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Procedures Available to Examine the Immunotoxicity of Chemicals and Drugs

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I. Introduction

APPLICATION of immunological and host resistance assays to study the effects of exposure to a drug or chemical in rodents has indicated that certain chemicals and drugs produce immune dysfunction [for reviews see (72), Vos et al. (74) and Faith et al. (17)] and alter host resistance to bacteria, viruses, parasites, and transplantable tumor cells as well as produce an increased frequency of spontaneous tumors [reviewed by Dean et al. (13)]. Many cancer chemotherapeutic agents likewise produce immune dysfunction as a consequence of their general toxicity.

Awareness of immunobiology as a subspecialty of toxicology assessment was provided by a comprehensive review (72) dealing with environmental chemicals known to alter the immune response in experimental animals. Observations that residents of Michigan or of Japan and China accidentally exposed to polybrominated biphenyls or polychlorinated biphenyls exhibited immune dysfunction (4, 61, 9) has caused concern about the effect of other agents on the immune system. Numerous reports in the recent literature have increased our awareness of the immunotoxicity of xenobiotic agents and extended the number of chemicals that are known to produce immunotoxic effects (17, 30, 74, 3). To immunologists the assessment of immune function as an adjunct to routine toxicity assessment of a suspect chemical or drug seems a logical and overdue approach. Safety assessment scientists and toxicologists have begun to recognize that the immunologists' repertoire of assays contain many sensitive in vivo and in vitro methods for examining cellular injury and functional impairment resulting from general or target organ specific toxicity. Some immunologists are beginning to recognize the potential of chemical agents to dissect and better define the intricacies of the immune system. The information gained from cellular and molecular approaches provided by immunology in the safety assessment of a drug or chemical should facilitate improved human risk assessment.

There are several factors that might explain the apparent increased sensitivity of the immune system for detecting toxic injury and the advantages offered by immune assessment: 1) Many cell types of the immune system continually undergo rapid proliferation and differentiation. 2) The immune system is a tightly regulated and highly organized network of cellular components. An imbalance of this regulatory network resulting from cellular injury can be expressed as an immune alteration. Immune alterations may be represented either as *immune enhancement*, which might ultimately result in autoimmunity, contact hypersensitivity, or allergy; or as *immune suppression*, which might result in decreased resistance to an infectious agent or impaired surveillance of a neoplastic event. 3) The cell types involved in the immune response can easily be removed or isolated and purified, and their function can be assessed in vitro to provide more sensitive endpoints for detection of cellular injury than are currently available by a routine toxicology evaluation.

It has been known for some time that there is an association between the therapeutic use of chemical immunosuppressants and an increased incidence of infectious and neoplastic diseases. The evidence for increased bacterial, viral, fungal, and parasitic diseases in patients on chronic immunosuppressive chemicals has been well documented (2). Infections are also a major cause of postsurgical complication believed to result from transient postoperative immunosuppression caused by stress and anesthesia (41). McKhann et al. (42) reported that the incidence of cancer in renal transplant recipients on prolonged immunosuppressive chemotherapy was 4.6 to 61 times higher than in the general population. Others have confirmed this relationship (56, 57). In McKhann's study in which only lymphoreticular cancers were considered, the incidence of tumor development was 333% higher than in the general population. Penn and Starzl (56) found the incidence of malignant tumors in renal transplant recipients approximately 80 times greater than in an equivalent control population. Gatti and Good (19) observed a higher frequency of neoplasia of the lymphoreticular type in patients with primary immunodeficiency, although most of these patients died of bacterial or fungal infections before they were considered old enough to express cancer.

Studies in laboratory animals (31, 51, 11a) support the clinical observations and demonstrate an enhanced frequency of ultraviolet-induced or benzopyrene-induced tumors in mice concomitantly treated with immunosuppressive agents. Although the mechanistic relationship between carcinogenesis and immune alteration is com-

plex, much data support the hypothesis that immune dysfunction may be cocarcinogenic in the etiology of certain types of tumors (3, 33).

Immune dysfunction as evident by depressed humoral-mediated immunity (HMI) and/or cell-mediated immunity (CMI) has also been observed in toxicology studies on rodents dosed with sublethal levels of several chemicals of environmental concern. Chemicals that have produced immunological alterations in rodent studies include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB), polycyclic aromatic hydrocarbons (PAH), hexachlorobenzene (HCB), diethylstilbestrol (DES), pentachlorophenol (PCP), certain organometals, and most heavy metals [see reviews (72, 74, 17, 30, 13)]. Some studies have also indicated that exposure to certain chemicals can alter resistance to bacteria, viruses, parasites, and transplantable tumor cells [see reviews (72, 13)]. Of major concern is the correlation between these immunological findings and altered host susceptibility as well as the extrapolation of these chemically induced immunotoxic effects to humans. For example, inadvertent exposure of humans to PBB's (4) was associated with immune dysfunctions similar to those observed in rodents (37).

The methods selected to measure altered immune function following chemical or drug exposure are variable between laboratories, require better standardization, and generally lack host resistance considerations. This leads to difficulty in interpreting immunotoxicity studies for human risk assessment. In some cases, several methods are available for the measurement of similar immunological parameters. A variety of apparently satisfactory methods are also available to measure altered host resistance to infectious agents. These methods, however, must be improved to give more sensitive and easily reproducible endpoints and to provide data that better correlates with changes in immune dysfunction measurements.

The purposes of the studies described here are to clarify differences among several methods for measuring the same immunological parameter(s) and to identify the most sensitive and reproducible testing panel for assessing immunotoxicity and altered host resistance. At present, we are developing, refining, and applying methods for measuring delayed hypersensitivity as well as T lymphocytes, B lymphocytes, macrophage, and bone marrow cell function to define chemically induced immunotoxicity and altered host resistance with suspect or known immunotoxicants. To demonstrate the utility of the testing panel, studies with one drug (DES) and three chemicals (benzo[a or e]pyrene and phorbol ester) will be described.

II. Comprehensive Panel for Evaluating Immune Alterations following Chemical Exposure

A comprehensive panel of in vivo and in vitro assays is currently being utilized by our group as part of a methods selection and validation approach to assess immunological and host resistance alterations following chemical exposure. The assays and host functions examined by this panel are described in table 1. These procedures involve the evaluation of pathotoxicology, host resistance models, delayed-type hypersensitivity, CMI, HMI, macrophage function, and bone marrow progenitor cells. The reader is referred to Luster et al. (40) for a detailed discussion of the methodologies employed. Procedures that can be performed in a toxicology setting after chemical exposure are given in table 2. This is also the current assay panel being evaluated by the National Toxicology Program in the chemical bioassay program.

TABLE 1

Procedures for defining immune alterations currently being evaluated at the National Institute of Environmental Health Sciences

Parameter	Procedure Performed*
Pathotoxicology	Hematology profile—hemoglobin, red blood cell count, white blood cell count, differential Clinical chemistries—CPK, αHBDH, SGPT, BUN, creatinine, acid and alkaline phosphatase, LDH, cholinesterase Serum proteins—albumin, globulin, A/G, total proteins Weights—body, spleen, thymus, liver, kidney Histology—liver, thymus, adrenal, lung, kidney, heart, spleen
Host resistance	Tumor assays—tumor cell challenge TD10–20 and radiometric tumor mass *Listeria monocytogenes* LD10–20 challenge Endotoxin hypersensitivity, LD10–20 Expulsion of *Trichinella spiralis*
Delayed hypersensitivity	Radiometric assay with T cell dependent antigen
Lymphocyte proliferation	One-way mixed leukocyte culture Mitogens—PHA, Con A, LPS
Humoral immunity	Immunoglobulin levels (IgG, IgM, IgA) Antibody response to T-dependent (SRBC) and T-independent (LPS) antigens
Macrophage function†	Resident peritoneal cell numbers and nonspecific esterase staining Phagocytosis Lysosomal enzymes-5′-nucleotidase, acid phosphatase, leucine amino peptidase Cytostasis of tumor target cells RES clearance by using ^{125}I-triolein
Bone marrow colony forming units	CFU-S-multipotent, hematopoietic stem cells CFU-GM-granulocyte/macrophage progenitor Cellularity ^{59}Iron incorporation in bone marrow and spleen

* Abbreviations used are: CPK, creatinine phosphokinase; α-HBDH, α-hydroxybutyric dehydrogenase; LDH, lactic dehydrogenase; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; SRBC, sheep red blood cells; RES, reticuloendothelial system; CFU, colony-forming unit.

† Employs both resident peritoneal cells and pyran-activated macrophages.

If in vivo and in vitro immunology assay data obtained from such studies with the described panel are negative, there can be reasonable confidence regarding the safety of the drug or chemical under the conditions and doses defined. A major concern in risk assessment has involved extrapolation of dose response data from effect to no-effect levels or from rodent model systems to humans. However, if conservative extrapolations are made with data from appropriate and clinically relevant immune function assays, the most accurate estimate possible of chemical safety will be obtained. At present, alterations in host resistance provide the best indicator to predict significant enough immune dysfunction to be health threatening.

A. Pathotoxicology

Routine pathotoxicology aids in assessing the immunosuppressive potential of a chemical toxicant. Since immune suppression may occur either secondary to generalized toxicity or be a specific attribute of a chemical, morphological evaluation of various tissues and organs helps determine the site(s) of a chemical insult. Further, should the immune system be a primary target organ, then morphological changes may suggest which component of the immune system is being affected. Thymus and spleen weights are useful indicators of immune dysfunction. Thymic atrophy occurs after exposure to many chemicals and appears to be an extremely sensitive indicator of chemical assault on the immune system. Thymic atrophy should not, however, be taken as a specific indicator of immunosuppression since stress or general toxicity will induce similar thymus lesions. Histological studies on bone marrow, lymph nodes (mesenteric and peripheral), spleen, and thymus are recommended and may aid in the diagnosis for B or T cell deficiency. A paucity of lymphoid follicles and germinal centers in the spleen is indicative of B cell deficiency whereas lymphoid hypoplasia in the paracortical areas is characteristic of T cell deficiency. In the thymus, the cortical elements rather than the medulla appears to be the target site and are characterized by depletion of cortical lymphocytes. Medullary lymphocytes are considered immunocompetent T cells while the cortical elements contain cells that are not yet fully immunocompetent (immature T cells).

Body weight/organ weight ratios are useful in determining the degree of organ toxicity, and relative thymic weight changes appear to be an even more sensitive parameter than morphological evaluation when only subtle changes have occurred. Thymic weight losses of less than 20%, while significantly different from control subjects, are difficult to discern on routine histopathology.

Splenic weights may be decreased as a result of lymphoid depletion or markedly increased by extramedullary hematopoiesis since the spleen retains its hemopoietic potential during adult life in the mouse. Morphological evaluation of the spleen is often helpful in determining the nature of the weight change. The relationship between lymphoid organ weights and specific immune alterations following exposure to the chemicals described in this study are summarized in table 3. These data show that thymus weight reduction correlates quite well with CMI alterations but not with humoral alterations. This is not to suggest that thymus weight alone can predict immune alterations, but certainly thymus weight is a useful index in overall immune assessment.

TABLE 2

*Minimum screening panel for defining immune alteration after chemical exposure in rodents**

Parameter	Procedures Performed
Pathotoxicology	Hematology profile—CBC and differential Weights—body, spleen, thymus, liver, kidney, brain
Host resistance	Susceptibility to transplantable syngeneic tumor at LD10–20
Delayed cutaneous hypersensitivity	T cell dependent antigen with radiometric assay
Lymphocyte function (CMI)	Lymphocyte blastogenesis to PHA or Con A, LPS, and allogeneic leukocytes (MLC)
Humoral immunity (HMI)	Immunoglobulin levels (IgG, IgM, IgA) Antibody plaque response to sheep erythrocytes

* Abbreviations used are: CMI, cell-mediated immunity; HMI, humoral-mediated immunity; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture.

B. Bone Marrow Progenitor Cell Assays

The bone marrow is an integral part of the immune system since it contains multipotent progenitor cells capable of differentiating along hemopoietic lines or giving rise to lymphoid stem cells that further differentiate into either of the two lymphoid cell populations. Primary immunodeficiency disease of man has been described where the block occurred at the multipotent stem cell level. Swiss-type agammaglobulinemia and Di George syndrome are two genetic variants that reflect such a block at the lymphoid stem cell level (55). Since chemical toxicity to either of these two progenitor cells would be

TABLE 3

*Relationship of body and lymphoid organ weights to changes in immune function parameters**

Chemical	Highest Dose Level (mg/kg)	Weights				Host Resistance Parameters	Immune Alterations	
		Body	Liver	Thymus	Spleen		Cellular	Humoral
Diethylstilbestrol	8	NE	I	D	I	D	D	D
Benzo(a)pyrene	400	NE	NE	NE	NE	NE	NE	D
Benzo(e)pyrene	400	NE	NE	NE	NE	NE	NE	NE
Phorbol ester	40	NE	I	D	I	D/I	D	D

* Abbreviations used are: D, significantly decreased ($P < .05$); I, significantly increased ($P < .05$); NE, no effect.

expected to result in an immune alteration, we have chosen to include bone marrow progenitor cell assays as part of our overall immune assessment.

Additionally, as one of the most rapidly renewing cell populations, hemopoietic cells have proven very sensitive to cytotoxic agents (14, 26, 7). Bone marrow failure is a significant complication of cancer chemotherapy and has also been implicated as a result of exposure to certain drugs (45, 16, 49, 48), and environmental agents (62, 25, 63). During the past decade a variety of in vitro culture techniques in a semisolid matrix have been developed for most of the hemopoietic cell lines. Examination of colony formation of the hemopoietic cells after exposure to various agents has proven to be a sensitive indicator of toxicity as well as a means for mechanistic study of the toxicity of various drugs (63).

C. Cell-Mediated Immunity

During the past decade, many assays have been developed to examine CMI functions. These include both in vivo (e.g., delayed hypersensitivity, graft vs. host, rejection of skin grafts) and in vitro techniques (e.g., lymphoproliferation, T cell cytotoxicity, and lymphokine production). We routinely assess CMI by examining delayed hypersensitivity responses (DHRs), in vitro lymphoproliferative response to mitogens and allogeneic leukocytes (MLCs), and enumeration of splenic T lymphocytes.

Although in vitro assays are commonly employed, DHRs remain the most widely accepted means of clinical or experimental assessment and correlate in humans with decreased host resistance to infectious agents (41). Radiometric assays can best be used in laboratory animals since they provide greater sensitivity than measuring skin reactions (69, 35). We use a modification (40) of a method originally described by Lefford (35) in which ^{3}H-TdR is administered before antigen challenge to enable radiolabeling of monocyte precursors in the bone marrow. A T dependent antigen such as purified protein derivative (PPD) in rats or keyhole limpet hemocyanin (KLH) in mice can be employed and we prefer to sensitize the mice before chemical administration in order to approximate that which may occur in a human population.

Lymphoproliferative (LP) responses are a widely known correlate of CMI and can be defective in the absence of lymphopenia (53). In the microculture LP assay, general mitogens (e.g., plant lectins, bacterial products), specific antigens, or alloantigens (i.e., mixed leukocytes) are used to selectively or polyclonally activate splenic lymphocytes to proliferate, as measured by ^{3}H-TdR incorporation into DNA. Depressed LP responses in humans or animals with a normal number of lymphocytes are usually interpreted as failure of cell activation. Recent studies have indicated that altered responsiveness may also occur through tolerance or suppressor substances produced by regulatory subpopulations of macrophages and T lymphocytes [reviewed by Oppenheim and Rosenstreich (53) and Katz (28)]. Other factors, however, should be considered as causing depressed LP responses in immunotoxicity studies. Some of these factors include: a chemically induced lymphocytotoxicity, as may occur with TCDD (38); redistribution of lymphocyte subpopulations (i.e., B, T, or null cells), as may occur with phorbol ester; and/or an early maturational defect in lymphocyte development, since mature lymphocytes respond differently to mitogens than less mature lymphocytes [e.g. PHA, Concanavalin A (Con A) ratio]. This does not mean that LP assays are unreliable predictors of immune dysfunction, but rather that they may prove to be extremely sensitive indicators of immunotoxicity, if appropriately interpreted.

D. Humoral Mediated Immunity

HMI is best assessed in chemically treated animals by determining antibody plaque-forming cell (PFC) responses, serum immunoglobulin concentrations, splenic lymphocyte responses to *Escherichia coli*, LPS, and enumeration of splenic B cells. Cunningham's modification (10) of the Jerne and Nordin plaque assay (27) has been extensively used to examine T lymphocyte dependent and independent antibody responses of the IgM type following chemical exposure. To a lesser extent, ELISA and RIA procedures have been used with similar sensitivity (73) to measure antibody response in serum.

Quantification of serum immunoglobulins (Ig) may be a useful parameter to examine, particularly in chronic studies where sufficient time is available for the natural decay of existing (preexposure) Ig ($T_{1/2} \cong 20$ d). Furthermore, increases or decreases in specific classes of Ig are associated with chronic or sinopulmonary infections, liver diseases (e.g., cirrhosis), and allergic diseases [reviewed by Gell and Coombs (20)]. A variety of techniques are available for quantitative assessment of Ig levels ranging from extremely sensitive techniques such as RIA and ELISA to less sensitive but reliable methods such as automated immune precipitation (AIP), electroimmunoassay (EIA), and radialimmunodiffusion [reviewed by Davis and Ho (11)].

The expression of specific surface markers and receptors on lymphocytes, lymphocyte subpopulations, and macrophages has been described [reviewed by Katz (28)]. These markers or receptors have been clinically employed to assess immunodeficiency through enumeration of the various cell populations, [reviewed by Oppenheim and Schector (54)]. We routinely determine splenic B and T lymphocyte numbers with FITC conjugated antisera against specific B cell (Ig^+) and T cell ($Thy\text{-}1^+$) surface markers as described by Seligman et al. (60) and Press (58), respectively. These staining procedures, which can be used in combination with various rosette procedures (e.g., E, EAC) offer sensitive techniques to quantify cell populations, but not their functional capacity.

E. Macrophage Function Assays

During the past few years we have learned that macrophages (MØ) not only provide nonspecific phagocytic

functions but are also directed and regulated by lymphokines. In addition, they provide interactions as well as products that have feedback and regulatory roles (i.e., prostaglandins and monokines) in immune responses. Thus, an understanding of MØ function is becoming central to our understanding of immune responses and any assessment of a chemical's immunotoxicity would not be complete without examining some MØ function parameters.

MØ are known to provide a variety of functions including phagocytosis, intracellular killing, antigen presentation, interferon production, as well as cytostasis and cytolysis or virally infected or neoplastically transformed cells. Most MØ can be enumerated by staining for nonspecific esterase activity as described by Koski et al. (32). Most of the functional parameters described above can be quantified and suggested methods are provided in table 4.

III. Immunotoxicology Studies with Diethylstilbestrol

DES is a synthetic nonsteroidal compound possessing estrogenic activity. It has been extensively employed as a therapeutic agent in humans [reviewed by McLachlan and Dixon (43)] as well as a growth-promoting agent in sheep and cattle [reviewed by McMartin et al. (44)]. While DES is no longer used in the treatment of threatened abortions, it is currently administered to women for hormonal replacement, as estrogen replacement in gonadal dysgenesis, and in men for treatment of prostatic cancer. A number of pathological effects have been attributed to DES administration in humans (43). The most notable of these findings include an increased incidence of latent clear-cell adenocarcinoma of the vagina in young women exposed to DES in utero (24).

Studies previously reported from this laboratory with DES (6, 39, 12) are highlighted in table 5. In our studies, adult B6C3F1 mice were given s.c. injections with 0.2, 2, and 8 mg of DES per kg of body weight (Sigma Chemical Co., St. Louis, MO) in 0.1 ml of corn oil for 5 consecutive days. Control mice received corn oil alone. Immunological studies were performed on all mice 3 to 5 days after the last exposure. All animals survived the DES exposure regimen without clinical evidence of toxicity as determined by body weight loss. Table 6 summarizes body and selected organ weight data from DES-exposed mice. While DES appeared to cause a slight increase in body weight, there was a striking decrease in thymic weights ranging from a 50% decrease in the 0.2-mg/kg dosage group to greater than 75% decrease at the highest dose. Histologically, all DES-treated mice showed marked thymic alterations characterized by progressive reduction in thymic cortical lymphocytes with increasing DES exposure. At the 8-mg/kg exposure the majority of cortical lymphocytes were destroyed.

Renal weights remained unchanged while liver weights were increased with increased DES exposure. Mice in the high DES dosage group had liver weights double that of control mice (2,070 mg vs. 1,063 mg in control mice). Histologically, the liver showed increased cytoplasmic vacuolization of hepatocytes that were centrilobular in distribution. The spleens of DES-exposed mice showed both increased weight and hypercellularity. Histologically, the splenic changes were characterized by a shift in the red pulp to white pulp ratio with increasing DES exposure. At the higher doses the splenic follicles were

TABLE 5

Effect of in vivo exposure to diethylstilbestrol (DES) on immune function and host resistance parameters

Parameters Assessed	Observed Effect
Resistance to *Listeria* challenge	D
Resistance to tumor challenge: PBY6	D
Trichinella expulsion	D
Thymus weight	D
Delayed-type hypersensitivity	D
Lymphocyte responses	
PHA	D
Con A	D
LPS	D
MLC	D
T cell quantification	D
Spontaneous lymphocyte cytotoxicity (NK)	NE
Antibody plaque response (19 S): PFC/10^6 cells	D
Immunoglobulin levels (M, G, and A)	NE
Macrophage phagocytosis	I
Macrophage cytostasis	I
Reticuloendothelial system clearance time	I
Bone marrow cellularity, CFU-S and CFU-GM numbers	D

Abbreviations used are: D, significantly decreased ($P < .05$); I, significantly increased ($P < .05$); NE, no effect; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture; NK, natural killer; PFC, plaque-forming cell; CFU, colony-forming unit.

TABLE 4

Assessment of macrophage function

Procedure	Function Assessed	Reference to Method
In vivo clearance: Uptake of ^{125}I-triolein or colloidal carbon	Measures clearance capacity of reticuloendothelial system	DiLuzio and Riggi (15)
In vitro assessment: Phagocytosis of particle	Phagocytic capacity and rate	Van Furth et al. (71)
Intracellular killing	Determine bactericidal capacity	Van Furth et al. (71)
Cytostasis	Capacity of macrophages to inhibit growth of leukemia target cell	Dean et al. (13a)
Cytolysis	Capacity to kill and lyse tumor target cell	Mantovani et al. (47) Adams et al. (1)
Biochemical	Measurement of activation of lysosomal enzyme system (i.e., 5'-nucleotidase, acid phosphatase and leucine amino peptidase)	Rhodes et al. (59)

TABLE 6
Body and selected organ weights in mice exposed to diethylstilbestrol (DES)

Dose of DES (mg/kg)	Body Weight* (g)	Thymic Weight (mg)	Thymic/Body Weight Ratio ($\times 10^3$)	Liver Weight (mg)	Right Kidney Weight (mg)
0	20.78 ± 0.35	49.2 ± 1.6	23.35 ± 0.87	1063 ± 59	133 ± 3
0.2	21.60 ± 0.59	21.8 ± 1.1†	10.21 ± 0.62†	1294 ± 24‡	146 ± 6
2.0	22.78 ± 1.00	14.5 ± 1.1†	5.98 ± 0.53†	1666 ± 66†	135 ± 7
8.0	22.10 ± 0.48	12.1 ± 0.7†	4.66 ± 0.27†	2070 ± 51†	139 ± 3
Dose response	$P = .07$	$P < .01$	$P < .01$	$P < .01$	NS

* Mean value ± SE of 7 animals/group except thymus in which 8 animals/group were examined.
† $P < .01$.
‡ $P < .05$.

small with an increase in red pulp showing marked erythropoiesis and myelopoiesis.

The effects of DES exposure on bone marrow cellularity and CFUs are summarized in table 7. There was a significant ($P<.01$) dose related hypocellularity of the bone marrow. The percentage of bone marrow CFU-GM as measured by in vitro cultures also exhibited a dose-dependent reduction. The number of hematopoietic pluripotent stem cells (CFU-S) likewise demonstrated significant ($P<.01$) dose-response reduction with DES exposure.

The effect of DES exposure on resident peritoneal cell numbers and function is depicted in table 8. Significantly more resident peritoneal cells were lavaged from DES-exposed mice than control mice. At the highest DES exposure a 100% increase in peritoneal cells was found, with both macrophages and lymphocytes being increased. Increased phagocytosis by macrophages of sheep red blood cells (SRBC) was observed in the group exposed to DES, 2 and 8 mg/kg. MØ from DES-treated mice were also cytostatic for MBL-2 leukemia target cells, which is indicative of MØ activation.

The ability of estrogens to stimulate phagocytic activity has been well documented (23, 52). DES has caused increased hepatic phagocytic activity of macrophages (29, 36). These studies indicate that the increased reticuloendothelial system (RES) activity from DES treatment was due at least in part to increased macrophage activation and hyperphagocytosis, although an additive contribution by increased RES organ weights could not be excluded since livers and spleens were enlarged at the high dosage levels of DES.

Direct antibody (IgM) PFC response to SRBC were reduced approximately 25% below control values in mice given the 2.0 and 8.0 mg/kg doses of DES as evaluated by PFCs/10^6 spleen cells and by over 40% based upon PFC/total spleen. Antibody responses in the lowest DES dosage groups were similar to those of control mice. Serum antibody titers to the T-independent antigen (LPS) were reduced 10%, 15%, and 45% from control values in the 0.2, 2.0, and 8.0 mg/kg dosage groups, respectively; this suggests that the effects of DES on B lymphocytes were moderate (39). The effect on CMI was evaluated by examining the parameters of delayed cutaneous hypersensitivity to KLH and LP responses to mitogens and allogeneic leukocytes in one-way MLC responses.

TABLE 7
Mouse bone marrow cellularity and CFUs after diethylstilbestrol (DES) exposure

Dose (mg/kg)	Nucleated Cells/ Femur ($\times 10^{-6}$)*	CFU-GM/1 × 10^5 Cells	CFU-S/5 × 10^4 Cells
0	19.1 ± 1.6	54.5 ± 2.9	6.78 ± 0.67
0.2	15.7 ± 0.6	40.9 ± 4.1†	6.56 ± 0.70
2.0	13.7 ± 0.9†	34.6 ± 2.2‡	6.02 ± 0.64
8.0	12.9 ± 0.7‡	33.5 ± 4.6‡	4.00 ± 0.36‡
Dose response	$P < .01$	$P < .01$	$P < .01$

* Mean ± SE of 7 animals tested/group.
† $P < .05$ vs. control group.
‡ $P < .01$ vs. control group.

TABLE 8
*Effects of diethylstilbestrol (DES) on resident peritoneal cells**

Dose of DES (mg/kg)	Total No. of RPC ± SE	SRBC Phagocytic Index	Cytostasis of MBL-2 Target Cell (%)
0	2.76 ± 0.12	47.6 ± 7.9	
0.2	3.50 ± 0.32†	44.0 ± 7.2	28
2.0	4.40 ± 0.22‡	74.6 ± 2.8†	86
8.0	5.62 ± 0.30‡	71.5 ± 4.7†	90
Dose response	$P < .01$	$P < .05$	$P < .01$

* Mean value ± SE of 8 animals/group. Abbreviations used are: RPC, resident peritoneal cells; SRBC, sheep red blood cells.
† $P < .05$.
‡ $P < .01$.

Figure 1 summarizes the effects of DES administration on in vitro splenic LP responses. LP responses to the T lymphocyte mitogens PHA and Con A, were severely depressed in treated mice. Suppression of these LP responses ranged from approximately 30% in the 0.2-mg/kg dosage group to 70% at the highest dosage group. A dose response effect was not seen in the LP response to LPS, the B cell mitogen. While the LPS response was augmented in the 0.2- and 2.0-mg/kg dosage groups, there was an apparent 29% decrease (not statistically significant) in the highest dosage group compared to control values. Suppression of MLC responses occurred in mice from the 2.0- and 8.0-mg/kg dosage group. Likewise, a series of coculture experiments were performed

and an adherent MØ population was identified as partially responsible for depressed responses (39).

Nicol et al. (52) found that exposure of Swiss mice to low levels of DES appeared to increase phagocytic activity and extend mean survival following challenge with *Pneumococcus* type I and *Pasteurella septica*. In our study adult B6C3F1 mice exposed to DES had increased susceptibility to *Listeria monocytogenes*, *Trichinella spiralis*, and transplantable syngeneic tumor cells (12). The assay endpoints included mortality after *Listeria* challenge, expulsion of *T. spiralis* from the gut, and increased susceptibility to tumor development after challenge with a low cell concentration of PYB6 cells (fig. 2). DES-exposed animals had markedly depressed resistance to tumor cell and *Listeria* challenge and reduced expulsion of *T. spiralis*. These data indicate that exposure to DES at the doses utilized resulted in thymic atrophy, depressed CMI and HMI, and altered host resistance.

IV. Immunotoxicology Studies with Benzo(a)Pyrene and Benzo(e)Pyrene

PAHs represent a ubiquitous class of environmental chemicals found in coal tar and soot as well as other combustion products. As a class, PAHs consist of three or more fused benzene rings in linear, angular, or cluster arrangements that contain only carbon and hydrogen atoms.

Malmgren et al. (46) observed that exposure of mice to the PAHs, 3-methylcholanthrene (MCA), 1,2-benzanthrene (BA), and 1,2,5,6-dibenzanthracene (DBA), at dosage levels equal to or less than one fourth of the MTD produced a marked depression in sheep cell antibody

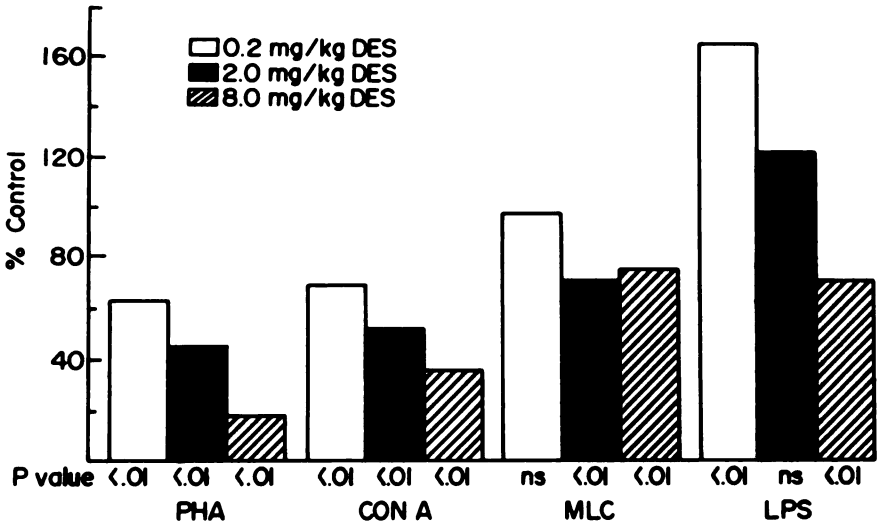


FIG. 1. Effect of DES exposure on lymphoproliferative response to mitogens and allogeneic cells. DES, diethylstilbestrol; PHA, phytohemagglutinin; Con A, concanavalin A; MLC, mixed lymphocyte culture; LPS, lipopolysaccharide.

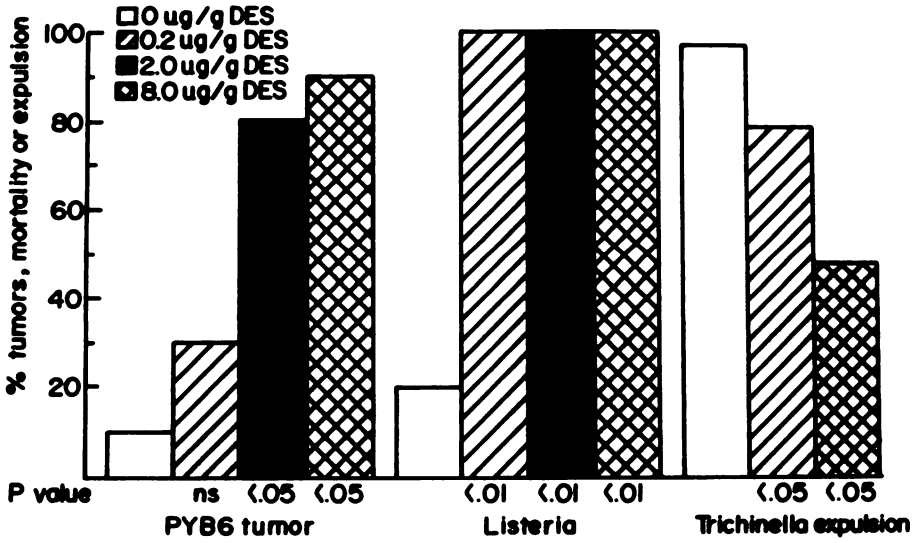
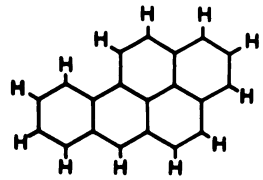


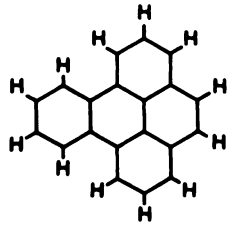
FIG. 2. Effect of diethylstilbestrol (DES) exposure on host resistance parameters.

(IgG) hemolysin production. Later studies by Stjernsward (64) confirmed that MCA suppressed immune responses with >50% reduction in the antibody PFC response (IgM) to sheep erythrocytes that persisted for a period corresponding to the latency period for tumor formation. Although prolongation of skin graft survival also occurred after administration of MCA, the data are ambiguous since prolongation was only noted if the grafting was performed 11 or more weeks after MCA exposure, a time that corresponded to the appearance of tumors. In a subsequent report, Stjernsward (65) found a similar reduction in the PFC response to sheep erythrocytes in CBA mice exposed to 7,12-dimethylbenz(a)-anthracene (DMBA) and benzo(a)pyrene (B(a)P) that persisted for more than 32 days. Thus, although the effects on HMI are clear, the effects of PAHs on CMI or host resistance has not been well characterized and might be of extreme importance since this class of chemicals are potent carcinogens.

Adult B6C3F1 mice were exposed to B(a)P or B(e)P (fig. 3) dissolved in corn oil by daily s.c. injections to give a total dose of 50, 200, or 400 μg/g of body weight over a 14-day period. Immune function and host resistance parameters were examined 3 to 5 days after the last exposure. Results from immune function evaluation with the carcinogen B(a)P and the noncarcinogenic congener B(e)P in adult B6C3F1 mice are summarized in table 9. B(a)P exposure did not significantly ($P>.01$) alter the DHR response at any dosage level examined. Splenic lymphocyte proliferative responses to the T cell mitogens in B(a)P exposed mice (data not shown) were depressed by over 50% in both the 200 μg and 400 μg dosage groups. The response to the B cell mitogen LPS was suppressed by 54% at the high B(a)P dose and exhibited enhancement at the low dose levels. Exposure to comparable doses of B(e)P did not alter any of the mitogen responses. The MLC response was likewise not significantly altered by B(a)P or B(e)P exposure.



Benzo (a) Pyrene



Benzo(e) Pyrene

FIG. 3. Structure of the carcinogen benzo(a)pyrene and its noncarcinogenic congener benzo(e)pyrene.

TABLE 9

*Effect of in vivo exposure to benzo(a) and (e)pyrene (B(a) or (e)P) on immune function and host resistance parameters**

Parameter Assessed	Observed Effect	
	B(a)P (Carcinogen)	B(e)P (Noncarcinogen)
Resistance to *Listeria* challenge	NE	—
Resistance to PYB6 tumor challenge	NE	—
Trichinella expulsion	D	—
Thymus weight	NE	NE
Delayed type hypersensitivity	NE	—
Lymphocyte responses		
PHA	D	NE
Con A	D	NE
LPS	D	NE
MLC	NE	NE
Natural killer cytotoxicity	NE	—
Antibody (19 S) plaque response (PFC/spleen)		
T-dependent	D	NE
T-independent	D	NE
Immunoglobulin levels (M, G, and A)	NE	NE
Macrophage phagocytosis	NE	—
Macrophage tumor cytostasis	NE	—
Reticuloendothelial system clearance time	NE	—
Bone marrow cellularity (CFU-S and CFU-GM)	D	NE

* Abbreviations used are: D, significantly decreased ($P < .05$); I, significantly increased; NE, no significant effect; —, not done; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture; PFC, plaque-forming cell; CFU, colony-forming unit.

B(a)P, but not B(e)P, severely suppressed the direct antibody (IgM) PFC response to both T-dependent and T-independent antigens at all dose levels up to 75% and 86% per 10^6 spleen cells, respectively (fig. 4). The effect of B(a)P on the secondary antibody (IgG) PFC response was less clear since significant suppression occurred only at the medium B(a)P dose (26%). Our observation of a depressed direct antibody PFC responses after B(a)P exposure confirmed the earlier observation of Stjernsward (65). In those studies, exposure of adult (2- to 3-month-old) CBA mice to 1 mg of B(a)P suppressed direct anti-SRBC PFC/10^6 spleen cells by 66% at 2 days, 69% at 7 days, and 47% at 32 days after exposure. More recently, Urso and Gengozian (68) found that a single exposure of pregnant C3H/ANF mice to B(a)P (100 or 150 μg/gm of body weight) induced a severe suppression of the anti-SRBC PFC response in offspring that persisted throughout life. Our studies have confirmed that in utero exposure to B(a)P results in depressed anti-SRBC PFC responses that persist for up to 12 weeks (table 10). Parameters other than HMI were not affected.

Host susceptibility parameters were assessed by challenge of B(a)P exposed mice to syngeneic tumor cells (PYB6) or *L. monocytogenes*. B(a)P exposed mice had a tumor incidence comparable to control mice after PYB6

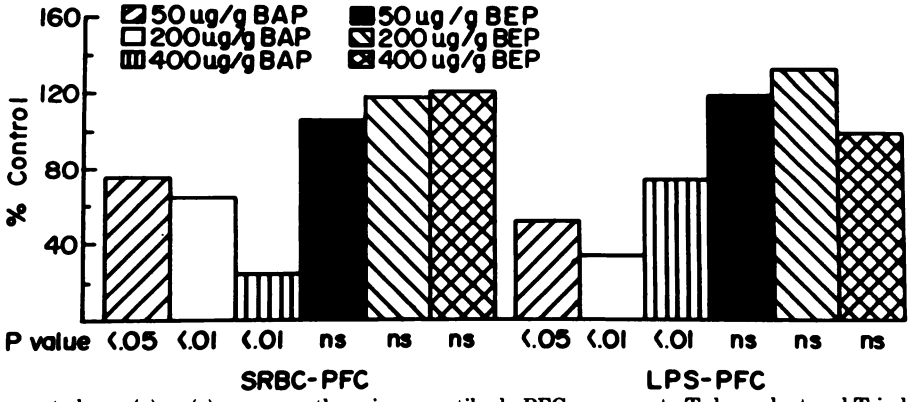


FIG. 4. Effect of exposure to benzo(a) or (e)pyrene on the primary antibody PFC response to T-dependent and T-independent antigens. BAP, BEP, benzo(a) or (e)pyrene; SRBC, sheep red blood cells; PFC, plaque-forming cell; LPS, lipopolysaccharide.

challenge (data not shown). Susceptibility to *L. monocytogenes* challenge (LD10) was likewise unaltered in B(a)P exposed mice.

In our study, DHR and host resistance responses were unaffected after B(a)P, which suggest that T cell immunocompetence was preserved in spite of some depressed splenocyte responses to PHA and Con A. Severe depression in the primary antibody PFC response to both T-dependent and independent antigen was the most striking observation. Zwilling (75) observed unaltered skin graft rejection in Syrian hamsters after exposure to 1 mg of B(a)P given by inhalation as B(a)P-Fe_2O_3, in spite of severely depressed humoral antibody responses. Recent evaluation of the effect of B(a)P on skin graft rejection confirmed that B(a)P exposure does not alter skin graft rejection (table 11) and provides further evidence that CMI is spared following B(a)P treatment.

Data from several laboratories, including our own, support the contention that immune alterations induced by carcinogenic PAHs in general, and in particular B(a)P, are lined to the carcinogenic potential of this structure since these effects were not induced by the noncarcinogenic congener. However, our data suggest that B cells and the humoral immune component rather than T cells and CMI are the primary target for the toxicity induced by this class of chemicals.

V. Immunotoxicology Studies with Phorbol Esters

Phorbol esters are chemicals of immunological concern because of their tumor-promoting ability. 12-O-Tetradecanoyl phorbol 13-acetate (TPA) [or phorbol myristate acetate (PMA)] (see fig. 5 for structure) is a representative of a phorbol ester with potent tumor-promoting activity derived from croton oil (22). In this respect, TPA is a potent promoter of multiple skin tumors in vivo (5, 70) and enhances transformation of fibroblast and rat embryo cell cultures in vitro after exposure to PAH carcinogens (34, 50) or oncogenic viruses (18, 56). TPA has also been reported to induce multiple activities on leukocytes after in vitro exposure, including induction of lymphocyte mitogenesis (67; Baxter, personal communication), macrophage membrane alterations, enhanced pinocytosis, and tumor cell cytostasis (21).

TABLE 10

*Effect of in utero benzo(a)pyrene exposure on T-dependent antibody plaque-forming cell (PFC) response**

Time after Exposure (Wk.)	Dose (μg/g)	SRBC PFC Per 10^6 Nucleated Spleen Cells	(% Change)	Spleen ($\times 10^4$)	(% Change)
7–8	0	891 ± 57†		28.2	
7–8	120	399 ± 42‡	(55%↓)	10.0‡	(59%↓)
7–8	240	333 ± 48‡	(63%↓)	8.3‡	(71%↓)
12	0	1034 ± 94		24.6	
12	120	813 ± 116	(21%↓)	16.0§	(35%↓)
12	240	860 ± 86	(17%↓)	16.5§	(33%↓)

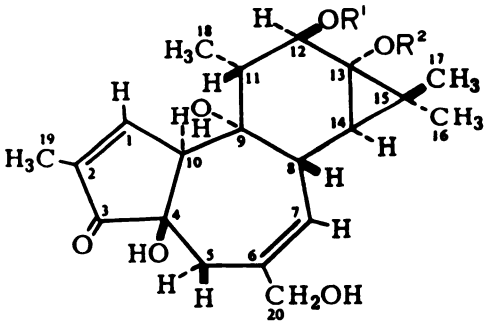
* Abbreviation used is: SRBC, sheep red blood cells.
† Mean ± SEM of 6 mice/group
‡ $P < .01$ vs. control group.
§ $P < .05$ vs. control group.

TABLE 11

Effect of benzo(a)pyrene exposure on rejection of DBA skin grafts

Dose (μg/g)	Mean No.* Days Graft Retained ± SEM	Significant Level
0	13.0 ± 0.6	—
400	13.6 ± 0.8	NS

* Five benzo(a)pyrene exposed mice and control B6C3F1 group were grafted with 1 sq cm of tail skin from DBA mice. The mice were observed daily with a dissecting microscope from day 10. Control DBA mice grafted with DBA skin had well healed grafts when the experiment was terminated on day 18.



Phorbol
Myristate Acetate
$R^1 = CO(CH_2)_{12}CH_3$ $R^2 = COCH_3$

FIG. 5. Structure of tumor promoter phorbol myristate acetate.

Since immune function has been suggested to be an important cofactor in neoplasia and tumor development (8), we examined the effects of TPA exposure in vivo on host resistance and immune function parameters. Adult B6C3F1 mice were exposed to a total dose of 2, 20, and 40 μg/g of TPA dissolved in corn oil given by four s.c injections over a 2-week period.

Preliminary results of these studies are summarized in table 12. Thymus weight, T cell numbers, and responses to T and B cell mitogens or allogeneic leukocytes in one-way MLC were also reduced. The lymphoproliferative response to Con A, LPS, and PHA were suppressed by 51%, 45%, and 31%, respectively, at the 40 μg/g exposure dose (table 13). The MLR was suppressed at this dosage level. Evaluation of splenocytes for T and B cell surface markers revealed approximately 60% loss in cells bearing T cell surface markers at the highest exposure level (table 13). Assessment of changes in spontaneous natural killer (NK) cellularity revealed that profound effects occurred (>90% reduction) at all dosage levels (table 14).

Serum immunoglobulin levels in the three major classes were depressed at the high dose only. Although the number of PFC/10^6 cells was depressed at this same dose, the number of PFC/spleen were unaffected as was bone marrow cellularity and progenitor cell numbers. Macrophage phagocytosis in vitro, tumor cytostasis, and in vivo RES clearance of ^{125}I-triolein were enhanced, which was consistent with the known effects of TPA exposure on in vitro macrophage function. As can be determined from the host resistance models, a rather profound and selective decrease in resistance to the two syngeneic transplantable tumors examined was observed following TPA exposure at all dosage levels with a concomitant increase in *Listeria* resistance (table 15).

Recently, evidence has been presented for a cell surface receptor for TPA on murine T lymphocytes that may account for the selective toxicity of TPA for T cells. It is interesting to speculate that the corrective T cell dysfunction (i.e., depressed PHA, Con A, MLC, and NK activity) observed following TPA exposure in vivo helps to account for the altered tumor resistance. The importance of macrophage activation underlying some of these effects has not been fully evaluated and may account for the increased resistance to *Listeria*, although there is no

TABLE 12

*Effect of in vivo exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) on immune function and host resistance parameters**

Parameters Assessed	Observed Effect
Resistance to *Listeria* challenge	I
Resistance to tumor challenge	
PYB6	D
B16 Melanoma	D
Trichinella expulsion	NE
Thymus weight	D
Delayed-type hypersensitivity	NE
Lymphocyte responses	
PHA	D
Con A	D
LPS	D
MLC	D
T cell quantification	D
Spontaneous lymphocyte cytotoxicity (NK)	D
Antibody plaque response (19 S): PFC/10^6 cells	D
Immunoglobulin levels (M, G, and A)	D
Macrophage phagocytosis	I
Macrophage cytostasis	I
Reticuloendothelial system clearance time	I
Bone marrow cellularity, CFU-S and CFU-GM numbers	NE

* Abbreviations used are: D, significantly decreased ($P < .05$); I, significantly increased ($P < .05$); NE, no effect; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture; NK, natural killer; PFC, plaque-forming cell; CFU, colony-forming unit.

TABLE 14

Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on natural killer cells

Dose of TPA (μg/g)	Cytolysis of YAC-1 Target Cells (%)
0	20.3*
2	2.7†
20	3.3†
40	4.4†

* Mean % cytolysis of six mice per group at 100:1 ratio against ^{51}Cr labeled YAC-1 target cells.

† Significantly different from control group at $P < .01$.

TABLE 13

*Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on lymphoproliferative response to mitogens**

Dose of TPA (μg/g)	Control ×10^{-3}	CPM ± of SEM of ^{3}H-TdR Incorporation ×10^{-3} (% Change)				Surface Markers (%)	
		PHA	Con A	MLR	LPS	IgG	Thymocyte
0	0.5 ± 0.1	41.5 ± 2.7	66.3 ± 2.8	14.0 ± 1.2	10.7 ± .8	44.0 ± 2.6	50.3 ± 4
2	0.8 ± .1‡	47.4 ± 2.9 (14%↑)	69.0 ± 6.1 (4%↑)	15.4 ± 2.0 (10%↑)	11.3 ± 1.5 (5%↑)	ND	ND
20	1.7 ± .5‡	36.3 ± 3.4 (13%↓)	53.1 ± 7.2 (20%↓)	15.0 ± 1.0 (7%↑)	9.3 ± 0.9 (13%↓)	ND	ND
40	2.5 ± .3§	26.6 ± 3.8‡ (36%↓)	32.5 ± 3.2‡ (51%↓)	9.6 ± 1.2‡ (31%↓)	5.9 ± 0.5‡ (45%↓)	36.0 ± 2.1‡	20.5 ± 2
Dose response	$P < .001$	$P < .005$	$P < .01$	$P < .05$	$P < .01$	—	—

* Abbreviations used are: ^{3}H-TdR, tritiated thymidine; PHA, phytohemagglutinin; Con A, concanavalin A; MLR, mixed leucocyte response; LPS, lipopolysaccharide.

† Mean CPM ± SEM of 6 mice/group.

‡ Significantly different from control at $P < .05$.

§ Significantly different from control at $P < .01$.

TABLE 15

Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) exposure on host resistance parameters

Dose of TPA (μg/g)	PYB6 Challenge		B16 Melanoma Lung Nodules		Listeria Challenge	
	No. Tumors / No. Challenged	Tumor Takes (%)	No. Nodules ± SEM	CPM of ^{125}IUDR Incorporation	Mortality	
					No. Challenged	%
0	8/35	23	15 ± 2	1509 ± 296	17/19	89
2	11/30	37	41 ± 5*	2673 ± 392†	6/10	60
20	17/30	57†	68 ± 4*	9805 ± 1803*	0/10*	0
40	17/28	61†	81 ± 7*	9355 ± 1530*	0/6*	0
Dose response	$P < .01$		$P < .01$		NS	

* Significantly different from control at $P < .05$.
† Significantly different from control at $P < .01$.

direct evidence that suppressor cell activity exists (Dean, unpublished data).

VI. Summary

The discipline of immunology should provide toxicology with sensitive models to assess toxicity as well as provide better safety assessment. More information on correlations between immune function and host resistance changes and a more standardized panel of methods for immunotoxicity screening will add further credence to the already impressive immunotoxicology data base. Research efforts are needed to evaluate newer in vitro methods with microsome activation systems to screen chemicals for immunotoxicity, to evaluate chemicals for induction of hypersensitization or allergy, and to better determine whether there are chemical structure activity relationships for chemically induced immunotoxicity. The studies described here represent preliminary examples of these approaches.

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