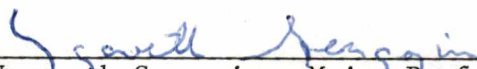


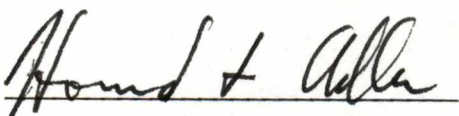
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I am submitting herewith a thesis written by Randall M. Rossi entitled "The Effect of Benzo-pyrene on Humoral Antibody Formation in Vitro." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

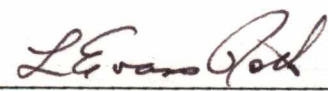

Dr. Nazareth Gengozian, Major Professor

We have read this thesis
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Accepted for the council:


Vice Chancellor
Graduate Studies and Research

THE EFFECT OF BENZO-PYRENE ON HUMORAL ANTIBODY FORMATION IN VITRO.

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Randall M. Rossi

March 1982

3058016

To VC ... with LOVE

ABSTRACT

The significance of chemical carcinogen-induced immunosuppression of the anti-sheep red blood cell response in C3H mice was investigated. The humoral immune response to a T-dependent antigen was simulated using a technique in which splenic lymphocytes were incubated in vitro (3). When the potent carcinogen benzo(a)pyrene (BP) was added to this system, the ability of these spleen cells to respond to a foreign cell, sheep red blood cells (SRBC), was greatly decreased. BP at concentrations as low as 10^{-8} molar was capable of suppressing the immune response. Maximum inhibition, (app. 100%), was obtained at a concentration of 4×10^{-4} molar; this however, may have been partially due to the inherent toxicity of the BP. A characteristic dose response curve occurred in the range 10^{-8} - 10^{-5} molar; lower concentrations showed no inhibition or a slight enhancement of the plaque forming cell response (PFC). When the non-carcinogenic analog of benzo(a)pyrene, benzo(e)pyrene (B(e)P), was tested, no inhibition was seen in the range 10^{-7} - 10^{-5} molar. This supports the existing evidence that BP suppresses the immune system in mice (4,26).

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LIST OF SYMBOLS

Benzo-pyrene	benzo(a)pyrene, benzo(e)pyrene, and all of their metabolites
BP	benzo(a)pyrene
B(e)P	benzo(e)pyrene
MEM	minimum essential medium
FCS	fetal calf serum
GPC'	guinea pig complement
SRBC	sheep red blood cells
BSS	balanced salt solution
RPM	revolutions per minute
'	minute(s)
ACE	acetone
DMSO	dimethyl sulfoxide
PFC	plaque forming cell(s) (response)
PAH	polyaromatic hydrocarbon(s)
MFO	mixed function oxidases
AHH	aryl hydrocarbon hydroxylase
EH	epoxide hydratase

INTRODUCTION

Polyaromatic hydrocarbons (PAHs), represent one of the largest groups of chemical compounds that pollute the environment (12,13,128). These chemicals, which naturally occur in vegetation, arise from the incomplete combustion of fossil fuels, in particular, coal and fuel products (122). Over 300 metric tons of benzo-pyrene alone are released into the atmosphere yearly from coal fired residential furnaces (13,14). In addition to the undesirable effects of this quantity of particulate matter being released into the atmosphere, an ever growing body of experimental evidence has linked PAHs with cancer. In 1775, the English surgeon, Percivall Pott, observed that chimney sweeps had a higher incidence of scrotal cancer due to their occupational exposure to coal dust (15). In 1916, Yamagiwa and Ichikawa were the first to induce skin cancer in rabbits by exposing them to coal tar (16). From this same coal tar, Cook isolated the chemical benzo(a)pyrene in 1933 (17,18), (FIGURE 1) (6). Although BP makes up only a small percentage of the total PAHs that are released into the environment, this carcinogen has been extensively studied as a model compound because of its ubiquitous nature (122,123,128,145). In addition to its carcinogenic properties (14,69,70), it is also mutagenic and teratogenic (14,19,121). Since several tons of BP are released into the environment annually, it is important to understand the effects that this chemical might have on the incidence of cancer in humans and animals.

Over twenty years ago, Thomas proposed that a host possessed a normal homeostatic cellular mechanism active in eliminating nonself

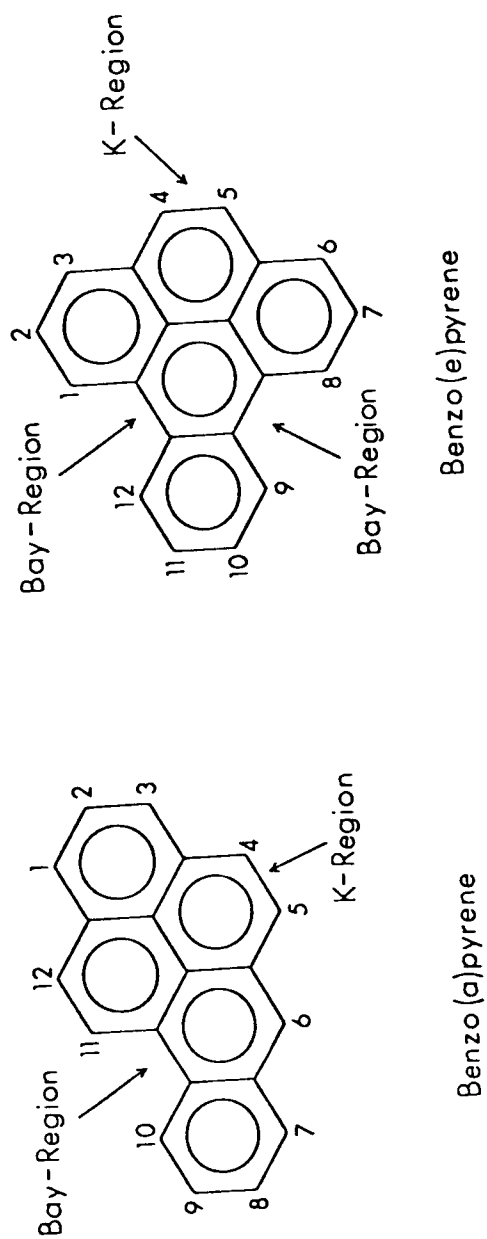


FIGURE 1

STRUCTURE OF BENZO(a)PYRENE AND BENZO(e)PYRENE

antigens such as parasites, mutated, and neoplastic cells (28). The concept implied that immunodeficient individuals would not only be more susceptible to infection, but also to the development of malignant diseases (2). A critical question is how these immunogenic tumors can grow progressively in their immunocompetent synergistic hosts (110). Many attempts have been made to explain this paradox (102-109). It is now generally believed, though not universally accepted, that in order for a malignant cell to survive within a host, either the host's immune system is already depressed by age (23), genetic defects (124), environmental insults (4,125), etc., or the cancerous cell itself is capable of either depressing the immune system (104) or in some manner 'masking' itself from it (120,103). In this way, the neoplasm will be able to propagate within the host.

The main role of the lymphoid system is to defend the host against invasion by viruses, bacteria, fungi, and parasites and to react against foreign cells or material and some neoplasms (129). Non-specific immune mechanisms (e.g. phagocytosis) are enhanced by the development of adaptive immunity characterized by memory, specificity, and recognition of non-self (1). Two major sets of lymphocytes can be distinguished: the B (bursodependent) and the T (thymodependent) lymphocytes. B lymphocytes produce antibody forming cells which release antibody into the surrounding area where it reacts specifically with the antigen; and in conjunction with complement and macrophages (21,146), causes the destruction or inactivation of the antigen (humoral immunity) (1,115-118,129). In the case of SRBC, the cells are lysed. In this process, T cells act initially as helper cells; in the presence of macrophages, they cause B cells to differentiate into antibody forming cells. T

cells are also involved in all cell mediated immunity without B cells. T helper cells may be looked upon as inducers of the effector cells for humoral and cell-mediated immunity and they are also responsible for the generation of T-suppressor cells which exert negative feedback control on the helper cells (1). One concern of these experiments is the effect of BP on the in vitro antibody response involving T and B cells, and macrophages.

CHAPTER I

MATERIALS AND METHODS

ANIMALS

Virgin, male C3H mice, (Cumberland View Farms, Clinton, TN), between the ages of three and six months, were used as the source of splenic lymphocytes for the in vitro cultivation. The sheep red blood cells were obtained from stock animal #838, under sterile conditions; fresh cells were collected each month.

CHEMICALS

Spectroscopically pure benzo(a)pyrene and benzo(e)pyrene, (Sigma Chemical Company, St. Louis, MO), were dissolved in reagent grade acetone or dimethyl sulfoxide, (Fisher Scientific Company, Fair Lawn, NJ). The tissue culture reagents were obtained from the Grand Island Biological Company (GIBCO), Grand Island, NY, except for the following: NaCO_3 , (8.4%, Abbott Laboratories, Norht Chicago, IL, and 7.5%, Microbiological Associates, Bethesda, MD), penicillin G sodium, 5,000,000 units per vial, and mycostatin from E. R. Squibb and Sons, Inc., Princeton, NJ. The fetal calf serum (FCS) was purchased in 500 ml lots from Reheis Chemical Company, Berkeley Heights, NJ and pretested in our lab to determine its suitability for use in the in vitro PFC response. These lots were aliquoted into 10-ml screw top test tubes and frozen until needed.

GUINEA PIG COMPLEMENT ABSORPTION

The lyophilized guinea pig complement (GPC'), (Capelle Laboratories, Cochranville, PA), was absorbed as follows: each vial of GPC' was solubilized with 5-ml of Eisen's solution (23). Sufficient SRBC were washed three times in Eisen's solution for 10' at 1500-2000 RPM at 4° in order to obtain 5-ml of packed cells. Twenty-five ml of solubilized GPC' were carefully added to the SRBC and gently mixed. This solution was allowed to stand for 30' on ice with occasional gentle mixing, then centrifuged for 30' at 2000 RPM, 4°C. The supernatant was removed and added to a fresh 5-ml of packed SRBC and the above procedure repeated. The supernatant, absorbed GPC', was aliquoted into 2-ml portions on ice and frozen until needed.

CELL CULTURE TECHNIQUE

The cell cultures were prepared according to the technique of Mishell and Dutton (20) as modified by Cosenza et al. (21), Hübner and Gengozian (23), and Urso and Gengozian (22). The spleens of decapitated, exsanguinated mice were obtained under sterile conditions. Each spleen was cut five or six times with a sharp scissors and then gently squeezed through a fine nylon mesh with the flat end of a plunger from a plastic 1-mm Falcon, (Falcon Plastics, Los Angeles, CA), tuberculin syringe in Hanks' balanced salt solution (BSS), (10X concentrate diluted 1:10 with sterile 2X distilled water), in order to disperse the cells. The suspension was allowed to settle for 3-5 minutes before the supernatant was gently removed with a Pasteur pipette and diluted with 10-ml of Hanks' BSS. The suspension was washed once in Hanks' BSS, (centrifuged at 1350 RPM, 4°C, for 10'), and once in 10-ml of minimum essential medium (MEM)

and centrifuged as above. The cell pellet was diluted with MEM, counted with a white blood cell diluting pipette, and adjusted to 12 million cells per ml. At this point, the experimental insult, acetone control, BP, or B(e)P, was added. Initially, the spleen cells were pipetted into Falcon #3001 tissue culture dishes, 1-ml per dish, and the insult added to the dish; this however, resulted in a prohibitively high localized concentration of acetone or acetone-benzo-pyrene that killed a significant portion of the cells. To minimize this, the insults were added to the MEM-spleen cell suspensions before the 1-ml aliquots, containing 12×10^6 spleen cells, were placed in the tissue culture dishes. The spleen cells were mixed with SRBC, (centrifuged at 1400 RPM, at 4° for 10' in Hanks' 1X twice, and MEM once) and incubated at 37°C in Lucite boxes under an atmosphere of 7% CO₂, 10% O₂, and 83% N₂. The cultures were rocked continuously, 7-10 oscillations per minute, on a rocker platform, (Bellco Glass Company, Vineland, NJ), and fed daily with 1 drop of FCS and 2 drops of nutritional mixture (21) from a 1-ml Falcon plastic pipette.

PLAQUE FORMATION

The presence of antibody forming cells in the in vitro culture was demonstrated by the technique of hemolysis in gel of Jerne et al. (3,25) as modified by Plotz et al., for use on microscope slides, (24), Hübner and Gengozian (23), and Urso and Gengozian (22). The lymphocytes were gently scraped from the culture dish with a rubber policeman and the suspension was pipetted into a 15-ml conical test tube and centrifuged at 1350 RPM, 4°C for 10'. After removing the supernatant, the cell pellet was resuspended in 1-ml of Eisen's solution and recentrifuged.

The supernatant was removed to 0.3-ml and the cell pellet resuspended in the remaining volume. One-tenth ml of the suspension, containing the desired nucleated cell concentration, (50,000 to 200,000 cells as determined by Coulter Counter, model Z_B, and trypan blue viability test), was pipetted into 0.5-ml of 0.5% agarose, (Bio-Rad Laboratories, Richmond, CA), in Eisen's solution containing 0.05-ml of 7% SRBC solution and maintained at 45°C. This mixture was agitated and quickly poured onto 25- by 75-mm glass slides previously coated with 0.1% agarose solution. As soon as the agar gelled, the slides were placed in a Lucite box maintained at 37°C. A 1:10 dilution of GPC' in Eisen's solution was added to each slide. After three hours, the PFC were scored with the aid of a binocular dissection microscope at a 10X magnification. Triplicates of each experimental insult were cultured and duplicate slides for each culture were scored. The anti-SRBC PFC activity of the cultured lymphocytes was represented as the number of plaques per million viable cultured cells on day five of incubation.

CHAPTER II

RESULTS

I. PRELIMINARY STUDIES

EFFECTS OF FETAL CALF SERUM ON THE PFC RESPONSE

Each lot of fetal calf serum was pretested in our laboratory in order to determine its suitability for in vitro PFC studies; #p32112 proved the most satisfactory for our needs (TABLE 1).

TABLE 1

PRODUCTION OF PFC BY SPLEEN CELLS CULTURED WITH VARIOUS LOTS OF FETAL CALF SERUM.

EXPT.	PFC/10 ⁶ Spleen Cells Cultured in FCS Lot #: ^a			
	L24306	S45006	S45809	P32112
1	836	368	1000	1743
2	857	285	1111	1940

a PFC assayed on day five.

Previous studies in this lab have shown an increase in the effectiveness of the FCS in supporting in vitro antibody formation after it has been incubated for 30' at 56°C in a water bath. This process is thought to deactivate the complement component of the FCS. A similar increase in effectiveness was also seen with the FCS lots pretested for

this study, therefore, all FCS samples were deactivated before being used in the experiments.

Recent studies by Kristensen et al. (93) have shown that the FCS can have a significant effect on the cell viability, particularly during the initial period of incubation. It also appears as though the FCS contributes a factor(s) that increases the transition of G_1 cells to S phase cells. This may have an important effect on the PFC response since this transition is necessary for splenic lymphocytes to synthesize antibodies (1, 115-118).

EFFECTS OF DIMETHYL SULFOXIDE AND ACETONE ON THE BEHAVIOR OF SPLEEN CELLS IN CULTURE

Since benzo-pyrene is virtually insoluble in water, (0.0038 mg/Kg H_2O (127)), it was necessary to find a suitable solvent to transport the benzo-pyrene into the cells. Previous experience and a literature search resulted in the choice of dimethyl sulfoxide (DMSO) and acetone (ACE) as two possible solvents.

The effects of various concentrations of the two solvents on the spleen cells in vitro were determined with respect to three experimental parameters: cell recovery, cell viability, and PFC on day five. The gradual decrease in the cell recovery, representative of in vitro cultivation of lymphocytes, is shown in FIGURE 2. Cell losses on days 1 and 2 comprise over 70% of the total cell loss while in culture. The ratio of 12 million cells on day 1 to 2 million cells on day 5, was consistent throughout the study regardless of the solvent or concentration used.

The cell viability with respect to the time of incubation is also represented in FIGURE 2. With the exception of day 1, the viabilities

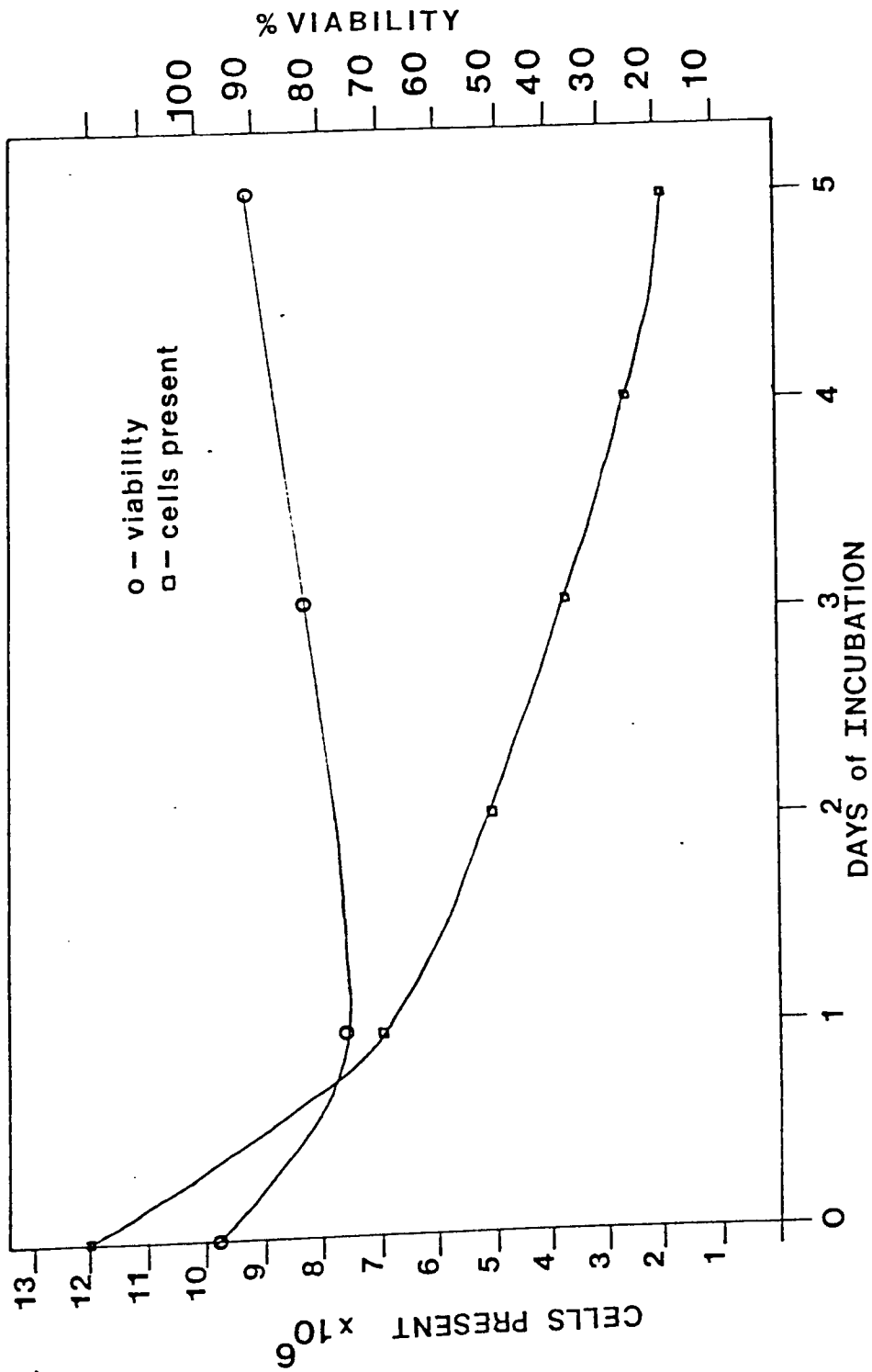


FIGURE 2

EFFECTS OF ACE AND DMSO ON THE CELL RECOVERY AND VIABILITY DURING INCUBATION

were consistently high during the incubation period. FIGURE 1 is characteristic of the MEM control, and MEM with from 0.1% to 5% concentrations of either DMSO or ACE dissolved in it.

The final parameter tested was the effect of DMSO/ACE on the PFC response in vitro (FIGURES 3 and 4). It is evident that DMSO has a dramatic effect on this parameter depending on the concentration; greatly enhancing the PFC response at 0.1%, while virtually eliminating it at 1.0% (FIGURE 4). The ACE response was consistently within 5% of the MEM control. Since both DMSO and ACE are presumed to be 100% permeable to all cellular membranes, the results with DMSO were unexpected. In addition, DMSO does not appear to effect the carcinogenicity of BP (69,70), or its ability to suppress the anti-SRBC PFC response in vivo.

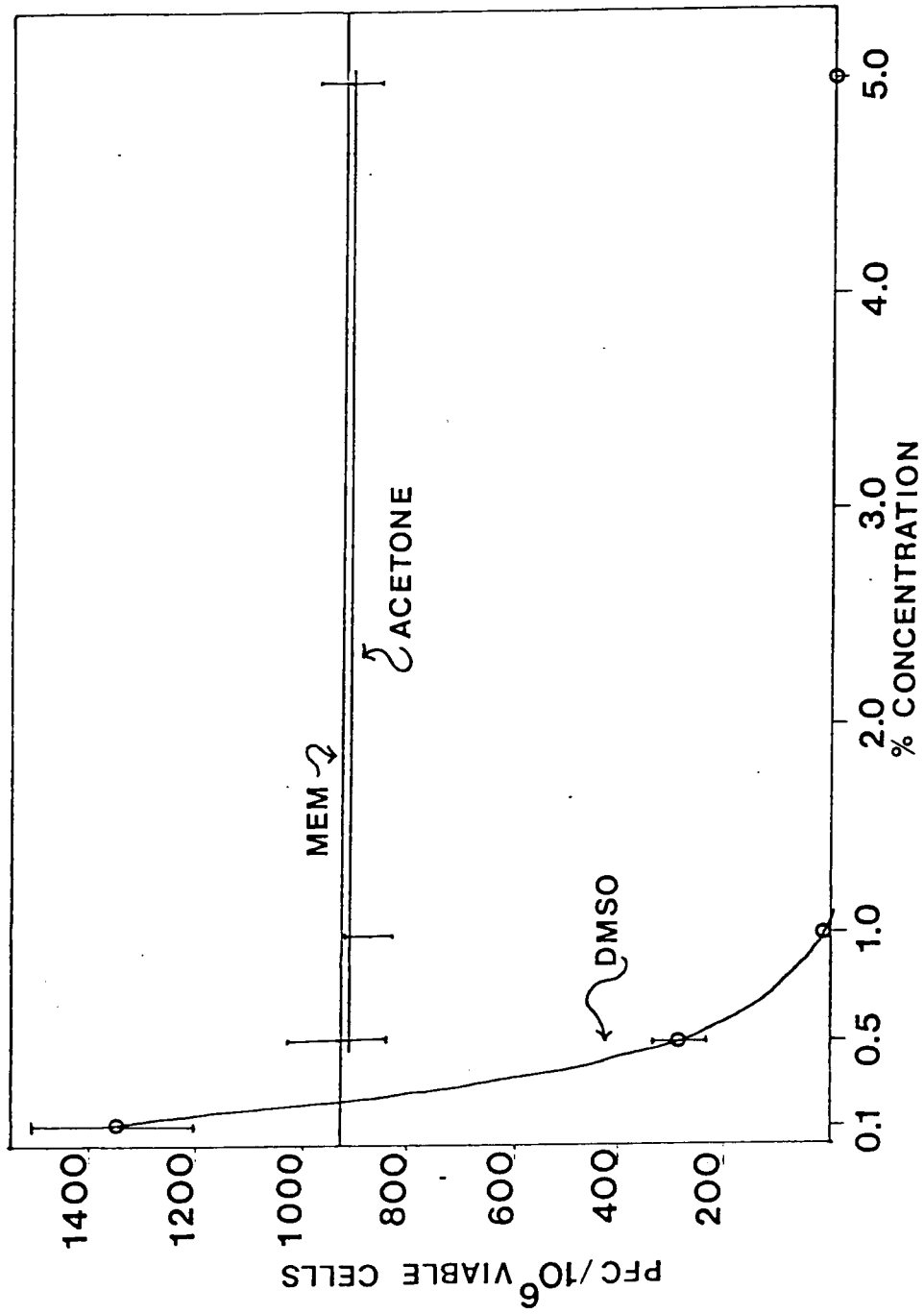


FIGURE 3

EFFECTS OF ACE AND DMSO ON THE PFC RESPONSE

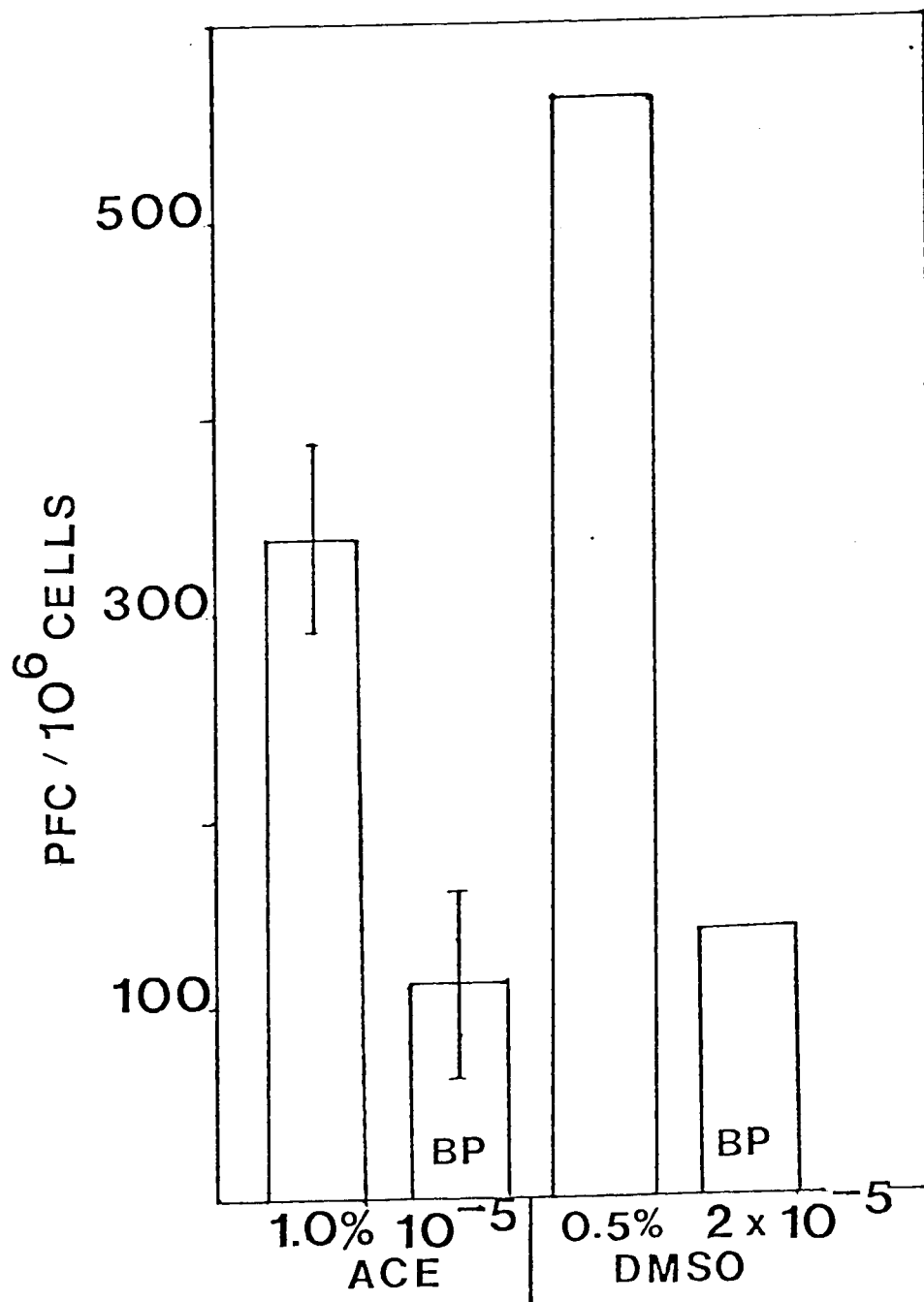


FIGURE 4

EFFECT OF DMSO-BP ON THE PFC RESPONSE AGAINST SRBC

II. EXPERIMENTAL RESULTS

EFFECTS OF BENZO-PYRENE ON THE EXPERIMENTAL PARAMETERS

Having shown that 1.0% ACE did not have any adverse effects on this system, it was necessary to determine the effect of BP and B(e)P on the cell recovery and viability. Although not shown, the cell recovery after treatment with benzo-pyrene was the same as the ACE control. Benzo-pyrene concentrations lower than 10^{-4} molar resulted in no noticeable effect on the number of cells recovered. The effect of BP and B(e)P on the cell viability during incubation is presented in TABLE 2. There is no significant difference when compared to the 1.0% ACE control at concentrations as high as 10^{-5} molar BP, ($p < 0.05$). The last two points were significant at the 0.1 level. Concentrations of BP greater than 10^{-3} molar were virtually 100% toxic in vitro to splenic lymphocytes. The viability when using B(e)P will decrease in a similar fashion as BP, but at much higher concentrations (personal communication, J. Selkirk). This may be due to the differences in metabolism between BP and B(e)P (6,7). The cell viability was used to convert the cell recovery into the number of viable cells present, i.e., PFC per million viable cells on day five.

INHIBITION OF THE PFC RESPONSE BY BP

Although the addition of benzo(a)pyrene to the incubation media on day one did not produce any adverse effects on the previously mentioned parameters, a striking reduction in the number of plaques was observed on day five (FIGURE 5A). FIGURES 5A and 5B represent two typical

TABLE 2

EFFECT OF BENZO-PYRENE ON CELL VIABILITY IN CULTURE.

MEDIA	[BP] ^e	DAY	% VIABILITY ^a	n ^d
MEM	-	0 ^b	97 ±2	30
MEM	-	5	92 ±4	46
1.0% ACE	-	5	90 ±5	46
1.0% ACE	1 x 10 ⁻¹⁰	5	91 ±5	20
1.0% ACE	1 x 10 ⁻⁸	5	89 ±6	4
1.0% ACE	1 x 10 ⁻⁷	5	92 ±4	11
1.0% ACE	1 x 10 ⁻⁶	5	92 ±4	9
1.0% ACE	1 x 10 ⁻⁵	5	84 ±8	20
1.0% ACE	4 x 10 ⁻⁵	5	76 ±3 c	3
1.0% ACE	4 x 10 ⁻⁴	5	54 ±12 c	2

MEDIA	[B(e)P]	DAY	% VIABILITY	n
1.0% ACE	1 x 10 ⁻⁷	5	92 ±2	5
1.0% ACE	1 x 10 ⁻⁵	5	93 ±3	10

a The percentage of viable cells was determined using the Trypan blue exclusion test. At least 100 cells were counted for each value

b Day 0 values were obtained within 30' of removal from the animal.

c Significant at the 0.1 level.

d Number of values used to determine % viability.

e Molar concentrations.

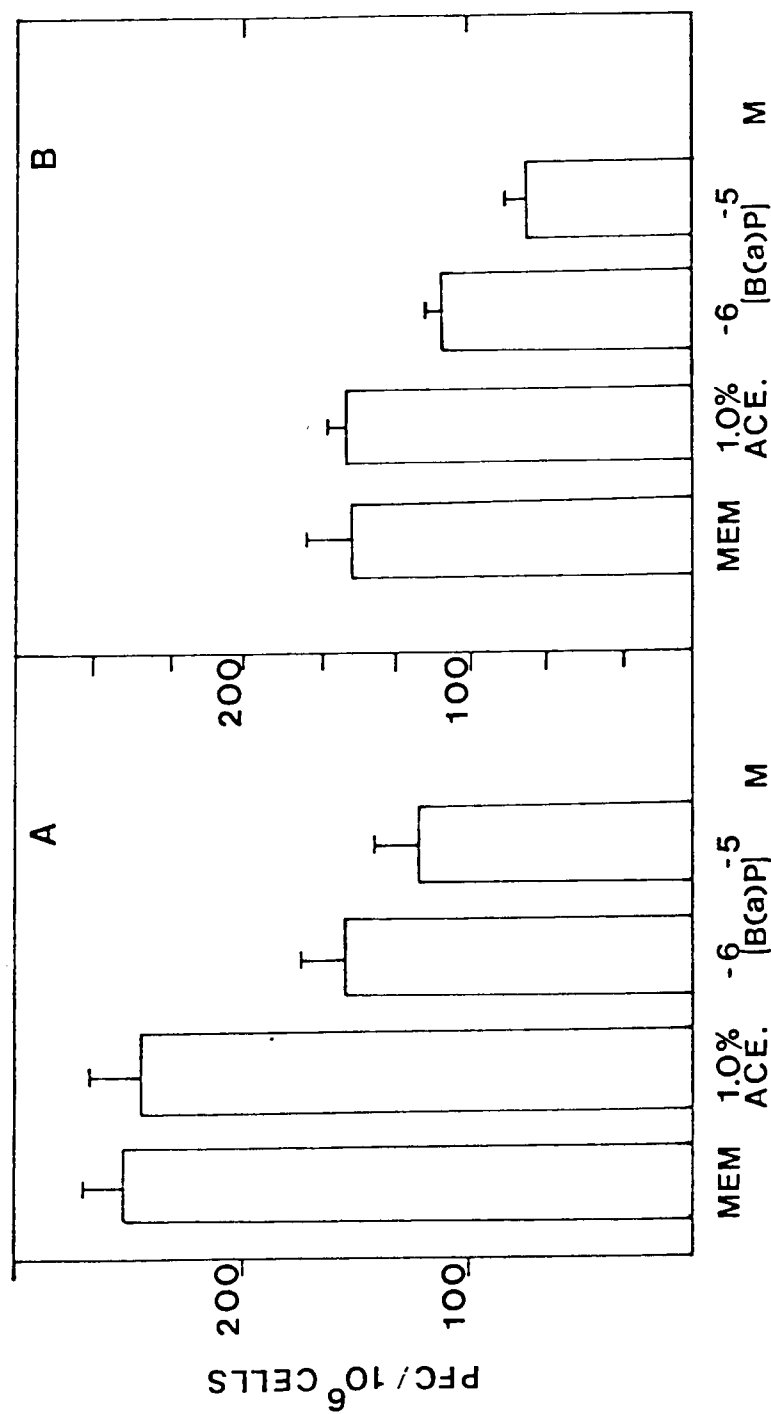


FIGURE 5

BP INHIBITION OF THE PFC RESPONSE

experiments done on consecutive days; the animals for both experiments were obtained from the same litters. Even though the actual number of PFC per million cells differ, the percent inhibition by BP for each experiment are in close agreement (51% vs. 49%). Each point represents triplicate samples with the standard deviations calculated from duplicate slides for each sample. The molar concentrations of BP are the final concentrations to which the lymphocytes were exposed during the cultivation.

DOSE RESPONSE CHARACTERISTICS OF BP TREATMENT

FIGURES 6 and 7 show that the effect of BP on the PFC response is dose dependent. A gradual reduction of the inhibition occurred from 10^{-5} to 10^{-8} molar, while at 10^{-10} molar and lower, there was no significant suppression. The large standard deviations in FIGURE 7 are a result of logistical difficulties in handling nine test groups. Fewer test samples, as shown in FIGURE 6, resulted in much smaller standard deviations. At any rate, the dose dependent pattern is clearly visible.

EFFECT OF B(e)P ON THE PFC RESPONSE

FIGURES 6 and 7 also illustrate the important difference between the effect of BP and B(e)P on the PFC response. The addition of B(e)P to the spleen cells in the presence of SRBC, did not reduce the number of plaques produced on day five. In fact, at times B(e)P appeared to enhance the PFC response.

SUMMARY OF THE EXPERIMENTAL RESULTS

An overall view of the effects of both BP and B(e)P on the in vitro anti-SRBC PFC response of C3H splenic lymphocytes is presented in

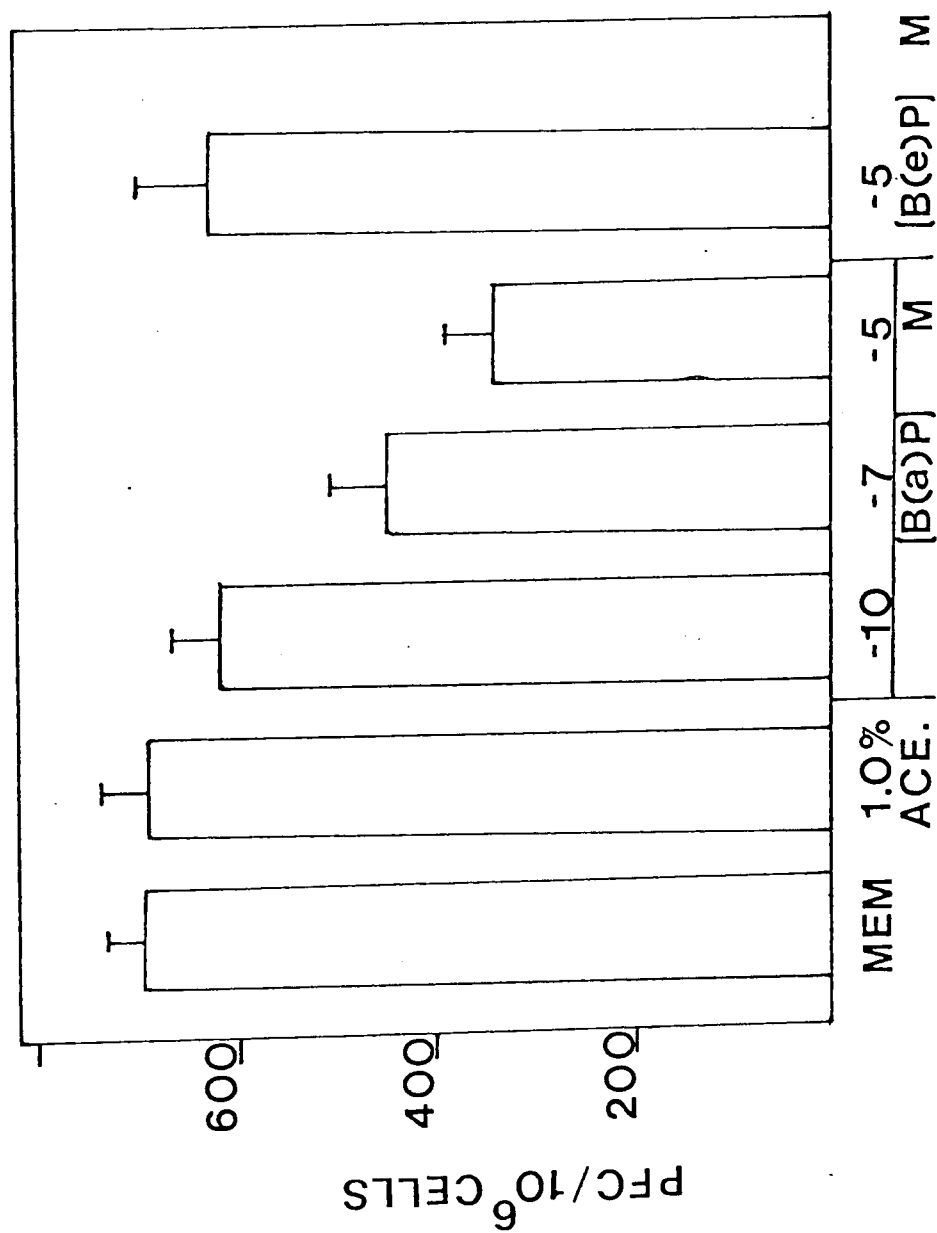


FIGURE 6

BENZO-PYRENE CONCENTRATION VS. PFC RESPONSE

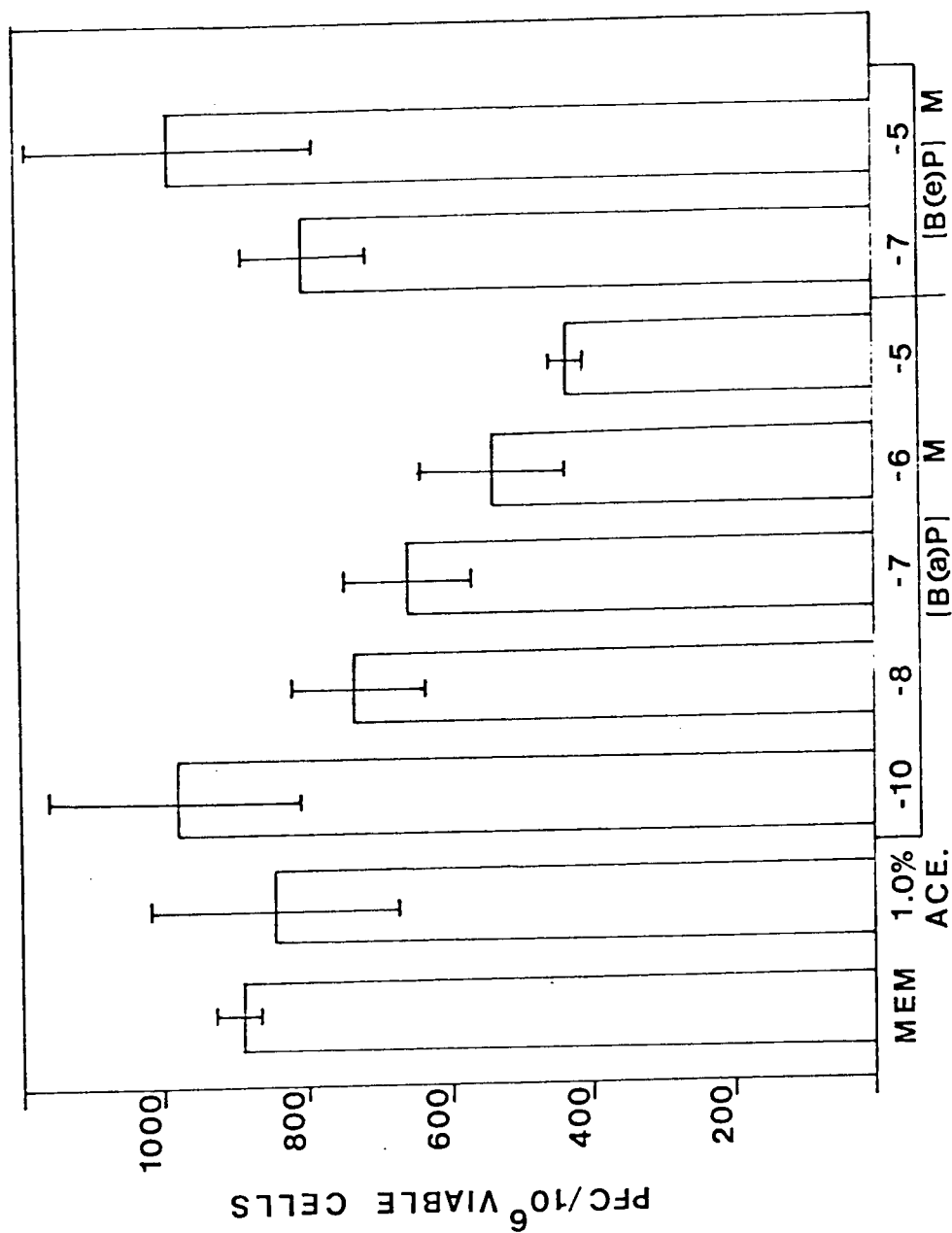


FIGURE 7

DOSE EFFECT OF BENZO-PYRENE ON THE PFC RESPONSE

FIGURE 8. The values expressed in this figure and in TABLE 3, were obtained by dividing the number of plaques counted for a given experimental insult, for example, 10^{-10} M BP, (1050 plaques), by the number of plaques counted for the ACE control slides, (1083), and multiplying by 100 in order to obtain the percent of the ACE control, (app. 97%). The numbers shown are the mean and standard deviation for all of the experiments combined. In addition to all of the values shown in FIGURE 8, TABLE 3 contains the number of experiments done per sample, the total number of determinations used to calculate a given percentage, and the statistical significance of the point with respect to the ACE control (100%). With the exception of the values marked (*) in TABLE 3, all the other points were shown to be significantly different from the control.

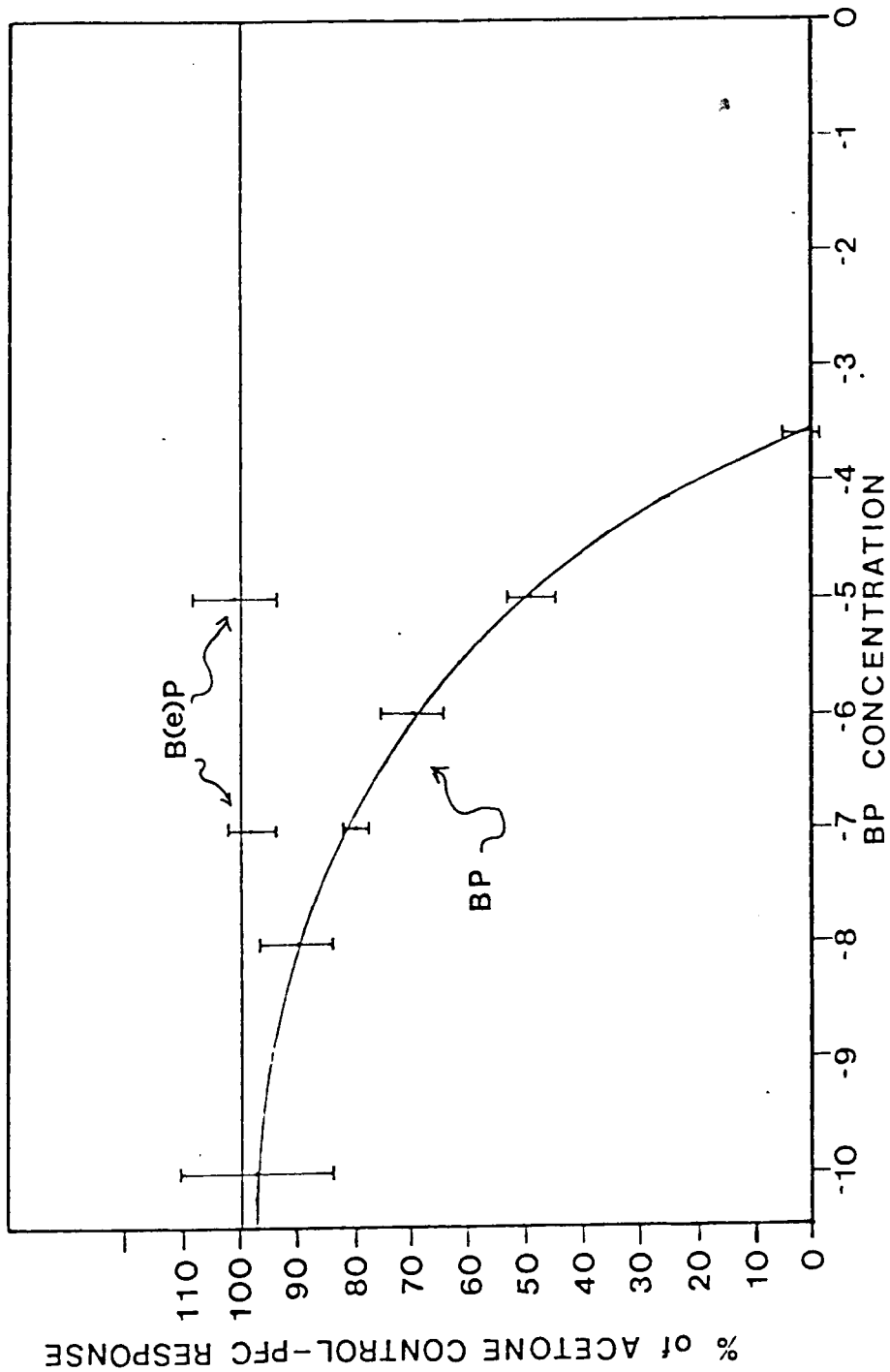


FIGURE 8

DOSE RESPONSE CURVE FOR BENZO-PYRENE

TABLE 3

INHIBITION OF THE PFC RESPONSE BY BENZO-PYRENE

[BP]	# of EXPTS.	n	PFC (% CONTROL)	ρ^a
1×10^{-10}	5	15	97 $\pm$ 13	0.89*
1×10^{-8}	4	12	90 $\pm$ 6	5.77
1×10^{-7}	2	6	80 $\pm$ 2	24.49
1×10^{-6}	6	18	70 $\pm$ 5	25.46
1×10^{-5}	11	33	49 $\pm$ 4	73.24
4×10^{-5}	2	6	2 $\pm$ 3	80.02
[B(e)P]	# of EXPTS.	n	PFC (% CONTROL)	ρ
1×10^{-7}	2	6	98 $\pm$ 4	1.22*
1×10^{-5}	4	12	101 $\pm$ 7	0.49*

a Statistical significance with respect to the ACE control, (T test).

* No significant difference from control.

CHAPTER III

DISCUSSION

It is evident from the data presented, that benzo(a)pyrene, but not benzo(e)pyrene is interfering with the ability of the mouse splenic lymphocytes to respond to a foreign body, (SRBC), in vitro. The factors that could cause a decrease in the number of plaques observed after treatment with BP are many and diverse, but they can be divided into two basic categories: those causing a disruption in the antigen recognition phase or those resulting in a malfunction in the production phase of the humoral immune response.

The reduction of the PFC response was not a result of cell killing since neither BP or B(e)P had any detrimental effects on the cell recovery or viability over the effective concentrations. The experimental results present no reason to think that any selective killing is occurring, although the possibility can not be excluded. The size and shape of the lymphocytes after treatment with benzo-pyrene were the same as the controls as observed through a light microscope. Therefore, it seems unlikely that the mere presence of BP and its metabolites would provide sufficient cause for the observed results.

BP has been known to be highly carcinogenic for many years, but it was not until 1959 that evidence for the metabolic activation of BP, and for that matter, any PAH, to reactive intermediates was found (46). BP applied to mouse skins formed metabolites that covalently bound to the skin protein. Since then, numerous reports have

documented the metabolic pathways of BP (6,7,50,128) and its binding to DNA, RNA, and nuclear protein (33,34,128,143,144). The literature contains several reviews that more than amply describe this material (35,50,128). Appendix II contains a brief discussion of the portions of the metabolic pathway that are of importance to this study.

Recognition, as well as inhibition of specific, immunocompetent lymphocytes constitute central activities within the immune response. Through these mechanisms, the immune system may evolve with select immunity against a given set of 'foreign' substances or, alternately, develop specific unresponsiveness to the same group of molecules (141). Macrophages are the initial cells that take up the antigen prior to their presentation to immunologically specific T-cells (136). The lymphocytes then form clusters around the macrophages (135). These clusters are an essential prerequisite for the subsequent antigen specific T-cell proliferation suggesting that physical interaction between macrophages and lymphocytes is required in an immune response (135).

This interaction results in the activation of T-helper cells (141). These cells subsequently stimulate the differentiation of antigen stimulated B-cells into plasma cells (1,116,118). In order for T-dependent antigens, (SRBC (1,116)), to trigger the activation of B-cells, it seems likely that the antigens must exert mitogenic properties of the B-cell. This may occur via combination between antigen and antigen-specific T-cells. The T-helper cells also stimulate the proliferation of T-suppressor cells which result in a negative feedback on the antibody production. The balance between the T-helper and T-suppressor cells strongly effects the degree of response toward the SRBC.

It is apparent that during the recognition phase, a reduction in the PFC response can be caused primarily in two ways, either by interfering with the surface interaction between any of the cells involved, or by disturbing the balance between T-helper and T-suppressor cells. These effects could be caused either on the cell surface, intercellularly, or both.

During the course of the B-cell's conversion into a plasma cell, (production phase), numerous periods of synthesis occur. It has already been established that BP metabolites, in particular, 7,8-9,10-diol-epoxide, actively bind to DNA and RNA. BP has also been shown to cause sister chromatid exchange when cultured with human lymphocytes (98). Although it has also been demonstrated that the BP-DNA adducts can be excised and the defect eliminated by the DNA repair process (40,41), it is conceivable that this binding could effect the production of antibodies, or the regulatory functions of the T-cells. One possibility would be the complete or partial reduction in the synthesis of the antibody; this could be due to a BP-adduct disrupting an initiation or termination sequence, by the removal of a necessary regulatory protein, or by a change in the ratio of T-helper/T-suppressor cells.

Another possibility that could result from a change in the amino acid sequence is the disruption of any one or more of the structural interactions that occur during the PFC response. Some of the obvious interactions include the specificity of the antibody for SRBC, which is highly dependent on the three dimensional configuration of the binding site (1,115-118), the interaction of complement with the antigen-antibody complex, (binding of the initial component

of complement is required for the reaction to occur (1,115-118)), the aggregation of the five inactive IgG molecules to form one active IgM molecule (1,115-118), and the loss of substrate affinity by the active IgM molecule (1,115-118). The last possibility can probably be ruled out since an extension of the period of incubation with GPC' did not result in an increase in the number of plaques observed after treatment with BP.

It is clear that the majority of possible explanations for the reduction of the PFC response during the production phase are a result of a change occurring at the nuclear level, (physical interaction of the BP metabolites with the active sites or structural proteins is also a possibility). The number of possible disruptive manifestations resulting from the BP insult are related to the number of reactive residues on the DNA/RNA. If the BP adduct is present at an early stage of differentiation of the T- or B-cell, the damage to the integrity of the antibody response could be faithfully passed on to the following generations, resulting in a clone of ineffective or counter productive cells. This could occur even if the initial BP adduct has been excised.

It is also conceivable that the metabolism of benzo-pyrene in the three lymphoid cell types, T, B, and macrophages, differs significantly such that one or more of them are being preferentially removed or inhibited. These possibilities are not readily apparent from the experimental data.

The disruption of the PFC response by PAHs is not limited to BP. Benz(a)anthracene, 7,8-dimethylbenz(a)anthracene, and 3-methylcholanthracene have all been shown to inhibit the anti-SRBC PFC

response in vivo (26,27). From these experiments and others, there appears to be a correlation between the degree of carcinogenicity of a PAH, and its effect on the immune system (14,26,27,70,128).

The question still remains as to how these findings are related to the alrger picture of neoplastic development resulting from an environmental insult with the carcinogen BP. Bp, and other PAHs, are capable of producing a local suppression of the immune system in addition to causing a neoplastic transformation; this could provide the means for the nascent ca-cer cells to establish themselves within the host. A continued, heavy exposure could result in a more general depression of the total immune system that would have a profound effect not only on neoplastic growth, but on the survival of the host in general.

In summary, splenic lymphocytes, simultaneously exposed to antigen and the potent carcinogen benzo(a)pyrene, expressed a greatly reduced humoral immune response in vitro against SRBC; furthermore, the non-carcinogenic analog of BP, benzo(e)pyrene, produced no such inhibition. The difference in the metabolic pathways of these two PAHs, in particular, the production of reactive intermediates capable of forming adducts with DNA, was suggested as a possible reason for this difference. This demonstrates a possible mechanism through which BP can prevent the host's natural defenses from destroying a neoplastic growth.

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APPENDIXES

APPENDIX I

BAY REGION THEORY

Many attempts have been made to correlate the structure of poly-aromatic hydrocarbons with their carcinogenicity (78,79,89,92). Since carcinogenic activity depends on chemical activity, quantum chemical calculations of the electronic structure and chemical reactivity should be capable of providing important information regarding this phenomena (79). Initially, little was known about the metabolism of these compounds, so early studies attempted to correlate their carcinogenicity with some physical property or quantum mechanical parameter calculated for some region of the molecule, for example, Pullmans' K and L region theory (78). It is now accepted that PAHs only exert their effect after metabolic activation to reactive metabolites, in particular, bay region diol epoxides (31,80-83). Diol epoxides are formed via oxidation of a terminal benzo ring of a hydrocarbon to form an arene oxide, hydration of the arene oxide to form a trans-dihydrodiol and oxidation of the adjacent double bond to form a diol epoxide (84). The 'bay region' of BP and B(e)P is illustrated in FIGURE 1 (6).

Both diastereometric 'bay region' diol epoxides of benzo(a)pyrene are among the most mutagenic compounds ever tested (85-88,121). The unique structural feature of the diol epoxides appears to be that the epoxide is on a saturated angular benzo ring that forms part of a bay region of the PAH. The key chemical feature of such epoxides is that they are highly susceptible to attack by nucleophiles (84). The high chemical reactivity of bay region diol epoxides can be attributed to

their unique electronic properties. Pertubational molecular orbital calculations (79,95), which predict π -electron energy changes, indicate that epoxides on saturated benzo rings that form part of the bay region of a hydrocarbon, undergo ring opening to form a carbonium ion much easier than do non-bay region epoxides (89-91,95). The calculations give the π -electron energy change in terms of the parameter ΔE_{deloc} , in units of the resonance integral β (79). Higher calculated values of ΔE correspond to a predicted greater ease of conversion of the diol epoxide to the carbonium ion, which should result in a greater alkylating ability for the diol epoxide and its being a more efficient mutagen and carcinogen (95). These calculations indicate that the more carcinogenic members of a series of PAHs tend to form carbonium ions from their bay region diol epoxides more readily than weaker carcinogens. It is important to keep in mind though, that the confirmation and application of the bay region theory depends on whether or not the derivatives in question are actually formed in the species in which the parent hydrocarbons are carcinogenic (90).

APPENDIX II

BENZO-PYRENE METABOLISM

The metabolic interface between environmental PAHs and humans and animals are the mixed function oxidase enzyme systems (MFO) that contain cytochrome P-450 as the terminal oxidase (50). The MFO are present in most tissues of all mammalian species (48). They metabolize and are inducible by a wide spectrum of xenobiotics (29,43,45), including pesticides, drugs, food additives, PAHs (29), and some endogenous substrates (43,45). Enzyme purification studies have shown that there are at least six different forms of cytochrome P-450 (P-450) (51). When BP is the primary substrate, P-450 is termed aryl hydrocarbon hydroxylase (AHH). AHH is genetically controlled and its inducibility varies greatly in different mouse strains (47-49,111,147). The level of enzyme activity is effected by age, sex, and hormonal status of the animal (48). Different forms of P-450 are preferentially induced by different inducers of MFO (29). The changes in the relative amounts of different metabolites after induction or inhibition of the MFO are clearly due to an alteration in the amount of different P-450 forms active in catalysis (50,119). Each P-450 exhibits positional specificity in the metabolism of BP (32,67,68), and stereospecificity in the metabolism of BP(-)-trans-7,8-diol to diol epoxides I and II (29, 68), the ultimate carcinogens (56,100,120,121,128). This may regulate the balance between activation and detoxification pathways of BP and may determine the tissue and individual susceptibility to the carcinogenic action of BP (29).

Other enzymes that are involved in BP metabolism subsequent to the initial interaction with MFO are epoxide hydratase, epoxide reductase, glutathione epoxide transferase, UDPglucuronate transferase, and sulfo-transferase (29). Of these, only epoxide hydratase (EH) plays a direct role in the activation of BP to reactive diol epoxide intermediates capable of covalently binding to cellular macromolecules such as DNA, RNA, and nuclear proteins (FIGURE 9) (148). EH is a widely distributed enzyme (53). EH is genetically controlled (112), inducible (113), and has multiple forms much like P-450 (114). It is found in both the smooth and rough endoplasmic reticulums (53). EH converts the epoxides formed in the initial reaction with P-450, 2,3-, 4,5-, 7,8-, and 9,10-epoxides of BP, into their respective dihydrodiols (30,31,54,55). It preferentially hydrates the epoxide that is the specific precursor of the (-)-trans-7,8-diol (29,56,57), the precursor of the ultimate carcinogen, 7,8-diol-9,10-epoxide (83,97,100). These dihydrodiols are then acted on by the same MFO as in the first step, P-450. This results in the formation of the diol-epoxides; these highly reactive electrophiles form covalent bond with nucleophilic residues in macromolecules (36,37). Of particular interest are the two diol-epoxide isomers of (-)-trans-7,8-diol-BP, BP diol epoxides I and II. These form covalent adducts primarily with guanosine residues of DNA and RNA and to a lesser extent, with adenosine and cytidine residues (35,143,144). Kakefuda and Yamamota have suggested two types of base modification that result from these bonds: one with adenine producing local unwinding and base-pair cleavage, and the other with guanine producing few secondary structural effects (38). It has also been proposed that the aromatic portion of BP intercalates with the bases (10,39,150).

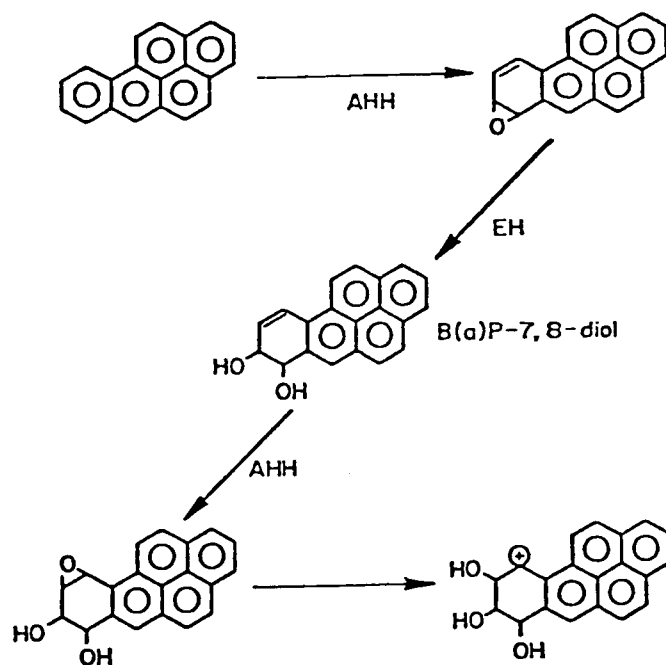


FIGURE 9

BP METABOLISM TO DIOL EPOXIDES I AND II

Several other metabolites of BP also bind to DNA, but the diol-epoxide is clearly the predominate interaction (29). An overall view of the metabolic pathways of BP are shown in FIGURE 10 (149).

It is important to note that BP, like most PAHs, induces the MFO responsible for its metabolism (42). In addition, the MFO are located in the endoplasmic reticulum, which is greatly enlarged in activated B lymphocytes. It is quite conceivable that these two effects have a synergistic effect on the amount of P-450 available to metabolize the BP. It would be of interest to determine if such an increase actually occurs and what effect it has on the amount of BP metabolites bound.

Benzo(e)pyrene, the non-carcinogenic analog of BP, which for many years was thought to be a potentially more potent carcinogen than BP due to theoretical considerations of its bay regions (79,84), is also metabolized by the cytochrome P-450 system (6,7). Recent studies have shown that even though the rates of metabolism of BP and B(e)P are very similar, the precursor of the ultimate carcinogen 9,10-dihydroxy-11,12-epoxy-9,10,11,12-tetrahydro-B(e)P, B(e)P-9,10-dihydrodiol, was found to constitute less than 1% of the total metabolites (7). In addition, the 9,10-dihydrodiol is preferentially metabolized at the 1,2,3 ring positions instead of the 11,12 double bond necessary for the formation of the ultimate carcinogen (7). In this manner, B(e)P metabolism is steered away from the formation of reactive compounds capable of binding to cellular constituents. Restated, BP forms a dihydro-diol which may be metabolized on an adjacent olefinic double bond to a reactive diol-epoxide adjacent to the bay region. It is apparent that B(e)P does not enzymatically favor this mechanism and forms an inert K region dihydro-diol instead (7). The much higher binding of BP than B(e)P

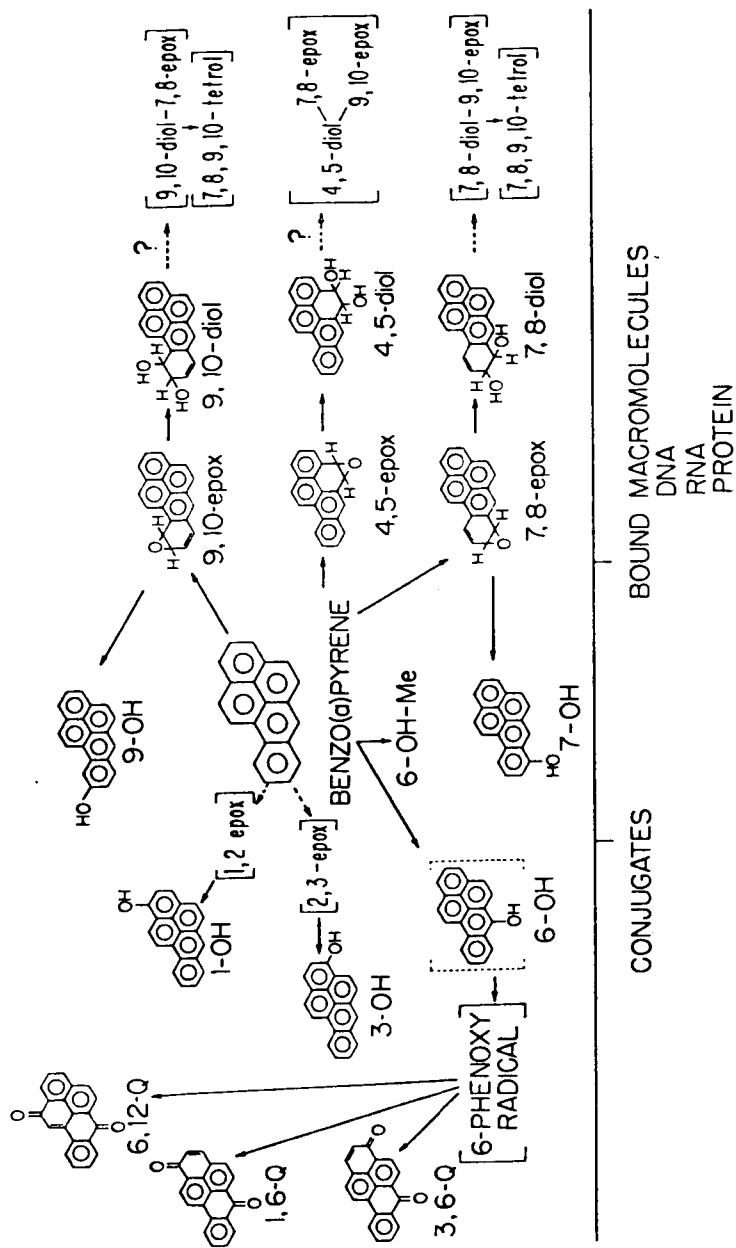


FIGURE 10

OVERALL BP METABOLISM

to both DNA and RNA of isolated nuclei from hamster embryo fibroblasts treated with the parent hydrocarbon, reflects the higher biological activity of BP (6). The difference in binding to cellular constituents may explain the disparity in their effects on the PFC response.

VITA

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