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Advances in
Pharmacology

1. Exp.
2. Pharmacol.
3. Immunol.
4. Toxicol.
5. Pharmacol.
6. Pharmacol.
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Assessment of Immunotoxicity Induced by the Environmental Chemicals 2,3,7,8-Tetrachlorodibenzo-p-dioxin, Diethylstilbestrol and Benzo(a)pyrene

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ABSTRACT

A testing battery will be described for assessing alterations in immunological function and host resistance following exposure of B₆C₃F₁ mice to TCDD, DES and BP. TCDD is a major contaminant of the defoliant 2,4,5-T (agent orange); DES is a potent synthetic estrogen used therapeutically to prevent menopausal symptoms and post-coital implantation, and as a weight promotor in cattle; and BP is the principal carcinogen in cigarette smoke and coal tar. Mice exposed perinatally to TCDD (5, 1 µg/kg) exhibited marked thymus atrophy and depressed mitogenic responses. Resistance to *Listeria monocytogenes*, gram-negative endotoxin and transplantable syngeneic tumor cells were likewise impaired. The mice appeared to have a specific T-cell defect associated with a receptor for TCDD present at high levels in thymic tissue. Mice exposed to DES at 3.0, 2.0 and 0.2 mg/kg of body weight for 5 days exhibited severe thymus atrophy accompanied by depression of the following parameters: antibody PFC response to SRBC and LPS; delayed cutaneous hypersensitivity; lymphoproliferative response to T- and B-cell mitogens and alloantigens; bone marrow cellularity; and stem and granulocyte-macrophage progenitor cell proliferation. Macrophages were activated relative to phagocytic, proliferative and tumoricidal responses with evidence of suppressor cell activity. Host resistance parameters were likewise impaired. Thus, DES exposure resulted in RES stimulation, severely depressed immunological and host resistance function, due in part to macrophage suppressor cells and possibly a T-cell defect. Finally, BP was dosed at 400, 200 and 50 mg/kg of body weight over 10 days. BP exposed mice demonstrated depressed antibody PFC responses; lymphoproliferative responses to mitogens; and depressed numbers of bone marrow macrophage-granulocyte progenitors. Macrophage function and host resistance to *Listeria* and tumor cells were unaffected. These data demonstrate the sensitivity of the immune response for detecting selective or non-specific chemical toxicity and support the hypothesis that certain carcinogens may operate via immunological mechanisms.

INTRODUCTION

During the past decade, the immune system has been examined in experimental animals for its functional integrity following exposure to a variety of chemicals of environmental concern. Certain chemicals have been found to produce alterations in

either humoral-mediated immunity (HMI), cell-mediated immunity (CMI) or host resistance (HR) parameters at levels where overt toxicity was not observed (see review by Vos, 1977; Faith, Luster and Vos, 1980; Dean, Padarathalingh and Jarralls, 1979b,c). Likewise, immune alterations have been observed in certain populations of individuals exposed to chemicals through accidental or occupational exposure. Although a few chemicals may be selectively toxic to a particular sub-population of lymphoid cells of the reticuloendothelial system (RES), such as the thymocyte, in the case of the aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Luster and co-workers, 1980b), it is our hypothesis that examination of immune function in most cases adds an extra level of sensitivity for defining cellular toxicity. This increased sensitivity of the immune response for detecting cellular toxicity results from the rapid proliferation and dynamic nature of the cellular components of the immune system. This concept is supported by human chemical toxicity studies in the form of clinical chemotherapy in which cells of RES are the most frequent target organ for chemical injury. The functional integrity of CMI or the quantitation of bone marrow elements following chemotherapy is frequently determined to define the level of toxicity and to regulate dosage.

There are several considerations when designing a comprehensive immunotoxicity assessment (protocol) with chemicals of environmental concern, as follows: (1) The planned route of exposure should mimic as closely as possible the actual environmental exposure (i.e. oral, parenteral, inhalation, etc.); (2) The dosage employed should somewhat exceed the real life exposure levels with consideration of body weight to surface ratio; (3) The dosage selected should be below those which produce overt toxic effects (i.e. negative weight gain or gross alterations in liver enzyme levels); and (4) The age of the animal selected for exposure should be considered carefully since some chemicals (e.g. TCDD) have already been demonstrated to have more potent immunological alterations when administered during pre- and/or postnatal life while the immune system is undergoing maturation.

The prototype chemicals selected for immunotoxicity assessment by our laboratory represent three broad classes of chemicals that are not only carcinogenic but of immense environmental concern. These include halogenated aromatic hydrocarbons, specifically 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); synthetic hormones, specifically diethylstilbestrol (DES) and the more established carcinogen, benzo(a)pyrene (BP).

TCDD is the primary chlorinated dibenzo-p-dioxin contaminant produced during the synthesis of 2,4,5-trichlorophenoxyacetate (2,4,5-T), the latter being one of the major toxic constituents of the defoliant agent orange (Courtney and co-workers, 1979). TCDD has been found to alter the immunocompetence of rats, mice, guinea-pigs and monkeys as well as alter resistance to bacterial, parasite and syngeneic tumor cell challenge in mice (see review by Vos, 1977; Faith, Luster and Vos, 1979). The study with TCDD described here represents highlights from a detailed pre/postnatal exposure study in B₆C₃F₁ mice (Luster and co-workers, 1980). Studies on the presence of a specific receptor for TCDD in thymic tissue were also conducted.

Diethylstilbestrol is a non-steroidal estrogen initially synthesized by Dobbs and associates (1938) during a search for synthetic estrogens. Since its discovery it has been used therapeutically as an estrogen replacement to control menopausal symptoms, suppress lactation, prevent threatened abortions, and prevent postcoital implantation ("morning after pill"), as well as to treat breast and prostatic cancer patients (see review by Noller and Fish, 1974). DES has also been extensively used as a growth promotant in cattle, sheep and poultry (see review by McMartin and co-workers, 1978). It was implicated as a potential carcinogen or cocarcinogen in humans when Herbst, Ulfelder and Poskanzer (1971) observed that 7 of 8 girls with a very rare clear-cell adenocarcinoma of the vagina were the result of high-risk pregnancies in which the mothers received DES to prevent abortion. These observations have been extended and confirmed in over 300 cases of vaginal as well as cervical adenocarcinoma (Herbst and co-workers, 1977).

Assessment of Immunotoxicity

Recently DES has been shown to have a relatively potent immunosuppressive effect on cell-mediated immunity (CMI) and humoral immunity in mice following prenatal exposure (Luster and co-workers, 1979), postnatal exposure (Kalland and co-workers, 1978, 1980; Kalland, 1980a,b) or adult exposure (Boorman and co-workers, 1980; Luster and co-workers, 1980a; Dean and co-workers, 1980). Studies described in this report highlight our findings with DES.

Polycyclic aromatic hydrocarbons, of which benzo(a)pyrene is a major constituent, are formed by incomplete combustion of fossil fuels and tobacco as well as being by-products of coal conversion processes. They are acted upon by aryl hydrocarbon hydroxylase to metabolites with potent carcinogen potential. The significance of chemical carcinogen-induced immunosuppression in the tumorigenic potential of a carcinogen is not yet fully understood. Malmgren and associates (1952) observed that many carcinogens depressed the antibody response to sheep erythrocytes. Later studies by Prehn (1963) further demonstrated that carcinogens reduce homograft rejection and stimulate the growth of transplantable tumors as well as primary tumors. Further evidence for aromatic hydrocarbon-induced immunosuppression comes from studies by Stjernsward (1965, 1966) and Ball (1970). Recently, Urso and Gengozian (1980) observed that *in utero* exposure to benzo(a)pyrene (BP) resulted in severe depression of the antibody plaque forming cell response to SRBC and an increased frequency of tumors in adult life.

In studies reported here, adult B₆C₃F₁ mice were exposed to BP in corn oil by subcutaneous injections followed by an evaluation of immunological and host resistance parameters.

MATERIALS AND METHODS

TCDD, DES and BP Exposure

Pre/postnatal TCDD exposure was accomplished as previously described in detail by Luster and co-workers (1980). TCDD was synthesized by the LEC at NIEHS at greater than 99% purity. Virgin female C57Bl/6 mice were mated with male C3H mice and pregnancy was determined by the presence of a vaginal plug. Mothers were administered TCDD dissolved in corn oil by gavage at day 7 of gestation and again on days 1, 7 and 14 following birth at dosages of 1.0, 5.0 and 15.0 ug/kg of body weight. Mice used for controls received corn oil. Immunological assessment was initiated 7 days after weaning and host resistance parameters were examined 6-8 weeks after weaning.

Diethylstilbestrol (DES) was obtained from Sigma Chemical Company (St. Louis, MO). Six to eight week old female B₆C₃F₁ mice were injected subcutaneously (sc) with 0.2, 2 and 8 mg/kg body weight of DES dissolved in 0.1 ml of corn oil for five consecutive days. Control mice received corn oil alone. Immunological and host resistance studies were performed 3-5 days following the last dose.

Benzo(a)pyrene (BP) was likewise purchased from Sigma Chemical Company. Six to eight week old female B₆C₃F₁ mice were administered BP dissolved in corn oil by subcutaneous injection to a total dose of 50, 200 and 400 mg/kg body weight over a 14-day period. Three to five days after the last treatment dosage immunological and host resistance parameters were evaluated.

Comprehensive Evaluation of Immunological and Host Resistance Parameters

The assay panel employed in the immunological, pathotoxicological and host resistance evaluation of immunotoxicity has previously been described (Dean and co-workers, 1979b). The methods utilized have likewise been described in detail

(Luster and co-workers, 1980c; Boorman and co-workers, 1980; Dean and co-workers, 1979c).

TCDD Receptor Assay

In a typical assay, 3 ml of a 100,000 $\times$ g supernatant from tissue having a protein concentration of approximately 0.5 mg/ml in 20 mM Tris HCl buffer containing 0.2 M KCl and 1 mM EDTA (pH 7.4), was incubated with 2×10^5 dpm of 1-N-(5- 125 I-iodovalerylamido)-3,7,8-trichlorodibenzo-p-dioxin (125 I-dioxin), plus a 300-fold excess of unlabeled TCDD for 2 hours at 0°C in a shaker bath. The unbound 125 I-dioxin was removed by activated charcoal performed by incubating 0.8 ml aliquot of the incubation mixture with 0.4 ml of charcoal/dextran suspension, centrifuging at 300 $\times$ g for 10 minutes, and counting 0.6 ml of supernatant in a gamma counter. Each assay was performed in duplicate. Specific binding is the difference between 125 I-dioxin bound in the absence of any unlabeled TCDD and 125 I-dioxin bound in the presence of excess unlabeled TCDD.

Statistical Analysis

The Mann-Whitney U test or Chi-square test was employed to assess the significance of treatment effects. Dose-response trends were determined by Jonckheere's test. Differences between mitogen responses were determined by Student's t-test.

RESULTS

Table 1 summarizes the effect of chemical exposure on body and selected organ weights. A significant decrease in body weight gain was observed in the 5 μ g/kg TCDD dosage group. Thymus weight and thymus-to-body weight ratios were also significantly reduced in this dosage group. Histopathological evaluation of the thymus revealed severe thymus atrophy with depletion of lymphocytes from cortical regions in the 5 μ g/kg dosage group.

Table 1 also summarizes body and selected organ weights from DES exposed mice. No evidence of overt toxicity was observed at any of the DES doses. DES induced a slight increase in body weight as might be expected, while producing a dramatic decrease in thymus weights which ranged from 50 to 75% depending on dose. On histopathological evaluation, marked alterations of thymus size characterized by marked depletion of cortical lymphocytes as a function of DES dose was observed. Spleen and liver weights were increased. BP produced a dose response trend ($p < 0.01$) towards body and thymus weight loss which was not statistically significant at any of the concentrations tested (Table 1). Other organ weights were unaltered. Histopathological evaluation of other lymphoid organs revealed no marked pathology.

The evaluation of bone marrow cellularity, spleen colonies (CFU-S) in x-irradiated recipients, and the number of bone marrow cells forming macrophage-granulocyte colonies (CFU-GM) is summarized in Table 2. Bone marrow cellularity and the number of cells forming CFU-S colonies/ 5×10^5 cells was significantly depressed in the high TCDD dosage group. CFU-GM colonies were significantly reduced in the high dosage group. The effect of DES exposure on bone marrow cellularity and CFU-S is also summarized in Table 2. The observed hypocellularity of the bone marrow was significant ($p < 0.01$) and likewise dose-related. The number of bone marrow CFU-GM as measured by *in vitro* cultures exhibited a dose-dependent reduction. The numbers of hematopoietic pluripotent stem cells (CFU-S's) were likewise demonstrated to have a significant ($p < 0.01$) dose-dependent reduction.

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TABLE 1 Effect of TCDD, DES and BP on Body and Selected Organ Weights

Assessment of Immunotoxicity

Chemical	Dose	Body weight (g)	Thymus weight (mg)	Thymus/body weight ratio ($\times 10^3$)	Spleen weight (mg)	Liver weight (mg)	Right kidney weight (mg)
TCDD	0	13.6 ^a	61	4.5	84	840	ND
TCDD	1 ug/kg	12.6	58	4.6	82	846	ND
TCDD	5 ug/kg	10.9 ^c	36 ^b	3.3 ^b	70 ^b	714 ^b	ND
DES	0	20.8	49	2.3	120	1063	133
DES	0.2	21.6	22 ^c	1.0 ^c	140	1294 ^b	146
DES	2.0	22.8	14 ^c	0.6 ^c	160 ^b	1666 ^c	135
DES	8.0	22.1	12 ^c	0.5 ^c	205 ^c	2070 ^c	139
Dose response		NS	<0.01	<0.01	<0.01	<0.01	NS
BP	0	21.2	63.3	3.0	97	1050	144
BP	50	20.9	59.4	2.9	97	1040	137
BP	200	20.2	57.0	2.8	92	1050	138
BP	400	19.8	48.4	2.4	100	1150	152
Dose response		<0.05	<0.05	NS	NS	NS	NS

a - Mean value of 7-8 mice/group; b - significantly different from control at $p < 0.05$; c - significantly different from control at $p < 0.01$; NS - Not significantly different; ND - Not done.

TABLE 2 Effect of TCDD, DES and BP on Bone Marrow Cellularity, Progenitor Cell Colonies, and T-Cell Dependent (SRBC) and Independent (LPS) Antibody Plaque Forming Cells

Chemical	Dose	Nucleated cells/ femur $\times 10^{-6}$	CFU-S/ 5×10^{-4} cells	CFU-GM/ 10^5 cells	SRBC PFC/ 10^6 spleen cells	LPS PFC/ 10^6 spleen cells or HA titer (log 2)
TCDD	0	17.7	10.3	29.4	ND	ND
TCDD	1 $\mu\text{g/kg}$	18.4 (4X) ^a	ND	34.8 (18X)	ND	ND
TCDD	5	14.8 (16X) ^b	7.0 (32X) ^b	26.0 (12X)	ND	ND
DES	0	19.1	6.8	54.5	1003	6.67
DES	0.2 mg/kg	15.7 (18X)	6.6 (3X)	40.9 (25X) ^b	1039 (4X)	6.00 (10X)
DES	2.0	13.7 (28X) ^b	6.0 (12X)	34.6 (36X) ^c	742 (26X) ^b	5.67 (15X)
DES	8.0	12.9 (32X) ^c	4.0 (41X) ^c	33.5 (38X) ^c	775 (23X) ^b	3.67 (45X) ^b
Dose response		<0.01	<0.01	<0.01	<0.01	<0.01
BP	0	19.8	10.6	108	1510	729
BP	50 mg/kg	22.8 (15X)	8.5 (20X) ^c	92 (15X)	1133 (25X)	389 (47X) ^c
BP	200	21.1 (6X)	8.5 (20X) ^c	89 (18X) ^b	984 (35X) ^b	256 (65X) ^c
BP	400	24.3 (23X) ^b	5.7 (46X) ^c	64 (41X) ^c	378 (75X) ^c	99 (86X) ^c
Dose response		<0.05	<0.01	<0.01	<0.01	<0.01

a - Mean value of 6-8 mice /group with X change in parenthesis; b - Significantly different from control at $p < 0.05$; c - Significantly different from control at $p < 0.01$; d - Hemagglutinin/titer (log 2).

Assessment of Immunotoxicity

43

The evaluation of bone marrow cellularity and progenitor cells in BP-treated mice revealed increased cellularity in the highest dosage group accompanied by depressed levels of pluripotent stem cells (CFU-S) which were significantly reduced at all dosage levels, while the number of CFU-GM colonies was reduced at the 200 and 400 mg/kg level.

The IgM antibody plaque forming cell (PFC) response to sheep red blood cells (SRBC) was significantly reduced ($p < 0.05$) at the 2 and 8 mg/kg dosage of DES (Table 2). And PFCs per total spleen were likewise reduced over 40%. The serum antibody titer to the T-cell independent antigen *E. coli* lipopolysaccharide (LPS) was similarly reduced, up to 45% in the high exposure group.

The most striking effect observed in the evaluation of BP was the depressed PFC response to both T-dependent and T-independent stimuli. The response to SRBC was depressed from 25 to 75% and the response to LPS was depressed from 47 to 86%, depending on the treatment group examined.

Assessment of the lymphoproliferative (LP) response of spleen cells from TCDD exposed weanlings to mitogens, revealed significantly depressed proliferative responses to the T-cell mitogens PHA and Con A, which were dose-dependent, but not to the B-cell mitogen LPS (Table 3).

Table 3 also 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 responses ranged from approximately 30% in the 0.2 mg/kg dosage group to 70% in the highest dosage group. A dose response effect was not seen in the response to the B-cell mitogen LPS. The LPS response was augmented in the 0.2 and 2.0 mg/kg dosage groups with a 29% decrease in the 8 mg/kg dosage group, which was not statistically significant when compared to controls. Suppression of the mixed leukocyte culture (MLC) responses occurred in mice from the 2.0 and 8.0 mg/kg DES dosage (data not shown).

LP responses to the T-cell mitogens PHA and Con A were depressed in BP treated mice up to 60% and 54% respectively (Table 3). The B-cell response to LPS was enhanced at the lowest dose and depressed 54% at the highest dose. All mitogens exhibited dose-dependent depression.

Macrophage numbers and function was examined in mice exposed to all three chemicals. Function was evaluated using assays for SRBC phagocytosis, leukemia target cell (MBL-2) cytostasis, macrophage-mediated tumor cell cytolysis and suppressor cell activity. Animals exposed to TCDD and BP exhibited normal M ϕ function and numbers. In contrast, mice exposed to DES exhibited an enhanced number of peritoneal M ϕ (Table 4) which exhibited enhanced phagocytic, cytostatic and cytolytic potential indicative of M ϕ activation (Table 4).

In an attempt to understand the mechanism of TCDD-induced T-cell deficiency, mice were examined for the presence of a putative receptor for TCDD in the thymus and other tissues. These studies (Table 5) revealed a receptor for TCDD present at high concentrations in thymus (114 fm/mg of protein), to a lesser extent in liver and present at only a very low concentration in heart muscle. Competition experiments demonstrated the relative specificity of this receptor (Table 5). Thus, the receptor for TCDD in thymus probably helps account for the selective toxicity of the chemical for thymus-dependent lymphocytes.

Host susceptibility to challenge with 1.2×10^6 viable *Listeria* (LD20) and syngeneic PYB6 tumor cells (TD20) in TCDD exposed mice is summarized in Table 6. Increased mortality to *L. monocytogenes* and tumor take frequency following PYB6 challenge was observed in TCDD exposed mice even at the lowest level of TCDD examined. Endotoxin hypersensitivity was not performed.

TABLE 3. Effect of TCDD, DES and BP on the Lymphoproliferative Responses of Mice to T and B Cell Mitogens

Chemical	Dose	Control	Mean CPM $\times 10^{-3}$ -TdR Incorporation (% change)		
			PHA	Con A	LPS
TCDD	0	1.0	31.4 ^a	40.3	3.8
TCDD	1 μ g/kg	1.1	35.9 (14%)	41.2 (2%)	4.6 (21%)
TCDD	5	0.6	14.9 (52%) ^c	25.7 (36%) ^b	3.1 (18%)
DES	0	0.9	27.5	23.4	8.2
DES	0.2 mg/kg	0.8	17.3 (37%) ^b	16.1 (31%)	13.6 (65%) ^b
DES	2.0	1.2	12.2 (56%) ^c	12.1 (48%) ^c	10.0 (22%)
DES	8.0	1.0	4.8 (83%) ^c	8.3 (65%) ^c	5.8 (29%)
Dose response		NS	<0.01	<0.01	NS
BP	0	0.5	77.7	82.0	16.9
BP	50 mg/kg	0.5	71.3 (8%)	87.3 (6%)	22.4 (32%) ^b
BP	200	0.5	52.8 (32%) ^c	54.7 (33%) ^b	16.8
BP	400	0.3	31.3 (60%) ^c	37.9 (54%) ^c	7.7 (54%) ^c
Dose response		<0.05	<0.01	<0.01	<0.05

a - Mean value of 6-8 mice /group; b - Significantly different from control at $p < 0.05$; c - Significantly different from control at $p < 0.01$.

TABLE 4 Effect of DES Exposure on Peritoneal Macrophage Function

Dosage (mg/kg)	Total number of peritoneal cells $\times 10^{-6}$ /mouse	% SRBC phagocytosis relative to control	% Macrophage cytoastasis (20:1)	% Net Cytolysis (10:1)
0	2.76 ^a	-	-	-
0.2	3.50 ^b	92%	28%	-2%
2	4.40 ^c	155% ^b	86% ^c	25.2% ^b
8	5.62 ^c	150% ^b	90% ^c	24.7% ^b
Dose response	<0.01	<0.05	<0.01	<0.05

a - Mean value of 8 mice/group; b - Actual values were significantly different from control at $p < 0.05$; c - Actual values were significantly different from control at $p < 0.01$.

TABLE 5 Concentration of TCDD Receptor in Cytosol of Mouse Tissue

Tissue cytosol	TCDD Receptor (fmol/mg protein)		
	2,3,7,8-TCDD	2,3,7,8-TCDF ^a	1-NH ₂ -3,7,8-TRICDD ^a
Thymus	114	73	56
Liver	94	58	30
Heart	10	ND	ND

a - Compounds that compete for specific binding of 125 I-dioxin in cytosol fraction; ND - Not done.

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TABLE 6 Effect of TCDD, DES and BP Exposure on Host Resistance Parameters

Chemical	Dose	PYB6 Tumor Cells		Listeria Challenge		Endotoxin Challenge	
		No. tumors/No. challenged	(% tumors)	No. deaths/No. challenged	(% mortality)	No. deaths/No. challenged	(% mortality)
TCDD	0	4/18	(22%)	7/25	(28%)	ND	
TCDD	1 µg/kg	6/10	(60%) ^b	6/15	(40%)	ND	
TCDD	5	4/9	(44%)	11/15	(73%) ^c	ND	
DES	0	2/10	(20%)	2/10	(20%)	0/10	
DES	0.2 mg/kg	3/10	(30%)	10/10	(100%) ^b	6/9	(67%) ^b
DES	2.0	8/10	(80%) ^b	10/10	(100%) ^b	9/10	(90%) ^b
DES	8.0	9/10	(90%) ^b	8/8	(100%) ^a	4/9	(44%)
BP	0	0/10	(0%)	0/10	(0%)	1/10	(10%)
BP	50 mg/kg	1/10	(10%)	1/10	(10%)	3/10	(30%)
BP	200	0/10	(0%)	1/10	(10%)	7/10	(70%) ^a
BP	400	2/10	(20%)	0/10	(0%)	2/10	(20%)

a - Significantly different from control at p < 0.05 by Chi-square analysis or Student's T-test;
b - Significantly different from control at p < 0.01 by Chi-square analysis or Student's T-test.

Assessment of Immunotoxicity

Table 6 also summarizes the effects of DES exposure in adult female B₆C₃F₁ mice on the frequency of F344 tumors following a low challenge level (5×10^4) of tumor cells. There was a significant increase in the frequency ($p < 0.05$) of tumor takes (80-90%) which occurred following tumor cell challenge in mice administered 8.0 or 2.0 mg/kg of DES. The mean latency in days to tumor detection was not significantly altered in DES exposed mice nor was the mean tumor volume (data not shown). Likewise, Table 6 summarizes challenge and mortality studies with *Listeria* in mice following DES exposure. Mice from all three DES treatment groups demonstrated a significant mortality ($p < 0.01$) to a *Listeria* challenge dose of organisms that killed only 20% of the corn-oil treated controls. The mean latency to death in days was likewise reduced in the DES treated groups with an average latency of from 4.8 to 5.4 days as compared to 8.0 days in the control group.

DES exposed animals were also significantly ($p < 0.01$) more sensitive to a challenge dose of gram-negative endotoxin (*E. coli*) than were control mice (Table 6) with a 67-90% mortality frequency. A dose-response pattern was not observed since the highest DES dose (8.0 mg/kg) failed to demonstrate significantly increased endotoxin mortality.

BP exposure failed to alter host resistance to tumor cell or *Listeria* challenge, although increased mortality was observed following endotoxin challenge (Table 6).

DISCUSSION

The National Toxicology Program (NTP) is examining a variety of *in vivo* and *in vitro* approaches as well as the application of new research disciplines to the field of toxicology for the evaluations of chemicals of environmental concern. Immunology represents one of the disciplines that the NTP is employing to examine possible target organ toxicity as well as generalized toxicity at the cellular and molecular level. Furthermore, the effects of chemicals on host defence to bacterial, viral, fungal and parasitic diseases as well as the surveillance and destruction of neoplastically transformed cells are simultaneously being evaluated. It is our goal at NIEHS to systematically apply the disciplines of immunology and microbiology in a comprehensive manner to study immunotoxicity and altered host resistance as well as to define a panel of sensitive assays to screen for chemical-induced changes in immunological and host resistance parameters. Extensive studies conducted at NIEHS have suggested that the route of exposure as well as the time of exposure relative to the maturational development of the immune system should be of paramount consideration in the design of evaluation protocols. Several studies have demonstrated that pre- and postnatal exposure to certain chemicals (e.g. TCDD or DES) during the maturation of the thymus-dependent elements of the immune system often produces more profound immunological effects than adult exposure (see review by Faith and coworkers) or produce long-lasting effects through adult life (Kalland, 1980b). Thus, the focus of the discipline of immunology on the evaluation of chemical toxicities may provide beneficial information to facilitate better human risk assessment.

The evaluation of the prototype chemicals highlighted here substantiate these beliefs regarding the importance of evaluating immunological parameters. Profound reduction in thymus weights were observed following pre/postnatal exposure to TCDD and following adult exposure to DES at exposure levels which were not overtly toxic by classical toxicological parameters. A slight decrease in body weight gain occurred at the 5 μ g/kg TCDD dosage group, but not the 1 μ g/kg group, where normal liver weights and serum chemistries were found. These data substantiate previous reports of selective thymus atrophy following TCDD exposure. Recently, Carlstedt-Duke (1979) reported the presence of a receptor for TCDD in thymus tissue and liver which was recently confirmed by Chae of this laboratory and reported here. The presence of this receptor may explain the selective toxicity of TCDD for thymocytes,

Assessment of Immunotoxicity

although the exact location of this receptor still awaits definition. Significantly reduced thymus weights occurred in all DES dosage groups. Thymus histology revealed severe lymphoid depletion of cortical regions in DES treated mice. BP did not produce significant thymus weight loss or histological alteration, which may suggest primarily a B-cell defect rather than T-cell alterations.

Bone marrow cellularity and progenitor cell (CFU-S and CFU-GM) studies revealed general hypocellularity following TCDD and DES exposure and hypercellularity following BP treatment. In the case of TCDD the hypocellularity is probably due to a direct toxic effect on the pleural-potential stem cell since CFU-S values were depressed, while CFU-GM colonies (granulocyte/macrophage progenitors) were increased. In contrast, in DES treated mice, bone marrow CFU-GM colonies were depressed and at the highest DES dose CFU-S cells were also depressed. BP exposure caused increased BM cellularity accompanied by depressed CFU-S and CFU-GM colonies. This observation awaits further definition. Thus, BM cellularity and progenitor cell cloning capacity appear to represent sensitive endpoints for immunotoxicity evaluation especially since lymphoid elements are a source of proliferatory and inhibitory factors for BM hemostasis (Metcalf, 1978) and are believed to provide important regulatory circuits whose alterations are probably reflected in BM cellularity and progenitor cell levels.

DES treatment produced a moderate but significant reduction in the PFC response to SRBC and antibody titer to LPS. Since DES induces Mφ suppressor cells (Luster and coworkers, 1980) the depressed responses could result from altered Mφ function or a direct action of the suppressor cells on the AB producing cells. In contrast, BP produced a dramatic depression in IgM antibody PFCs to the antigens SRBC (75X+) and LPS (86X+). This observation extends the recent observations of Urso and Gengozian (1980) and earlier reports by others that BP and other polycyclic aromatic hydrocarbons depress the antibody response to SRBCs (Stjernswärd, 1965, 1966; Ball, 1970).

Examination of functional responses of lymphocytes to T- and B-cell mitogens are believed to represent both an estimation of T, B and null cell levels as well as monitor the functional integrity of these populations (Dean, 1979a). Recent studies in cancer patients have revealed the importance of the LP assay in the immunological staging of cancer patients relative to survival and recurrence of tumor (Cannon and coworkers, 1980). The significance of chemical-induced alterations in functional LP responses and the long-term consequence of such impairments awaits further definition. Studies with TCDD exposed weanlings suggested more severely depressed T-cell than B-cell functions. More detailed studies (Luster and co-workers, 1980a) revealed that the depressed responses to T-cell mitogens was due to an intrinsic defect and not related to suppressor cell activity since these cells failed to transfer depressed responses in co-culture.

The expression of suppressor activity from adherent cells in DES treated mice may be reflected in enhanced macrophage cytostasis occurring in DES exposed mice. Recent studies demonstrated that induction of adherent suppressor cell activity results from the stimulation of cyclic nucleotides by prostaglandins which estrogens have been shown to often mimic (Webb and Jamieson, 1976; Weissman and co-workers, 1975).

Phren (1963) observed that topical application of polycyclic aromatic hydrocarbons prolonged transplantation rejection of allografts. The route of exposure or dose of BP employed may explain the failure of BP to significantly alter host resistance in our study, since BP seemed to alter primarily B-cell responses (i.e. antibody formation), but not T-cell responses.

We observed increased mortality following *Listeria* challenge in TCDD and DES exposed mice. BP did not alter *Listeria* susceptibility. The increased mortality

observed following DES was associated with a significant increase in the number of viable *Listeria* in the spleens and livers at 4 days (Dean and co-workers, 1980), a time when T-cell immunity is thought to be expressed (Tripathy and Mackaness, 1969). bfc bacterial counts were similar to control mice at day 1, a time when Mφ are thought to exert their greatest effect. These data suggest that the increased *Listeria* susceptibility found following TCDD and DES exposure results from a T-cell defect. In previous studies Thigpen and co-workers (1975) observed increased mortality following *Salmonella* challenge in TCDD exposed mice, a fact which some feel could be related to the increased sensitivity of these mice to gram-negative endotoxin. Likewise, Heller (1960) found that synthetic estrogens increased phagocytosis, but that enhanced phagocytosis did not protect against microbial infection. In contrast, Nicol and coworkers (1964) found that DES stimulated phagocytosis and prolonged survival of Swiss mice against *Pneumococcus* type I and *Pasteurella septica*. Immunity to *Pneumococcus* is thought to be T-cell independent which may partially explain the previous results and the possible conflict with data reported here. Thus, the immunological defect which results in increased susceptibility to *Listeria* following TCDD or DES exposure strongly implicates a T-cell defect which is supported by functional assays of T-cells.

Tumor susceptibility studies following challenge with 5×10^3 viable syngeneic PYB6 tumor cells revealed that non-treated adult B₆C₃F₁ mice resisted tumor formation with only a 10-20% frequency of tumor formation. In contrast, mice exposed to TCDD in utero or DES as adults had a tumor frequency of up to 60% following TCDD and up to 90% following DES exposure. In all cases the tumors were progressive and resulted in death. BP did not affect the frequency of tumor takes following challenge in this model.

The mechanism by which the unimmunized host can resist tumor or allograft challenge is believed to involve "immune surveillance" mechanisms requiring immunocompetent thymus-dependent lymphocytes (T-cells) and macrophages (Burnet, 1970). Both TCDD and DES have been shown to alter T-cell immunocompetence in this report.

Previous studies (Gass, 1964) in both male and female C3H mice demonstrated a significantly increased dose-related incidence of mammary tumors following DES exposure. The incidence of mammary tumors following DES was enhanced over DES alone by radiation exposure (Segalhoff and Maxfield, 1971), another agent which alters T-cell function. In a later report, Gass and co-workers (1974) demonstrated a requirement for murine mammary tumor virus (MMTV) for the mammary tumor inductive effects of DES.

BP exposure failed to alter susceptibility to *Listeria* which is consistent with primarily a B-cell defect.

Sensitivity to gram-negative endotoxin was apparently increased following exposure to all three chemicals. These data suggest that the detoxification of LPS is related to an intact Mφ population and possibly even requiring B-cells.

The data presented here demonstrate the sensitivity of the immune system and the assay panel proposed for detecting cellular toxicity. Alteration of T-cell function appeared to correlate with increased susceptibility to bacterial and tumor cell challenge. Studies to correlate between assays of lymphocyte function and host resistance are currently in progress.

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