposed to the effects of the solvent (19). Data revealing the structure of the lesions produced in DNA is important not only in terms of learning new reaction mechanisms, but it may also lead to a better understanding of what types and degrees of physical damage are the most tolerated and which are more likely to lead to the formation of cancer. Part of the tolerance previously mentioned may include the various repair mechanisms responsible for restoring damaged DNA to its original form. DNA damage caused by benzo[α]pyrene is reversed by an excision repair mechanism. The first step in the repair mechanism is the removal of the altered base by DNA glycosylase which cleaves the glycosidic bond leaving the deoxyribose phosphate backbone in tact. The strand is nicked on either side of the apurinic or apyrimidinic base locus by apurinic/apyrimidinic endonuclease followed by removal and replacement of several residues by DNA polymerase then ligated by DNA ligase to complete the repair. Structural insights regarding BPDE-DNA adducts could lead to the discovery as to which adducts, if any, are preferrentially repaired. This in turn may lead to a better understanding of aspects underlying the repair mechanism itself.
Fig. 2. The covalent attachment of guanine to (+)-anti-BPDE.
B. RESEARCH OBJECTIVES

Once the link was established between higher numbers of xenobiotic-DNA adducts and cancer, it became a matter of determining the source of the xenobiotics. Individuals who held occupations where exposure to carcinogens such as benzo[a]pyrene was likely to occur were previously studied (2, 5). When compared to controls and corrections made for those who smoked cigarettes, subjects who where exposed to higher concentrations of benzo[a]pyrene (>0.2 µg/m³) had significantly greater adduct levels. These occupations include iron foundry workers and coke-oven workers where epidemiological studies indicate as much as a 16-fold relative risk increase for lung cancer for topside coke-oven workers with 15 years of experience. Data has also been published on the general population from a small region in central Italy (7). Samples were taken and assayed for the presence of serum antibodies against benzo[a]pyrene-DNA adducts. Data from questionnaires was interpreted and correlated to measured adduct levels using multivariate data regression analysis. It was found that 21% of the general population tested positive for the presence of anti-BPDE-DNA antibodies with no discrimination between males or females. Subjects falling within certain clusters, such as living in an urban area, active cigarette smoking, and families with histories of lung cancers had higher numbers of adducts found in DNA samples.

It has been hypothesized that exposure to benzo[a]pyrene suspended in the volatile gases produced from hot cooking oil fumes is the cause of the lung adenocarcinomas seen in women. It is customary for women to work in the kitchen in Taiwan and China preparing various deep-fried foods oftentimes without adequate ventilation. As a result, women have a much greater exposure risk than previously
realized. Studies have been published reporting on the amount of benzo[α]pyrene found in various cooking oils in addition to measurements of benzo[α]pyrene fumes found in ambient air at three catering shops located in The Peoples Republic of China (20). The ambient air concentrations of benzo[α]pyrene were found to be in the range of 0.01 to .04 µg/m³ which would have been assigned a medium exposure risk in the foundry worker study.

It is the goal of this research to produce at least one monoclonal antibody specific for the detection of BPDE-DNA adducts. During the hybridoma process, an attempt will also be made at isolating an antibody that is specific for unmodified DNA. The monoclonal antibodies will then be used as a probe in the development an ELISA procedure capable of detecting DNA adducts. In this procedure, modified DNA will be bound to a microwell composed of a synthetic polymer and then probed for the presence of adducts with the developed antibody.

C. OVERVIEW OF SANDWICH ASSAY

The assay that will be developed and used in these studies will be an ELISA (Enzyme Linked Immunosorbant Assay) otherwise known as a sandwich assay. It has been so named because of the use of layer upon layer of reagents. A sandwich assay was chosen because of its simplicity, cost effectiveness, and sensitivity. Finally, in addition to being very fast, the assay offers the ability to test large numbers of patient samples in a single screen.

The newly developed sandwich assay works by sequential immobilization of the antigen, first antibody, and second antibody-enzyme conjugate on a specialized plastic well. The plastic wells are manufactured using different polymers and offer a wide
variety of affinities for proteins and nucleic acids. The sandwich assay begins with a clean plastic well that is coated with the antigen in step one (Fig. 3). Excess, unbound antigen is rinsed away by washing the well with a mild detergent followed by water. To complete the first layer, any unbound areas of the plastic well are filled in with a blocking agent to prevent non-specific binding by other components of the assay. The second layer is made up of antibodies specific for the antigen in question, in this case, BPDE-DNA. A solution containing the antigen specific antibody is incubated in the presence of the previously immobilized antigen. Again, the excess of reagent is washed with mild detergent followed by water completing the second layer. The final layer, the antibody-enzyme conjugate, is immobilized by binding to the antigen specific antibody that makes up the second layer. This binding occurs because the first antibody was produced in a murine (mouse) system while the second antibody, produced in goats, is specific for murine class G immunoglobulins. To form the third and final layer, a solution containing the enzyme-conjugate is added to the plastic well. After washing away any unbound reagent, all three layers of the assay are in place and the well is ready for development. The second antibody contains the enzyme, horseradish peroxidase, which catalyzes the breakdown of hydrogen peroxide into oxygen free radicals. Therefore, hydrogen peroxide is added to the sandwich assay in the developmental and final step of the ELISA. In addition to hydrogen peroxide, tetramethylbenzidine is contained in the development solution that changes color upon oxidation. The color change can be measured by absorbance and compared to known standards to quantify the antigen bound to the plastic wells.
Fig. 3 Diagram of a typical Enzyme Linked Immunosorbent Assay (ELISA). Also known as a sandwich assay, the ELISA consists of three layers comprised of antigen (▲) modified and (■) unmodified DNA blocked with 0.3% ovalbumin; layer two is made up of the BPDE-DNA specific antibody(▲); Layer three is the goat anti-mouse IgG horseradish peroxidase conjugate(●).
A. MATERIALS

All chemicals were reagent grade unless otherwise stated.

1. Biochemical Materials

NS-1 (non-screening mouse myeloma) cells (American Type Culture collection), BALB/CByJ mice (Jackson Laboratories, Maine).

2. Chemical Reagents

BPDE (MW=302.31) was obtained from the National Cancer Institute (NCI) repository, triethylamine, sodium cacodylate, sodium acetate, bovine serum albumin (BSA), ovalbumin (OVA), goat-anti-mouse immunoglobulin G whole molecule-peroxidase conjugate (goat-anti-mouse IgG-HRPO), protein A-Sepharose affinity gels, dimethyl sulfoxide (DMSO), hypoxanthine (1 x 10^{-4} M) and thymidine (1.6 x 10^{-5} M) culture medium (HT), lipopolysaccharide (LPS), fetal calf serum (FCS), methyl cellulose, polyethylene glycol (PEG, MW 1500), sodium dihydrogen phosphate (NaH_2PO_4, MW 120.0), tris [hydroxymethyl] amino-methane (trizma base, MW 121.1), 2, 6, 10, 14-tetramethyl-decanoic acid (pristane), Tween-20 (polyoxyethylenesorbitan monolaurate) were purchased from Sigma, Penicillin/Streptomycin containing (10,000 units penicillin and 10 mg streptomycin per 1 mL of solution), L-glutamine (200 mM), HAT medium containing (5 mM hypoxanthine, 20 µM aminopterin, and 0.8 mM
thymidine) was obtained from JRH Biosciences. Sodium bicarbonate (NaHCO₃, MW 84.01), and sodium chloride were products of Mallinckrodt. Tetrahydrofuran (anhydrous inhibitor free, MW 72.11) was purchased from Aldrich. N₂ gas (BOC Gasses)

B. EQUIPMENT

The equipment used during research was an ultrafiltration system using a YM 10 membrane and Centricon-3 centrifugal microconcentrator (Amicon), Hemocytometer (Arthur H. Thomas Co.), Carey 3-Bio UV-VIS spectrophotometer (Varian), Model J2-21 refrigerated centrifuge (Beckman), Model TJ-6 centrifuge (Beckman) with Model TJ-R refrigeration unit (Beckman), Tuberculin syringes (1 cc, 3 cc, 5 cc, and 10 cc, Becton-Dickinson), Model EL 307C microplate reader (Bio-Tek Instruments), electrophoresis cell (Ciba-Corning), microliter sample dispenser (Ciba-Corning), 24-well microwell plates (Corning), 96-well microwell plate (Falcon), clinical centrifuge (Damin/IEC), model 110 expended scale pH meter (Digital), Immulon I, Immulon II, and Immulon IV removalwell strips (Dynatech Laboratories, Inc.), EC-400 power supply (E-C Apparatus), 4-0 Silk (1.5 metric, Ethicon), culture flasks (25-mL and 50-mL, Falcon), petri dishes (10 x 35 mm and 15 x 100 mm, Falcon), disposable cell scrapper (Fisher Scientific), sterilized disposable pipettes (1,5,10 and 25 mL, Fisher Scientific), suspension culture dishes (35 x 10 mm, Fisher Scientific), Model 3326 water-jacketed CO₂ incubator (Forma Scientific), microsyringe (10 µL, Hamilton), Type 6 optical unit detector with 280nm and 254nm filter (Instrumentation Specialties Co.), Model UA-5 Absorbance/Fluorescence monitor (Instrumentation Specialties Co.), Miltex stainless steel sterile surgical blade (Kai), 0.22 µm sterilized filter unit with filling bell
C. METHODS

1. Preparation of Antigen

Highly polymerized calf thymus DNA was dissolved in 0.05 M cacodylate buffer, pH 7.1 to a concentration of 0.3 mg/mL. This required overnight mixing at room temperature. 5 mg of benzo[α]pyrene-7,8-dihydrodiol-9,10-epoxide(+),(anti) (BPDE) was dissolved in 5 mL of tetrahydrofuran (THF) and stored in the dark at -80°C until use. All work with BPDE whether as a solid or in solution took place in the dark and under a nitrogen blanket due to BPDE’s reactivity with light and ambient moisture. A glove bag was sufficient for this purpose and also offered protection for the experimenter in the event of a spill. BPDE was reacted with DNA over a range of concentrations according to (Table 1). The reactions were carried out in Eppendorf 1.5 mL centrifuge tubes made of neutral plastic so that the charged DNA would not adhere to the sides of the vessel. These tubes were found capable of resisting solvents such as 33% THF, ethyl acetate, butanol, or diethyl ether under the experimental conditions required. After mixing, the reaction vessels were covered with aluminum foil and allowed to incubate at 37°C for one hour.
Table 1  Volumes of reactants and buffers combined for reaction of
BPDE with DNA

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>[BPDE] (uM)</th>
<th>Stock BPDE added (uL)</th>
<th>Triethylamine added (uL)</th>
<th>Cacodylate buffered DNA (uL)</th>
<th>0.05M Caco. buffer (mL)</th>
<th>Reaction Volume (mL)</th>
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<tr>
<td>1</td>
<td>100</td>
<td>30</td>
<td>1</td>
<td>800</td>
<td>169</td>
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</tr>
<tr>
<td>2</td>
<td>200</td>
<td>61</td>
<td>1</td>
<td>800</td>
<td>138</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>91</td>
<td>1</td>
<td>800</td>
<td>108</td>
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<tr>
<td>4</td>
<td>400</td>
<td>121</td>
<td>1</td>
<td>800</td>
<td>78</td>
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</tr>
<tr>
<td>5</td>
<td>500</td>
<td>152</td>
<td>1</td>
<td>800</td>
<td>47</td>
<td>1</td>
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The reaction mixtures were extracted ten times with a mixture of ethyl acetate and butanol (6:9) followed by two extractions with water saturated diethyl ether. The DNA was then dialyzed against 2 x 5 L milli-Q water, pH 7.0 at room temperature (RT) for twelve hours. The concentration of DNA was determined using a Cary 3Bio UV-Vis spectrophotometer and the extent of modification calculated as described earlier (1). To verify that the calf thymus DNA was exposed to BPDE, the ethyl acetate, butanol, and diethyl ether extracts were pooled and evaporated to dryness in a fume hood. The remaining off-white crystals were redissolved in an acetone-water mixture (1:1) and scanned in the Cary from 200-500nm.

2. Production of Antibodies

a. Immunization of Mice

A hypodermic syringe was prepared before surgery containing 100 µL of BPDE-DNA at a concentration of 400 µg/mL. The optimal age of the mice for fusion is two months and so the primary immunizations were administered at six weeks of age. The primary immunization was performed on day zero intrasplenically by anesthetizing the mouse with 60 mg/mL or 10X pentobarbitol. The amount used was 40-70 mg/kg body weight to induce sleep for approximately 45 minutes, however, mice must be monitored closely to avoid loss of antigen in the event of an overdose. The mouse was laid on its right side, inside of a glovebox for sterility, and the area just below the left ribcage was sterilized with 70% EtOH. The superficial skin was elevated from the peritonium by pulling upward until taught with a pair of tweezers and a small 1cm cut made with a pair of surgical scissors taking care not to penetrate the peritonium. The incision was moved transversely until the spleen, resembling an elongated kidney bean in shape and color,
was located (Fig. 3). The needle was inserted longitudinally into the organ and the antigen injected as the needle was retracted to insure an even distribution throughout the entire spleen. The incision was closed with two sutures followed by injection of 0.25 mL penicillin intraperitonially to minimize infection. The surgical area was again cleaned with 70% EtOH and the animal loosely wrapped in a napkin for warmth and cleanliness. On day 11, the mouse was administered a booster shot intravenous by concentrating the modified DNA to 1.0 mg/mL in a centrifugal concentrator and 20 µL of the concentrate was injected into the tail vein. This procedure required some skill on the part of the investigator. The needle was inserted into the tail vein at a very shallow angle and the antigen injected slowly. The area where the needle penetrated the tail vein was firmly pinched with the thumb and forefinger before retracting the needle to prevent the antigen from bleeding out of the wound. The antigen was forced into circulation by gently squeezing the tail with the thumb and forefinger of one's free hand and moving in the direction of the mouse's body holding the wound closed with the other hand. The booster procedure was repeated on day 13.

b. Titer Assay

A titer assay was performed 1-3 days following the last booster shot. Two strips, each containing 12 microwells, of Immulon II microwells were coated with 20 ng of BPDE modified DNA per well (Fig. 4). Modified and unmodified DNA were coated onto Immunowells by using concentrations of DNA that would yield the desired amount of DNA per well and minimize the volume of solvent used. Solvent minimization was desired since the DNA was coated onto the wells by evaporation of the solvent in a 37°C oven, an overnight process. The wells were then blocked for a
Fig. 4. Anatomy of a mouse relevant to the production of monoclonal antibodies. Note the location of the thymus and spleen. (21)
minimum of two hours with 200 µL of 3% ovalbumin in 50 mM Tris-HCl buffer, pH 8.5.

Blood from two mice, one immunized with BPDE-DNA and one unimmunized mouse, was obtained by puncturing the tail vein with a low gauge sterile needle. A drop of blood was formed and drawn into a heparin coated capillary tube by capillary attraction. If the blood began to clot prematurely it was forced out by gently applying pressure to the vein with the thumb and forefinger moving from the mouse body toward the wound until a drop of blood formed. The procedure was repeated until the capillary tube was 1/3 full. The capillary tube was placed, Critoseal side down, into a small glass test tube and spun at 500 rpm in a Beckman model TJ-6 centrifuge for ten minutes. The blood serum appreciable size had formed. This procedure was repeated until the capillary tube was about one-third full. The end of the tube was sealed with Critoseal and the serum was withdrawn into a 10 µL microsyringe and diluted into 10 mM sodium phosphate buffered saline (PBS), pH 7.3. Dilutions were made in the range of 400X to 8 x 10^5X and 100 µL of each incubated for one hour in the two strips of previously prepared BPDE-DNA coated microwells as depicted (Fig. 5). The wells were emptied and washed 5 times with PBS containing 0.1% Tween-20, 7 times with dH2O, inverted and tapped gently on a folded napkin to remove residual wash water, and dried for 5 minutes in front of a fan producing a gentle breeze. 100 µL of 3000X goat anti-mouse IgG (whole molecule) horseraddish peroxidase conjugate was pipetted into each well as a probe for the presence of bound anti-BPDE-DNA antibodies. The wells were again washed as above, inverted and tapped dry followed by the addition of 100 µL of enzyme substrate and 100 µL of a chromophore. The wells were allowed to develop for 15-20
Fig. 5. Titer Assay. Well configuration for the titer assay with one strip containing unimmunized mouse serum as a control (top) and rendering of the developed immunowells (bottom)
minutes and the reaction stopped with 50 µL of 2 N H₂SO₄. Finally, each wells absorbance was read at 450nm on a model EL307C microplate reader from Bio-Tek Instruments.

c. Cell Fusion

Just prior to the mouse immunization process, a culture of mutant mouse myeloma cells (NS-1) was revived from storage in liquid nitrogen. This was accomplished by defrosting 8 x 10⁶ cells by immersion of the cryogenic tube in 70% EtOH at 37°C. The contents were pooled into a 15 mL centrifuge tube and spun using a bench top clinical centrifuge. The supernatant was aspirated off and the pellet resuspended in 12 mL of complete medium which was distributed into four Falcon 25 cm² culture flasks each containing 3 mL. The cells were cultured in a water jacketed incubator at 37°C, 5% CO₂ and 100% humidity to insure a quantity of 20-50 million cells on the day of the cell fusion.

The day the cell fusion was to take place, mouse thymocytes and splenocytes were also prepared. All surgical tools were autoclaved in preparation of harvesting cells in addition to polyethylene glycol (PEG) contained in a covered glass tube.

A young unimmunized mouse (less than 8 weeks of age) was sacrificed by cervical dislocation and it’s throat and abdominal area sterilized with 70% EtOH. The thymus was removed and washed three times by immersion in 3 mL of serum free medium (SFM) contained in each of three small petri dishes. After the third wash, the thymus was pulled apart with tweezers forming a suspension that was filtered through a wire screen into a sterile test tube to remove excess tissue. A small sample of the filtrate was diluted 1:400 with SFM and the cells counted. This procedure was repeated to recover
the splenocytes from the spleen of the immunized mouse. The proper number of thymocytes for a cell fusion is $2 \times 10^8$ cells. The ratio of NS-1 to spleen cells was maintained between 1:2 to 1:5 so that the number of spleen cells required was $1 \times 10^8$.

The correct volume of each of the cell suspensions, in addition to medium containing the NS-1 cells, was centrifuged at 500 rpm for 10 minutes to yield the proper number of each cell type. The $2 \times 10^8$ thymocyte cells were resuspended in IMDM solution prepared by the addition of 1.4 mL of 2X IMDM, 10 mL fetal calf serum (FCS), 2 mL 50X hypoxanthine-aminopterin-thymidine (HAT), 0.2 mL 4 mg/mL Lipopolysaccharide (LPS), and 1.4 mL of sterile dH$_2$O. The total volume of the thymocyte containing IMDM solution was 15 mL. The splenocytes and the NS-1 cells were both resuspended in 5 mL each of SFM. The fusion was begun by mixing the 5 mL suspensions of splenocytes and NS-1 cells and centrifuging for 5 minutes at 500 rpm. The supernatant was aspirated and 50% PEG was added followed by 9 mL of SFM. The reaction mixture was centrifuged at 500 rpm for 5 minutes and the supernatant suctioned away by aspiration. The pellet was resuspended in 15mL of thymocyte containing IMDM previously prepared and kept at 37°C. This was then added to 25mL IMDM containing methylcellulose and the solution was mixed by inverting the tube. The solution was drawn into a 25mL sterile serological pipet, and dispensed into small petri dishes. The cultures were incubated at 37°C, 5% CO$_2$, and 100% humidity in a water jacketed incubator.

d. **Clone Picking**

Two to three weeks following the cell fusion the cells were monitored closely for the presence of colonies which, when held up to a fluorescent light, appeared as white
circular growths within the gel. Using direct light under the dissecting microscope, colonies appeared as dark spheres contrasted against the bright white gel. Once the colony was between 0.5 and 1.0 mm in diameter, homogenous, smooth with well defined edges, it was harvested for screening. If it was uncertain as to whether two colonies had grown together or if colonies were closer than 1.5 mm apart, these colonies were discarded. The colonies suitable for picking were counted and 200 µL of complete medium (see section 2e for preparation) containing 5 x 10^4 thymocytes was pipetted into the same number of wells in a 96-well microplate. Colonies, while being viewed through a dissecting microscope, were picked by suctioning into a 50 µL pipet taking great care not to disturb neighboring colonies. Any fragments of colonies left behind in the gel after clone picking were removed to avoid the possibility of unnecessary duplicate hybridomas. The colonies were pipetted into separate microwells of the sterile 96-well microplate previously prepared. The cultures were incubated at 37°C, 5% CO₂, and 100% humidity in a water jacketed incubator.

**e. Duplication of Clones**

The cultures were inspected daily for contaminants or to see if feeding was necessary. A pink (more basic) medium indicated no feeding was necessary while an orange colored medium indicated that cells would need to be fed within several hours. The cells were fed immediately if the medium was colored yellow (more acidic). All cultures were fed complete medium warmed to 37°C prepared by combining 375 mL of sterilized dH₂O, 5.2 g RPMI (10X) growth medium, 10 mL HT supplement (50X), 11 mL NaHCO₃ (7.5%), and 5 mL P/S (100X). The pH was adjusted to 6.8-7.0 by the addition of 1 N HCl and the volume brought to 450mL. The solution was then sterilized
by pumping the media through a 0.22 µm membrane into a sterile bottle. Finally, 50 mL of Fetal Calf Serum (FCS) was added to the medium which was stored at 4°C. If the wells were found to be 1/3 full, then excess medium was pipetted off and saved at -20°C in a labeled test tube leaving the well about ¼ full. New medium (containing 5 x 10^4 thymocytes if feeding occurred within one week of clone picking) was added dropwise.

If the cells occupied two-thirds to three-quarters of the bottom surface of the microwell, the cells were split into a duplicate 96-well microplate. The splitting served three purposes. First, in the event of contamination the clone line would not be lost, second, cell overgrowth leads to slower growth or death, and third, a larger volume of culture medium containing antibodies accelerated the screening process.

**f. Clone Screening**

After obtaining a minimum of 200 µL of used media from a cell line, the media was screened for the presence of antibodies against BPDE-DNA adducts. This was accomplished by coating 20 ng of BPDE modified DNA and unmodified DNA onto Immulon II microwells. 40 µL of 0.5 µg/mL BPDE-modified DNA was pipetted into Immulon II microwells and the solvent evaporated in vacuo at RT. Similarly, 40 µL of 0.5 µg/mL unmodified DNA was pipetted into a duplicate set of Immulon II microwells and the solvent evaporated. The wells were then washed 5 times with PBS containing 0.1% Tween-20, 7 times with dH_2O, inverted and tapped gently on a folded napkin to remove residual wash water, and dried for 5 minutes in front of a fan. Each well was then blocked with 200 µL of 3% ovalbumin for 2 hours and washed as above. Then 50 µL of medium from each hybridoma cell line was pipetted into separate wells and diluted with 50 µL of 10 mM PBS and incubated for one hour and the wells washed.
100 µL of 3000X goat anti-mouse IgG (whole molecule) horseraddish peroxidase conjugate was pipetted into each well as a probe for the presence of bound anti-BPDE-DNA antibodies. The wells were again washed as above, inverted and tapped dry followed by the addition of 100 µL of enzyme substrate and 100 µL of a chromophore. The wells were allowed to develop for 15-20 minutes and the reaction stopped with 50 µL of 2 N H2SO4. Finally, each wells absorbance was read at 450nm on a microplate reader.

**g. Amplification of Positive Clones for Antibody Production**

Clones that were determined to be positive during the screening process were selected for amplification both *in vitro* and *in vivo*. Positive cultures were taken from the duplicate microwell plate by gently suctioning the medium up and down using a sterile pipet until all the cells were suspended in a homogenous mixture. About ¼ of the medium was left in the well to propagate more clones whereas the remainder of the medium was pipetted into a 24-well microplate and fresh medium added as necessary. When the cell density of the well was such that 2/3 to 3/4 of the bottom of the well was covered with growth, the cells were split into empty, neighboring wells and feeding continued. This process was repeated until the cell density was sufficient to seed a 25 cm³ culture flask. The medium collected from feeding the positive cultures was saved by freezing at -20°C until antibody purification. The cells were continually split into new sterilized 25 cm² culture flasks until a total cell count of between 10-20 million was obtained. An eight week old Balb/c mouse was injected with 0.5 mL of pristane intraperitoneally 3-4 days prior to cell injection. The day of the injection the cells were counted and a sufficient volume of medium centrifuged to yield 10-20 million cells. The
supernatant was aspirated off and the cells resuspended in 2 mL of SFM. The cell suspension was drawn into a 3cc syringe with an 18 G needle to insure the cells were not lysed. The area of injection was sterilized with 70% EtOH and the injection given slowly. The progress of tumor growth was monitored beginning 1-2 weeks following the injection. By firmly holding the mouse and gently pushing on the area of the injection, it was possible to tell if the cancer was in the form of solid or liquid tumor. If the tumor was determined to be a liquid tumor, ascites fluid was removed from the mouse occasionally by inserting an 18 G needle into the swollen area and removing it quickly. The abdomen was gently squeezed to release the fluid into a centrifuge tube where as much as 0.5 to 5.0 mL was expected. Upon expiration of the mouse which has taken anywhere from 2 to 8 weeks, the remainder of the ascites fluid was removed via invasive surgery where all remaining fluid was aspirated and the body cavity extracted of any residual antibodies with 10 mM PBS. If the tumor was solid, no harvesting of ascites fluid was possible. Instead, upon expiration of the mouse, the tumor was surgically removed and placed in a test tube. The test tube was filled with 10 mM PBS until just covering the tissue which was ground in a tissue grinder until the solution was homogeneous. The cells were then sonicated in a small basin ultrasonicator using 60 second burst/rest intervals for 5 cycles. The homogenate was then centrifuged twice at 3000 rpm in a model TJ-6 centrifuge from Beckman to remove cellular debris and fat. All ascites and wash fluids were pooled and stored at -20°C until purification of the antibody.
h. Antibody Purification

Antibodies were purified from culture medium, ascites and wash fluids using Protein A Sepharose affinity chromatography. A small Protein A column was equilibrated at 4°C with 10 mM PBS, pH 7.4 until a baseline was reached at 280nm. The PBS buffer was discontinued and residual buffer pipetted off the top of the column. The sample was applied to the column followed by continued washing with PBS until a baseline was again reached. The buffer was then changed to 52.0 mM citrate buffer with 24.5 mM phosphate at pH 3.0. Within 15 minutes, a smaller peak eluted from the column which was collected in a clean flask. During this process, the pH of the collected fraction was adjusted with 1.0 N NaOH to maintain a pH of approximately 7.0 to avoid denaturation of the antibodies. The collected fraction was dialyzed against PBS in an Amicon Ultraconcentrator and concentrated to a final volume of 0.5 to 1.0 mL. The absorbance of the dialyzed and concentrated antibodies was measured at 280nm using a Cary 3-Bio spectrophotometer. The value of the absorbance at 280nm was divided by the extinction coefficient of 1.35 mL/mg resulting in the concentration of the antibody solution (22).

3. Development of Sandwich Assay

Following the production and purification of monoclonal antibodies specific for BPDE-DNA adducts, an assay was developed for use in probing human DNA samples. The assay involved the immobilization of DNA on stationary plastic microwells followed by incubation with the antibody. The parameters for which were determined as outlined in the following text.
a. Microwell Study

Both modified and unmodified DNA were coated onto immunowells from Corning, Dynatech, and Nunc. Dynatech produces several types of immunowells, three of which (Immulon I, Immulon II, and Immulon IV) were used in this study. All immunowells were coated with 50 ng of 1% modified DNA and 50 ng of unmodified DNA diluted with 5 mM K$_3$PO$_4$ buffer at pH 7.0, as well as water blanks by evaporation over calcium chloride at 25°C and approximately 0.5 torr. The wells were washed 5 times with PBS containing 0.1% Tween-20, 7 times with dH$_2$O, inverted and tapped gently on a folded napkin to remove residual wash water, and dried for 5 minutes in front of a fan producing a gentle breeze. The wells were blocked with 200 µL of 0.3% ovalbumin dissolved in PBS for 2 hours, washed as above, and each well incubated with 20 ng of Ab-A6 for one hour followed by another washing. The presence of antibody bound to the wells was detected using a goat anti-mouse IgG horseradish peroxidase conjugated antibody specific for whole IgG molecules. 100 µL of 3000X enzyme conjugate in 10 mM PBS was incubated in each well for 30 minutes and the wells washed and dried. The wells were again washed as above, inverted and tapped dry followed by the addition of 100 µL of enzyme substrate and 100 uL of chromophore. The wells were allowed to develop for 15-20 minutes and the reaction stopped with 50 µL of 2 N H$_2$SO$_4$. Finally, each wells absorbance was read at 450nm on microplate reader zeroed with a 250 µL volume of water. Each DNA assay was conducted in five trials. The blanks were coated with water, blocked and assayed in the exact manner as all other wells.
b. Blocking Study

To find a suitable blocking agent to use with Ab-A6 on the microwells determined from 3a, 0.3% solutions of Ovalbumin (Sigma 5503), BSA (Sigma 6003), (Sigma 7906), (Sigma 3059), (Sigma 4503), purified egg white (JAJ), and unpurified egg white diluted in 10 mM PBS (2X), (4X), (8X), (12X) were tested. Microwells were coated with 100 µL of 500 ng/mL Ab-A6 in Tris-HCl overnight. The wells were washed and dried as in 1a followed by the addition of 200 µL of each blocking agent (N = 4) and the plates incubated for 2 hours, washed, and dried. 100 µL of 3000X goat anti-mouse IgG Fab horseradish peroxidase conjugate was added as the second antibody. The plates were developed as in 3a.

c. Saturation Study

In order to determine the optimum amount of antibody that would result in the maximum obtainable signal, twelve serial dilutions of Ab-A6 were made in triplicate into microwells coated with 100 ng of modified DNA as in 3a and blocked with 0.3% ovalbumin (Sigma 5503) as in 3b. Dilutions began at a concentration of 5 µg/mL in 10 mM Phosphate buffered saline at a pH of 7.35. The volume of antibody used was 100 µL. The plate was incubated in a moist chamber at RT for one hour. Following the incubation the plate was washed 5 times with PBS containing 0.1% Tween-20, 7 times with dH2O, inverted and tapped gently on a folded napkin to remove residual wash water, and dried for 5 minutes in front of a fan. The microwells were then assayed for the minimum amount of antibody that would yield the greatest signal. This was accomplished by using goat anti-mouse IgG horseradish peroxidase whole molecule as in 3a.
d. Washing Study

To ascertain the optimal conditions for plate washing, one strip (12 wells) was coated with 400 ng of 1% modified DNA per well by evaporation over calcium chloride at RT and 0.5 torr. The wells were washed five times in succession with either 10 mM PBS containing 0.05% tween-20, 10 mM PBS, or deionized water. All wells were then washed five times in succession with deionized water, inverted and tapped gently on a napkin to remove excess water, and dried in front of a fan producing a gentle breeze. Each well was incubated with 100 µL of 1 µg/mL anti-BPDE-DNA antibody for one hour and washed as above. The second antibody, goat-anti-mouse IgG horseradish peroxidase, was added at a dilution factor of 5000 and volume of 100 µL to all wells for 30 minutes and washed. Development was carried out by adding 100 µL each of solution A and solution B to all wells and incubated at RT for an additional 30 minutes.

4. ELISA performance Characteristics

a. Linearity Study

Modified DNA of varying percent modifications (PM) were each assessed for their ability to bind to the anti-BPDE DNA antibody. The DNA concentration in each microwell was held constant by diluting modified DNA into wells containing unmodified DNA of the same concentration as 1X modified DNA. For a beginning concentration of 1 µg/mL, 100 µL of 1 µg/mL unmodified DNA was pipetted into all wells to be assayed. Then 100 µL of 2 µg/mL modified DNA was serially diluted into wells 1-12. The plate was then dried over calcium chloride at room temperature at less than 1 torr overnight until there was no evidence of moisture on the surface of the wells. The wells were blocked to a volume of 300 µL with 0.3% ovalbumin (Sigma 5503) for
two hours and washed 5/5 with 10 mM PBS containing 0.05% tween-20 followed by water. The wells were dried in a gentle breeze and incubated with 50 ng of antibody per microwell by the addition of 100 µL of 500 ng/mL anti-BPDE DNA antibody for one hour and again washed as above. Following was the addition of 100 µL of 5000X goat-anti-mouse horseradish peroxidase conjugate to each well. The incubation proceeded for 30 minutes at RT and the wells were washed and dried. Development was started by adding 100 µL each of solution A, the enzyme substrate, and solution B, the chromophore. The reaction was stopped by adding 50 µL of 2 N H₂SO₄ to all wells. The absorbancies of each plate were read on a microplate reader at 450nm. This study was performed with DNA in both double and single stranded forms. To assess the ability of the anti-BPDE antibody to bind adducts in single stranded DNA, modified and unmodified DNA was diluted to 4 µg/mL with 5 mM potassium phosphate buffer, pH 7.0. Single stranded (denatured) DNA was prepared by immersing the test tube into boiling water for 10 minutes followed by immediate immersion into an ice water slurry for 20 minutes with frequent agitation. This trial was not performed at constant DNA concentration so both modified and unmodified DNA were coated in duplicate by serially diluting 100 µL of 4 µg/mL 1% modified or 4 µg/mL unmodified DNA into 100 µL aliquots of 5 mM potassium phosphate buffer, pH 7.0, previously pippeted into the microwells. Twelve serial dilutions were done in all. Well washing, blocking, and the assay for the presence of mouse immunoglobulins was accomplished as in the previous experiment.
b. Conformation of DNA denaturation by agarose gel electrophoresis

A 1% agarose slurry was prepared by suspending 10 g of agarose per 100 mL volume of 40 mM Tris-acetate buffer containing 2 mM ethylene diamine tetra-acetic acid (EDTA) at pH 7.8. The solution was heated until the agarose completely dissolved and allowed to cool. When the temperature of the solution reached 50°C, a volume of 10 mg/mL ethidium Bromide dissolved in water was added to the gel solution to bring the concentration of the dye to 0.5 µg/mL. The gel solution was poured into a Bio-Rad mold to a depth of 3-4 mm and an appropriate gel comb added. The gel was allowed to congeal for 30 minutes and the comb removed followed by an additional 30 minutes cooling time. The gel was transferred to the electrophoresis chamber which was then filled with 40 mM Tris-acetate buffer containing EDTA and 0.5 µg/mL ethidium bromide at pH 7.8 until the level of the buffer was just covering the top of the gel.

Previously, the DNA samples were prepared for electrophoresis by an ethanol precipitation of sufficient volume to yield approximately 2-3 µg of DNA. The samples were centrifuged at 12,000 rpm for 20 minutes. The ethanolic overhead was decanted and the centrifuge tubes containing the samples were dried under vacuum at less than 0.5 torr. The dried DNA was dissolved in 10 µL of gel-loading buffer comprised of 40 mM Tris-acetate, 50% glycerol, and 0.25% bromophenol blue tracking dye at pH 8.0. The power supply was connected and the gel run at 70 volts until the tracking dye was at the edge of the gel. The power was discontinued and the gel visualized on a UV light examining table.
c. Precision study

To determine the ability of the anti-BPDE DNA antibody to repeatedly bind antigen at concentrations within the linear range, 1% modified DNA was coated onto 24 microwells (2 strips) at 150, 375, and 750 ng/well requiring a total of 6 strips. The sample volume was 100 µL per well which was dried at RT over calcium chloride and less than 0.5 torr. The wells were blocked, washed, and assayed as in the linearity study (4a).

d. Determination of the detection limit

The detection limit was calculated by data obtained from the following experiment. Immulon I microwells were coated with 1% modified DNA diluted into unmodified DNA. Unmodified calf thymus DNA was diluted with 5 mM potassium phosphate buffer, pH 7.0 to a concentration of 1 µg/mL. 100 µL of this solution was pipetted into each of 36 microwells. Next, an equal volume of 2 µg/mL 1% modified DNA was serially diluted twelve times (the equivalent of one strip of microwells). Three trials, at each of twelve concentrations were prepared. Likewise, 24 microwells were filled with 100 µL of unmodified calf thymus DNA to acquire the background signal and resulting noise. The entire plate was labeled and placed into a vacuum oven where it was dried at RT over calcium chloride at less than 0.5 torr. This process took overnight and the wells were carefully inspected for signs of moisture the following morning to determine if further drying was necessary. Dried plates were washed, blocked, and assayed according to section 4a.
CHAPTER 3

RESULTS

A. VERIFICATION OF REACTANTS

Due to the reactivity of the active metabolite benzo[α]pyrene diol-epoxide to light and moisture a pre-reaction scan of the compound was not attempted. Instead, to prove that the DNA was exposed to BPDE, the ethyl acetate-butanol mixture containing unreacted BPDE from the DNA extractions was evaporated in a fume hood. The residual off white powder was reconstituted in a 50% acetone:water mixture and the UV-visible spectrum taken from 200-450 nm. The results of the scan were compared to a spectrum obtained from the National Cancer Institute (NCI) repository. Both spectra displayed strong absorbances at 244, 375, and 344 nm characteristic of benzo[α]pyrene diol-epoxide (Fig. 6-7). The unmodified calf thymus DNA used in the reaction with benzo[α]pyrene was checked prior to its use. The UV-Visible spectrum was taken from 200-450 nm on a Carey 3-Bio spectrophotometer resulting in a broad peak with $\lambda_{\text{max}}$ of 260 nm corresponding to nucleic acids. No other peaks were observed (Fig. 8).

B. MODIFICATION OF DNA WITH BPDE

Following the modification of DNA with BPDE and removal of excess reactant, the absorbance at 260 and 350 nm was taken and an approximation to the percent modification (PM) was calculated according to the following published by (Weinstein et al. 1976): $\text{PM} = 100 \times \gamma_{260} \times (A_{350}/\gamma_{350}) \times \left[A_{260} - (0.2(A_{350}))\right]$ where $\gamma_{260}$, the extinction coefficient for denatured calf thymus DNA, was $8.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\gamma_{350}$, the
Fig. 6. UV-Visible scan of benzo[a]pyrene diol-epoxide. UV-Visible scan on Cary 3-Bio showing the major wavelengths of maximum absorbance of (+)-(7S, 8R, 9R, 10S)-7, 8-dihydroxy-9, 10-epoxy-7, 8, 9, 10-tetrahydrobenzo[a]pyrene
Fig. 7. Literature UV-Visible scan of benzo[α]pyrene diol-epoxide.
extinction coefficient for BPDE-dG adducts, was $2.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. It could be seen from a wavelength scan of post reaction DNA that the profile had changed as compared to the pre-reaction sample. Superposition of figure 5, BPDE, and figure 7, unmodified DNA, would account for the data obtained in figure 9. The formula above incorporated a correction factor of $A_{260} - (0.2(A_{350}))$ to account for the fact that deoxyguanosine adducts exhibit strong absorbance characteristics close to 260 nm as does the DNA polymer. The correction factor assured that the ratio of measured adducts to DNA was a reflection of the overall DNA modification as validated by isotopic labeling. The percent modification, has been defined as the "percentage of nucleotides in the polymer covalently modified by" BPDE". Reactions were then run exposing unmodified DNA to varying concentrations of BPDE which produced a sigmoidal curve (Fig. 10).

C. VERIFICATION OF ANTIBODY PRODUCTION BY TITER ASSAY

After modification of DNA with BPDE, the antigen was subjected to the immune system of a live mouse to produce an antibody of desired specificity. This was accomplished by injecting 40 µg of highly modified DNA directly into the spleen of the mouse followed by periodic booster shots, also of 40 µg, given intravenously. Verification of antibody production was determined by immobilizing the desired antibodies in the mouse serum by solid phase enzyme linked immunosorbant assay (ELISA). The titer of the particular assay shown in figure 11 was found to be approximately 2900.

D. CELL FUSIONS

After completion of two cell fusions, a total of more than 100 viable clones were isolated and cultured. Of the harvested clones some displayed more characteristics of
Fig. 8. UV-Visible scan of unmodified reactant DNA. UV-Visible scan of 6X unmodified reactant DNA at approximately 0.2-0.3 mg/mL in 0.05 M cacodylate buffer pH 7.1.
Fig. 9. Absorbance profile of DNA modified with BPDE-I.
Fig. 10. Percent modification vs. micromoles of BPDE used in the reaction. Plot of absorbance taken from 0.3 mg/mL samples of calf thymus DNA treated with varying concentrations of BPDE.
spleen cells including slower growth and a higher mortality rate. Other clones retained characteristics of the NS-1 fusion partner which generally divided more quickly and were more likely to survive. The colonies were allowed to grow until they produced enough antibody containing used medium to screen each colony for the presence of positive clones (Fig. 12-13). To assess whether or not a clone tested positive or negative for production of anti-BPDE-DNA antibodies, the used culture medium was incubated in the presence of modified and unmodified DNA. The y-axis response reflects the signal obtained from wells coated with modified DNA minus the signal obtained from wells coated with unmodified DNA where positive responses are characteristic of antibodies against BPDE-DNA. In many cases, colonies grew at a rate such that clone screens were able to be conducted weekly and each clone line was tested in duplicate. Because there was a distribution of cells, some slow growing and some fast growing, a method of testing was devised resulting in a value termed “clone effectiveness”, which accounted for both a clone lines number of positive screens as well as its rate of growth. In this way, only the fastest growing positive clones were selected for expansion for use in antibody production. Through experience it was found that in many cases the slower growing clones were unable to be amplified to a degree suitable for antibody production. During each clone screen, a clone was tested at least three times and clone effectiveness expressed as a decimal percent. Overall clone effectiveness was taken as the sum of clone effectiveness for all screens (Fig. 14). Therefore, clone lines that were slow growing were tested less frequently than faster lines and had much lower efficiencies. It was determined from the data that clone lines A2 and A6 tested positive most often and were the most viable in terms of growth rate and appearance. The growth was assessed
Fig. 11. Titer assay of a mouse immunized with BPDE-DNA. The absorbance at one-half the difference between the maximum and minimum indicating a titer of 2900.
by cell counts as well as identifying dividing cells to assure that the cells were undergoing all phases of growth. Healthy clones were those that were medium to large in size, translucent, and spherical.

E. AMPLIFICATION OF POSITIVE CLONES

All culture media containing the newly produced hybridoma cells was formulated with a colorometric pH indicator. Fresh media was a red-orange color in contrast to the spent media which was a less intense shade of this color to light yellow. When the cell media was determined to be depleated, clones testing positive were split into empty neighboring wells until a sufficient number of cells were available to translocate the cells. The media containing the cells of a single monoclonal cell line were then pooled into a larger culture flask. The order of progression was from the initial 96-microwell plate to a 24-microwell plate and finally to a 25 cm² culture flask. The medium was changed as necessary until 20-40 million cells were collected to use for the in vivo production of antibodies.

Mice injected with specific anti-BPDE-DNA hybridoma cells generally became symptomatic for carcinoma within two to three weeks with the appearance of easily visible abdominal tumors. These tumors were either solid tumors or the more preferable liquid tumors. Both types usually became systemic within approximately two to three weeks following the first discovery of cancer and it was observed that mice entering this stage of disease had greatly increased appetites with the gradual onset of lethargy, paralysis, and death. Immediately following the expiration of the animal the tumor was removed and kept frozen at -20°C until purification. Solid tumors removed were from 5
Fig. 12. Clone Screen for Fusion 1.
Fig. 13. Clone Screen for Fusion II.
Fig. 14. Clone effectiveness. Each clone was tested in triplicate per screen and clone effectiveness expressed as the decimal percent. Overall clone effectiveness was taken as the sum of clone effectiveness for all screens.
to 15 grams appearing as fatty tissue while the volume of ascities fluid from liquid tumors was from 0.5 to 10 mL having a milky white color sometimes mixed with blood from internal hemorrhaging.

F. ANTIBODY PURIFICATION

After obtaining ascities fluid from the mouse or solid tumor homogenate, the fluid was combined with used medium generated from the cell cultures used to amplify the hybridoma. The solution was then purified using Protein A affinity chromatography monitored at 280 nm. Each chromatograph resulted in two peaks (Fig. 15). The first and largest of the two peaks was representative of all protein matter having little or no affinity for the solid phase. This fraction was termed “pass”. Once the elution buffer was changed following a return to base-line, a second, usually much smaller, peak was obtained indicative of the elution of immunoglobulins. This fraction, which varied in amount from 1-10 mL of slightly turbid liquid, was collected and concentrated to a volume of 0.5-1.0 mL. Each concentrated fraction was measured at 280 nm. This value was divided by the extinction coefficient of 1.35 mL/mg for class G immunoglobulins and the concentration determined. Each purification generated anywhere from 1 to 5 mg of purified antibody specific for BPDE-DNA adducts which was kept frozen at -20°C until use.

G. DEVELOPMENT OF SANDWICH ASSAY

1. Microwell Study

Microwells of varying types were coated with 50 ng of modified DNA, 50 ng of unmodified DNA, as well as water blanks. Each type of immunowell was made of a
different plastic polymer to assess its ability to immobilize the DNA used in the assay. Immunowells produced by Dynatech (Immulton I and Immulton II and Immulton IV), Nunc, and Corning (Costar and “Corning Brand”) were tested and rated based on the signal to noise ratio obtained. Immulton I microwells generated the highest value followed by Costar and Corning Brand. All other well types produced no appreciable signal. The signal to noise ratios (S/N) were 6.183, 3.347, and 1.791 respectively.

2. Blocking Study

Solutions of 0.3% OVA(Sigma5503), BSA(Sigma 6003, 7906, 3059, and 4503), purified egg white (JAJ), fibrinogen, gelatin, and unpurified egg white diluted in 10 mM PBS (2X), (4X), (8X), and (12X) both denatured and non-denatured were tested for their ability to lower non-specific binding to the microwells determined in 7a.

3. Antibody Saturation Study

To determine the optimum amount of antibody to use in the assay, a constant amount of DNA was coated onto each well surface and blocked. The antibody was added over a wide range of concentrations to find the smallest amount that would generate the maximum signal. Figure 16 on the preceeding page indicates that the highest signal obtained was 1.36AU beginning at 100 ng of antibody per microwell. Using more antibody was not beneficial as it did not increase signal strength.

4. Washing study

A washing study was performed to ascertain the optimal conditions for the sandwich assay. Trials were conducted where the microplates were washed 5/5 with 10 mM PBS containing 0.05% tween-20 / deionized water, 10 mM PBS / deionized water, or deionized water / deionized water. It was found that the wells washed 5/5 with 10 mM
Fig. 15. Antibody purification elution profile. The profile above indicates that all non-IgG proteins from culture media and cellular debris are eluted in the "pass" (large peak) fraction with 10mM PBS buffer. When the buffer was changed to 52mM citrate, all IgG molecules were selectively eluted and collected (small peak).
PBS / deionized water resulted in the highest signal and greatest precision while offering acceptable background noise.

5. Linear range study

DNA was coated onto microwells at varying concentrations in an attempt to find a range over which the signal produced would be linear. DNA in both single (denatured) and double (native) stranded forms was used. In the native DNA experiment, the range of DNA concentrations was varied from 1 µg/mL to $4 \times 10^{-4}$ µg/mL corresponding to 100 ng to 0.04 ng/microwell. Figure 16 indicates that the signal appears to be linear from 20 ng/well to 100 ng/well with a correlation coefficient of 0.942. Further experimentation indicated that the upper bound of this range could be extended to 400 ng/well (data not shown). Another trial was conducted to study the effects of DNA denaturation on the assay for DNA-adducts. The DNA, both modified and unmodified, was denatured and two trials of each were prepared in lieu of three. Unmodified DNA produced an overall flat signal across the spectrum of concentrations tested albeit somewhat variable from 0.762 – 0.910 AU. At higher concentrations, modified DNA generated a positive deviation from the unmodified DNA baseline behavior as seen in Figure 17-18 resulting in the linear range. The DNA was checked to insure complete denaturation by agarose gel electrophoresis. A photo of the gel is illustrated in Figure 19. Lanes 1 and 2 were loaded with native and denatured calf thymus DNA respectively. Lanes 3 and 4 were duplicates of 1 and 2 except that the DNA was sheared five times through an 18G hypodermic needle to facilitate fragmentation of the sample. Lanes 1 and 3, containing the native DNA samples, resulted in banding patterns consistent with intact DNA as well as fragmented DNA. Lanes 2 and 4 did not produce
Fig. 16. Antibody saturation study using Immulon I microwells from Dynatech. The figure indicates that the smallest amount of antibody resulting in the highest signal is approximately 100 ng.
any detectable banding with the exception of very faint streaking throughout both lanes. The streaking was more pronounced in lane 2 containing the nonsheared sample.

6. Precision study

To ascertain the precision with which the anti-BPDE-DNA antibody binds to modified DNA, twenty-four microwells were coated with antigen and blocked in preparation for the assay. Unmodified DNA was used as a control and run alongside modified DNA also using 24 replicates. The concentrations used were 175, 350, and 750 ng/well. Figure 20 illustrates a plot of the data obtained in this study. The variability for the 175, 350, and 750 µg/well trials are 8.7, 16.5, and 9.6% respectively.

7. Determination of the detection limit.

To find the detection limit of this assay, 1% modified DNA was serially diluted into unmodified DNA at the lower end of the linear range. The results are shown in figure 21. The mean blank signal was calculated to be 0.986AU ± 0.086 (N = 24) and the minimum signal from which reliable data could be obtained was determined according to the following established by the International Federation of Clinical Chemistry: minimum usable signal = MBS(mean blank signal) + 2.63s.d. of MBS (21). This value on the y-axis intersected with the linear range at 50 ng of 1% modified DNA per well. The limit of linearity, therefore, was shortened from 20 – 400 ng/well to 50-400 ng/well.
Fig. 17. Linear range experiment using 1% modified DNA. Three trials were conducted at twelve adduct concentrations where modified DNA was serially diluted into 1 µg/mL unmodified DNA.
Fig. 18. Linear range experiment using 1% modified DNA and unmodified DNA as a control. Two trials were conducted at twelve adduct concentrations where modified (Δ) and unmodified (V) DNA was serially diluted into 5 mM potassium phosphate buffer, pH 7.0.
Fig. 19 Agarose gel electrophoresis of DNA for verification of the denaturation process. Lanes 1 and 2 contain native and denatured calf thymus DNA respectively. Lanes 3 and 4 are duplicates of 1 and 2 except that the DNA was sheared five times through an 18G hypodermic needle to produce fragmented DNA.
Fig. 20 Precision study. Plot of modified and unmodified DNA depicting the results of 24 trials of modified DNA (black) and unmodified DNA (white) at three concentrations. The variability of each experiment's modified DNA signal can be seen above each bar.
Fig. 21. Results obtained from the determination of the detection limit. The signal (corrected (▲)) was plotted versus modified DNA concentration. Also shown is the detection limit (------).
CHAPTER 4

DISCUSSION OF RESULTS AND CONCLUSION

A. PREPARATION OF ANTIGEN

1. Verification of reactants

The antigen used in these experiments was prepared synthetically by the addition of the active metabolite of benzo[α]pyrene to calf thymus DNA. Benzo[α]pyrene is metabolized in humans by cytochrome p450 to the diol-epoxide, which is the active carcinogen. The metabolite, benzo[α]pyrene diol-epoxide (BPDE), was acquired and reacted with unmodified DNA under an inert atmosphere in the dark to minimize hydrolysis of the activated epoxide ring by ambient moisture or UV radiation. Due to the sensitivity of BPDE to light, verification of the reactants by UV-Visible spectrometry was attempted on post reaction extracts of the modified samples. The absorbance versus wavelength profile for each extract matched that of a profile obtained from literature. It was concluded that the solid obtained commercially was BPDE and that the unmodified calf thymus DNA had come into contact with the carcinogen.

The unmodified calf thymus DNA was also verified using UV-Visible spectrophotometry in a similar manner. A pre-reaction wavelength scan indicated that the DNA was not modified with BPDE. Following the reaction, a similar scan revealed several peaks characteristic of BPDE.
2. Modification of DNA with BPDE.

Once both reactants had been verified, the unmodified calf thymus DNA was reacted with BPDE over a range of concentrations. This was to provide a standard curve whereby target adduct concentrations could be produced by using the appropriate volume of BPDE in the reaction. The concentration of BPDE was varied from 60 to 600 µM and resulted in a saturation type curve. Saturation began at approximately 400 µM after which there was no further increase in the percent modification with higher concentrations of the carcinogen. It has been reported that BPDE preferentially binds to guanosine residues, especially at the exocyclic amino group, making GC rich regions within DNA targets of BPDE binding. It may be that BPDE’s preference for particular residues over others leads to site specificity within the DNA molecule based on sequence context. This may help to explain the saturation curve generated in the reaction of BPDE with DNA.

B. HYBRIDIZATION OF CELLS

1. Verification of antibody production by titer assay

Following immunization of a mouse with the antigen, it was extremely important to determine whether an antibody against the antigen was being produced by the mouse’s immune system. This information was crucial in the planning and success of the cell fusion. Figure 10 illustrates that the animals titer against BPDE-DNA was approximately 2900. A titer of greater than 1000 has been deemed a sufficient indication that an organism possesses a large number of cells producing the particular antibody. It was imperative that this condition be met in order to increase the probability of selecting a positive clone during the clone picking process.
2. Cell fusion

The underlying condition that necessitates performing a cell fusion is the extremely limited time that antibody producing beta cells isolated from the spleen can be sustained in culture. The maximum life span of such cells is only about two weeks which is insufficient time to adequately screen and propagate the cells so that antibodies can be harvested. For this reason, the antibody producing spleen cells were fused to NS-1 cells, a murine myeloma cell line, two main characteristics being immortality and rapid growth. These characteristics are imparted, in whole or in part, to the beta lymphocytes upon hybridization with the NS-1 fusion partner.

Following the hybridization, beta lymphocytes, NS-1, and hybridized cells remained in the culture media. There was no apparent cell morphology to aid in distinguishing between the remaining cell types and so another method was used to separate the hybrid cells from non-hybridized cells. When the cell fusion was complete, the cells were resuspended and grown in HAT selection media containing aminopterin. Aminopterin arrests purine synthesis so that cells lacking the purine salvage pathway are unable to survive in the presence of this selection media. NS-1 : NS-1 fused cells or unfused NS-1 cells die during this process. Splenocyte : splenocyte or unfused spleen cells will continue to live in the media for about two weeks, their normal lifetime in culture. Fortunately, the growth phase, clone picking, and clone screening extend substantially longer than two weeks so that all non - (NS-1 : splenocyte) cell lines are selected against.

The culture media, in addition to selecting for the proper hybridized cell lines, was specially formulated to provide a thick matrix which retarded cell mobility to prevent mixing of the clones or their colonies but allowed the translocation of cell nourishment.
In this way, the clones were immobilized within the media so that clone mixing and the production of polyclonal antibodies was nominal.

Immobilization of the clones while in the initial stages of culture led to unforeseen problems that were easily eliminated in subsequent fusions. In the first fusion the initial observation of fast growing clones led to the assumption that the fusion was a complete success. Within several days it could be seen that the number of colonies growing in the media was too large and that some colonies would begin overlapping before they could be isolated. This fusion resulted in less than 100 clones harvested before significant colony overlapping suspended further clone picking. This was eliminated by taking due care during the cell counting process of the cell fusion so that the proper cell density could be established and maintained.

3. Clone picking

Clonal colonies were picked and isolated into individual wells of a 96 well culture plate starting approximately 14 days after the cell fusion. At this point the strategy was to amplify all clones so that each cell line would produce about 1 mL of antibody containing culture media per week to permit screening of the clones. During this time a definite distribution of cells could be identified with some cell lines growing rapidly, some moderately, and some slowly. The rapid growers were usually those that were harvested first while the slow growers were generally isolated later during the picking process. It was found that the rate of growth and cell viability were not intimately correlated. In other words, during the time the cell lines were being amplified, a process of cell stabilization was also in effect. It was not uncommon to find slow growing clones whose growth was substantial enough to permit screening and further
amplification while some of the fast growers would simply disintegrate. This factor was found to be very important and must be considered when making decisions based on the outcome of the clone screens. In order to crudely quantify the rate of cell growth and the clone line’s ability to discriminate between antigen versus non-antigen, a value termed clone effectiveness was defined as the sum of a cell line’s individual clone screens expressed as the decimal percent. In this way, faster growing, more viable clones were screened more often and typically had a higher clone effectiveness. Likewise, cell lines that may have been growing slowly but had a large ratio of positive tests to negative tests also scored higher clone effectiveness values. It was believed that, over time, clone effectiveness was a reliable indicator as to which cell lines were growing healthily and screening positive.

4. Amplification of positive clones and purification of immunoglobulin

After selecting the positive clones determined from clone effectiveness, the cell lines were seeded into 24 well microplates containing fresh growth media and allowed to propagate until the cells almost completely covered the bottom of the wells. One or two wells from the 24 microwell plate were pooled and placed into 3-5 mL of new media in a 25 cm² culture flask. These stocks of cell lines served as a source of cells that could be frozen or used for in vivo antibody amplification. Antibodies were produced in high quantities by injection into the peritoneum of a Balb/c mouse. Very large liquid or solid tumors could be isolated from mice treated with the hybridomas which was subsequently purified with protein A-Sepharose affinity chromatography. The amount of antibody obtained was a random quantity influenced by factors such as the growth rate of the hybridoma cell line used and the ability of the mouse’s immune system to resist the
cancer. Generally, the mice would die within 2-4 weeks and somewhere between 5-10 mg of antibody could be cultivated. When the antibody was concentrated into 10 mM sodium phosphate buffered saline pH 7.4, the concentration varied based upon how much antibody was generated and the final volume obtained during the concentration process. The concentrated antibody solution was clear, water like, having a slight milky appearance which frothed easily even when shaken gently.

C. DEVELOPMENT OF ELISA ASSAY

1. Microwell study

The ELISA used was an antibody capture sandwich assay whereby the antigen was first immobilized on the microwell surface. The antigen coated wells were then coated with a blocking agent to eliminate any unbound well surfaces that may bind components of the ELISA used in subsequent steps. The blocking step was followed by incubation with the anti-BPDE-DNA antibody where, during the incubation, the BPDE-DNA antibody was captured upon binding antigen immobilized on the microwell surface. The last incubation was with goat anti mouse horseradish peroxidase conjugate followed by development. The purpose of the microwell study was to identify the type of microwell having a binding capacity for modified DNA resulting in optimal sensitivity for the assay.

Factors that most influence antigen binding to the microwells are the properties of the polymer used in producing the plastic well plates as well as any residual curing agents that may have been used during their manufacture, and the properties of the antigen being used. Selection of the proper microwells for use with modified DNA was
difficult since most of the commercially available microwells have been designed for use with proteins that are more non-polar in character than DNA.

2. Blocking study

A blocking study was conducted using a total of 12 types of binding proteins both in native and denatured forms to find the best blocking agent for reducing non-specific binding of other ELISA components to uncoated portions of the wells. The blocking agent’s size, shape, and affinity for the microwell surface and the antigen are the variables in question when selecting the proper blocker for an assay. This aspect of the research required creative mental imaging to try and picture how the modified DNA was attached to the surface of the wells. For example if coating the microwells with antigen was to leave areas large enough to accommodate binding of the antigen specific antibody to the well surface yet to small for the blocking protein to fill the gap, that blocking agent would result in high background noise. The solution, in this case, would be to use a smaller blocking protein capable of filling the gaps produced during antigen coating. Currently, there is no information available concerning the structure of antigens bound to specific microwell types or the interaction of blocking agents with bound antigen. It was only possible to find the best blocking agent experimentally.

3. BPDE-DNA antibody optimum concentration study

Following the microwell and blocking agent study, it was also necessary to determine the smallest amount of antibody to use resulting in the largest obtainable signal. This parameter was important because at low concentrations the antibody was completely ineffective or resulted in inefficient antigen-antibody binding which lowered the overall assay sensitivity. At higher than optimal antibody concentrations, the anti-
BPDE-DNA antibody did not lose binding effectiveness as in some cases where high antibody concentrations resulted in competition for antigen binding sites. Instead, the BPDE-DNA antibody bound antigen with a saturation like effect whereby using a higher antibody concentration did not attenuate the highest obtainable signal but resulted only in the wasting of precious biochemical reagents.

4. Linear range determination

Over a broad range of antigen concentrations, there should be found at least one region where absorbance is linearly proportional to concentration. Modified DNA was coated onto Immulon I microwells over a range varying from 400 ng/well to 0.04 ng/well using ½ serial dilutions. After several trials, the results indicated that the linear range began at 3 ng/well and extended to 400 ng/well. It is important to state that the amounts expressed in ng/well that have been discussed thus far correspond to the amount of modified DNA added to each microwell and not the amount of benzo[α]pyrene-DNA adducts that could be found in each well. The DNA used in most trials throughout this project was 1% modified or less so that the DNA concentrations within the linear range rendered the direct measurement of BPDE-DNA adducts below the detection limit of most simple and conventional laboratory methods. Unfortunately, the modified DNA had to be standardized at an extremely high concentration of 0.3 mg/mL so that the absorbance of benzo[α]pyrene at 350 nm could be detected spectrophotometrically. Because the ratio of total nucleotides to modified nucleotides was at least 99:1, it was suspected that the preceding methodology may have been susceptible to rather large matrix effects such as extensive light scattering at the wavelength of interest. This phenomena would result in a greater than actual absorbance measurement giving rise to
an overestimated DNA percent modification. If this were the case, the assay could become more sensitive than previously disclosed. Within the linear range, the curve fit produced a correlation coefficient of 0.942.

5. Antibody performance

To measure the newly produced antibodies ability to repeatedly bind a single quantity of antigen, 24 microwells were each coated with modified and unmodified DNA. The study was conducted at three modified DNA concentrations of 175, 350, and 750 ng/well and the coefficients of variation were 8.7, 16.5 and 9.6% respectively.

The lower limit of detection for the BPDE-DNA antibody was determined using the International Federation of Clinical Chemistry guidelines resulting in a detection limit of 50 ng of 1% modified DNA.

D. SUMMARY AND FUTURE STUDIES

Following the production and testing of the anti-BPDE-DNA antibody, it was found that the antibody was selective for calf thymus DNA modified with BPDE. The usefulness of this antibody in an epidemiological study directed at elucidating the causation of an abnormally high lung cancer rate among Taiwanese women hinged on the antibodies ability to detect very low adduct levels found in human tissues. The current assay method required that predominantly negatively charged DNA adhere to an uncharged plastic polymer. Additionally, well coating was the first of four layers comprising the sandwich assay and between each layer was a washing step to remove any residual reagents. Solvents commonly used for well washing included 10mM phosphate buffered saline pH 7.4 (PBS), PBS containing 0.05% tween-20 (PBS-tween), and deionized water. All of the washing solvents used were high in ionic strength
relative to the plastic wells which may have served to partition the DNA into the solvent phase. Inadvertently removing DNA during any phase of the assay, whether DNA modified with BPDE or unmodified DNA, could have resulted in increased non-specific binding and higher background noise, increased signal variability, an increase in the detection limit, and an overall diminished performance from the assay.

Future work with the BPDE-DNA antibody will first focus on finding a buffering agent capable of binding both the plastic microwells as well as the charged DNA. More than likely this buffering agent will be a protein containing an overall charge of zero but perhaps containing a large number of histidine residues. This protein's non-polar character should facilitate the binding of the protein to the well surface whereas a large number of positively charged histidine residues may serve as a counter ion for immobilization of the DNA. As an alternative to finding a suitable buffering protein, commercially available kits for the biotinylation of nucleic acids could be utilized to tag the DNA. Biotin binds to DNA at the extreme ends so that the protein may act to anchor labeled DNA to the well surface. Furthermore, if the binding of biotinylated DNA to the microwell is found to be inefficient, avidin, a protein with a high affinity for biotin, could be coated on the wells and avidin-biotin binding would serve to anchor the DNA.

The other aspect of this study that deserves renewed attention is the spectrophotometric determination of the BPDE-DNA standards used to calibrate the assay. Although, matrix effects may be minimal at longer wavelengths where benzo[α]pyrene absorbs light, significant variability in the calculation of percent modification (PM) was observed as a function of concentration. The literature containing the only validated formula for calculation of percent modification of calf
thymus DNA modified with BPDE using spectrophotometry did not indicate a target DNA concentration at which the measurement of the absorbances at 260 and 350nm would result in a reasonable estimate to the percent modification. Therefore, the current formula used to calculate the percent modification obtained from the literature should be calibrated by another method before use, perhaps by use of radio-labeled BPDE as a control. Finally, when all other parameters of the assay have been optimized and it has been determined that the antibody functions well under the determined experimental conditions, the sensitivity of the assay can be increased. This can be achieved by altering the enzyme conjugate to contain a radioactive-label or may even include vanguard nanoparticle technologies which can offer chemiluminescent, fluorescent, or light scattering probes. In conjunction with improved enzyme conjugates, assay sensitivity can be enhanced even further by incorporating the use of a competitive ELISA system. Together, the preceding improvements may improve the sensitivity of the current method by at least three orders of magnitude.
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