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Application of Tumor, Bacterial and Parasite Susceptibility Assays to Study Immune Alterations Induced by Environmental Chemicals

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Model systems to study the effects of chemicals of environmental concern on bacterial and parasitic diseases as well as the immunosurveillance and destruction of transplantable tumor cells were described and evaluated. Studies were conducted in female B6C3F1 mice following adult or pre/postnatal exposure to several prototype chemicals. The prototype chemicals employed included the synthetic estrogen diethylstilbestrol (DES), the polycyclic aromatic hydrocarbon benzo(a)pyrene (B[a]P), and the carcinogenesis promoting agent 12-0-tetradecanoylphorbol-13-0-acetate (TPA).

The host resistance models employed depend primarily on functional thymus-dependent immunity, although humoral immunity is suggested to have a role in the parasite model as well. These models include: subcutaneous challenge with a dose of PYB6 tumor cell causing a 10-20% incidence (TD_{10-20}) of tumor; intravenous challenge with B16 melanoma cells; challenge with a dose of *Listeria monocytogenes* causing a 10-20% incidence of mortality (LD_{10-20}); challenge with a dose of *E. coli* lipopolysaccharide endotoxin causing a 10-20% incidence of lethality (LD_{10-20}); and challenge with larvae of *Trichinella spiralis* for parasite expulsion kinetic studies.

Increased mortality was observed following *Listeria monocytogenes* challenge in DES-exposed mice. B(a)P and TPA exposure did not alter host resistance to this organism. 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, a time when T-cell immunity is thought to be expressed, but 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 DES exposure may result from a T-cell defect, although the intracellular killing capacity of DES-treated Mϕ's has not been well examined.

Tumor susceptibility studies following challenge with 5×10^3 viable syngeneic PYB6 tumor cells revealed that nontreated adult B6C3F1 mice resisted tumor formation, with only a 10-20% incidence of tumor formation. In contrast, mice exposed to DES or TPA as adults had a tumor frequency of from 70-100% following TPA and up to 90% following DES exposure. In all cases the tumors were progressive and resulted in death. B(a)P did not alter the frequency of tumor incidence from controls in this model.

Preliminary data, using the B16 melanoma intravenous challenge model and ^{125}IUdR to quantitate tumor mass revealed this model was sensitive to non-specifically activated macrophage kill. DES treated mice with activated macrophages did not demonstrate increased tumor mass, while mice exposed to TPA or the potent immunosuppressive agent cyclophosphamide had a significantly increased tumor mass in their lungs.

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Expulsion of *Trichinella spiralis* adults from the gut also apparently required functional T-cells and possibly some element of humoral immunity. Mice exposed to DES and B(a)P exhibited increased numbers of adult worms in the gut at day 14.

Sensitivity to gram-negative endotoxin (LPS) was apparently increased following exposure to DES or B(a)P. These data suggest that the detoxification of LPS is related to an intact Mϕ population. The data presented here demonstrate the sensitivity of the host resistance assay panel proposed for detecting immune alteration. Alteration of T-cell function appeared to correlate with increased susceptibility to bacterial and tumor cell challenge.

Introduction

During the past few years there has been a renaissance of interest in the development of sensitive and reproducible rodent host resistance models to define immunological dysfunction following exposure to drugs or chemicals of environmental concern. These assays are needed to improve the data base for the safety assessment of these agents and to provide better correlates with the numerous *in vitro* assays of immunological function. This interest in providing better correlation and interpretation between an alteration in one or several measurable immunological parameters and host resistance effects, stems from the well known association between congenital immunodeficiency syndromes and an increased frequency of neoplasia (*1*, *2*) or infectious diseases. In addition, renal transplant patients maintained on chronic therapy with hydrocortisone or immunosuppressive agents to sustain an allograft have been shown to have a much higher (2.5 ×) frequency of solid tumors and an increased frequency of infectious disease (*3*, *4*). Several groups (*5*, *6*) have observed a correlation between depressed delayed cutaneous hypersensitivity responses (DHR) to recall antigens (i.e., partial or complete anergy) and an increased frequency of bacterial sepsis and mortality following major surgery. Collectively these clinical and epidemiological data support a strong association between immune dysfunction and altered host resistance.

Altered immunological function, as indicated by the inability to be sensitized for DHR to recall antigens and increased susceptibility to certain infectious agents have likewise been observed in congenital athymic nude (nu/nu) mice (*7*, *8*) and rats (rnu/rnu) (*9*). Likewise, Law et al. (*10*) observed that neonatal thymectomy increased the susceptibility of mice to polyoma virus induced tumors. The application of immunologic and host resistance assays to study rodents following exposure to chemicals has indicated that certain chemicals can result in immune dysfunction (*11-13*). In addition, such exposure often leads to altered host resistance to bacteria (*14*, *15*), viruses (*16-18*), parasites (*19*, *20*), or transplantable syngenic tumor cells (*20-22*) as well as spontaneous tumors (*23*, *24*).

Although there is general agreement that severely depressed cell-mediated immunity (CMI) or humoral-mediated immunity (HMI) results in altered host susceptibility to infectious agents, there is no general agreement regarding the effects of subtle or chronic immunosuppression nor is there agreement about which measurable immunological function(s) most consistently predicts altered host resistance. The recent emphasis on evaluating chemical induced immunotoxicity has resulted in a concerted effort to optimize the sensitivity and reproducibility of these models, to re-examine the application of these models and to improve the data base regarding their correlation with other immune function parameters which are readily measured *in vitro*. These pursuits have been a major goal of our laboratory and will be described here.

Operative Immunological Mechanisms for Resistance to Bacterial Infections and Neoplasia

Bacterial infections are classified as either acute, chronic or toxigenic. The former is illustrated by Staphylococcus or Streptococcus infections in which non-specific phagocytic mechanisms and later specific antibody mediated mechanisms are primarily operative (Fig. 1). Antibodies serves to enhance the phagocytosis and killing of pathogenic microorganisms by polymorphonuclear leukocytes (PMN) and macrophages (Mϕ) through opsonization or neutralization of specific bacterial toxins by preventing binding of the toxin to their specific receptors. Chronic infections, caused by organisms such as *Listeria monocytogenes* or *Mycobacterium tuberculosis*, that are facilitative intracellular pathogens multiply within the phagocytic cell and thus prevent PMN killing. CMI enhances the killing efficiency of the macrophage in these infections through lymphokines such as macrophage chemotactic factor (CF), macrophage migration inhibition factors (MIF) and macrophage activation factor (MAF). Ultimately, CMI plays the primary role in the control and eradication of these often persistent and chronic agents (Fig. 1). Toxigenic infections result from the production of toxins by certain

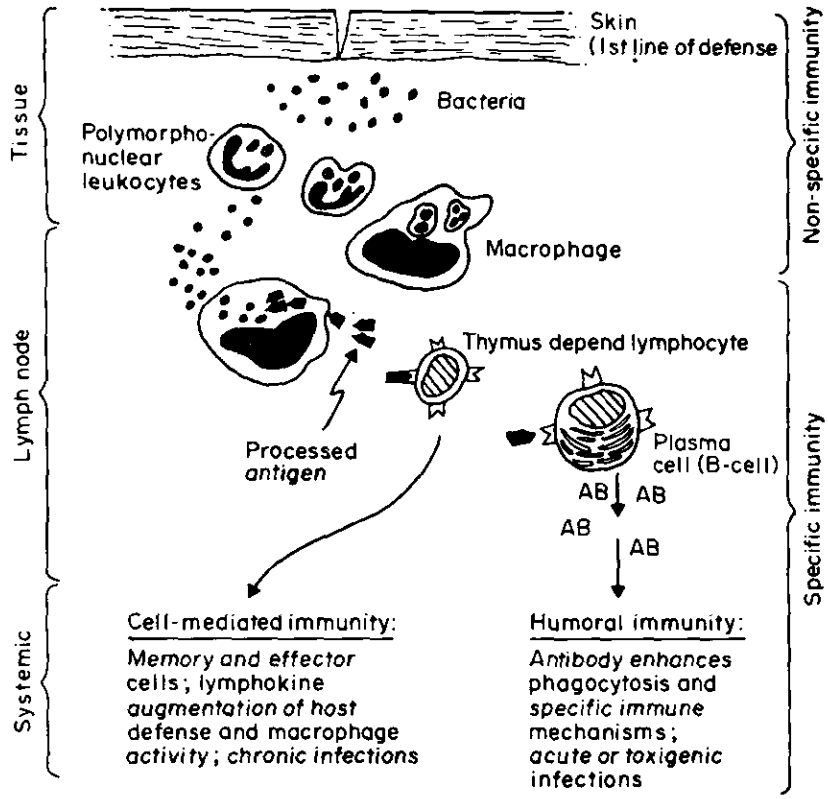

FIGURE 1. Diagrammatic representation of host resistance to bacteria indicating roles played by cell-mediated and humoral immunity.

bacteria and require the production of specific antibody for their neutralization (e.g., tetanus toxin) with little if any role played by CMI.

An imbalance or transient dysfunction of the immune surveillance mechanisms is believed to facilitate the development of neoplastic disease (*25*). For example, a newly transformed or transplanted tumor cell may present a foreign antigenic configuration to the host who in turn develops an immunological response designed to eradicate the tumor cell. This response is believed to be similar to that mounted against an allograft and its relatively weak antigens associated with the tumor cell membrane is predominantly T-lymphocyte-dependent, involves a cascade of cell types and events collectively known as cell-mediated immunity and culminates in the production of T-effector cells and activated macrophages (*26*).

Chemicals, Exposures and Assay Methods

We have investigated several chemicals of environmental concern by short term exposure in adult female B6C3F1 mice. Diethylstilbestrol (DES) was obtained from Sigma Chemical Company (St. Louis, Mo). Female B6C3F1 mice, 6 to 8 weeks old, exclusive of the control group were injected subcutaneously (sc) with 0.2, 2 or 8 mg/kg body weight of DES dissolved in 0.1 ml of corn oil for five consecutive days. Benzo(a)pyrene [B(a)P] was kindly supplied by Dr. Douglas Walters through the NTP chemical repository and administered in corn oil by sc injections. Adult female B6C3F1 mice received a total dose of 50, 200 or 400 mg/kg body weight of B(a)P over a 14-day period exclusive of controls. The phorbol ester, TPA (12-0-tetradecanoylphorbol-13-0-acetate) was obtained from Chemicals for Cancer Research (Eden Prairie, MN), dissolved in corn oil and administered by sc injections. Adult female B6C3F1 mice received a total dose of 40, 400 or 800 μg of TPA over a 14 day period exclusive of controls. Control mice for the dosage groups described above received corn oil (0.1 ml) on the same schedule as chemically treated groups in each case. Immunological and host resistance studies were performed 2-5 days following the last dose.

The battery of assays employed for host resistance evaluation has been previously described in detail by Dean and co-workers (*13*, *21*, *27*).

Altered Resistance to Tumor Cell Challenge Following Chemical Exposure

In normal mice most transplantable syngeneic tumors can be titrated to a cell concentration that will produce tumors in 100% (TD_{100}), 50% (TD_{50}) or 10% (TD_{10}) of the challenged mice. Treatment of mice with known immunosuppressive agents which alter thymus-dependent lymphocyte numbers or function prior to tumor cell challenge will result in a significantly higher frequency of tumors (*21*). Conversely, agents which stimulate immunologic function will facilitate resistance to tumor development in mice given a TD_{80-100} dose of tumor cells. These same agents will likewise facilitate or diminish the frequency or latency (i.e., time to tumor development) of spontaneous tumor in high risk mouse strains. Table 1 summarizes the effect of DES, B(a)P and TPA exposure in adult female B6C3F1 mice on the incidence of tumors following a TD_{10-20} challenge dose of 5×10^3 viable PYB6 tumor cells. A significantly increased frequency ($p < 0.05$) of tumors was observed in mice exposed to the medium (80%) and high (90%) dose of DES and all doses of TPA (70-100%). B(a)P exposure did not modulate the out-growth or development of this tumor. These results are consistent with the immunological alterations reported in the companion presentation (*28*) which indicated that DES and TPA effect predominantly T-cell mediated immune function while B(a)P exposure alters primarily the HMI response.

Table 1. Effect of chemical exposure on tumor frequency following challenge with PYB6 tumor cells.

Chemical treatment	Total dose	No. tumors[a] No. mice challenged	Tumor frequency, %
DES[b]	0	2/10	20
	1.0 mg/kg	3/10	30
	10.0 mg/kg	8/10[c]	80
	40.0 mg/kg	9/10[c]	90
B(a)p[d]	0	2/10	20
	50 mg/kg	2/10	20
	200 mg/kg	2/10	20
	400 mg/kg	1/10	10
	800 mg/kg	2/10	20
TPA[e]	0	4/20	20
	40 μg/mouse	8/10[c]	80
	400 μg/mouse	7/10[c]	70
	800 μg/mouse	8/8[c]	100

[a]Mice were challenged subcutaneously with 5×10^3 syngeneic PYB6 cells 3 days following the last exposure and palpated twice weekly for 60 days.

[b]Mice were treated with 0.2, 2 and 8 mg/kg diethylstilbestrol (DES) (Sigma Chemical Co.) in corn oil daily for 5 days and assays were performed on days 7-10.

[c]The tumor frequency was significantly different from corn oil treated controls by chi-square analysis at $p < 0.05$.

[d]Mice were given 5, 20, 40 and 80 mg/kg of benzo(a)pyrene (B(a)P) in corn oil by gavage for 10 treatments over a 14 day period.

[e]Mice were injected subcutaneously with 10, 100 and 200 μg of the phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-O-acetate) dissolved in corn oil twice weekly for 2 weeks.

Previous studies (*29*) in both male and female C3H mice demonstrated a significant increased dose-related incidence of spontaneous mammary tumors following DES exposure. They likewise (*29*) demonstrated a requirement for murine mammary tumor virus (MMTV) for the mammary tumor inductive effects of DES. The incidence of spontaneous mammary tumors following DES was additionally enhanced by radiation exposure, another agent which alters T-cell function (*30*). In addition, Bern et al. (*31*) found that neonatal exposure to estrogens likewise gave an increased incidence and earlier appearance of mammary tumors. McMartin et al. (*32*) has suggested that the effect of DES on mammary tumor incidence is via the stimulation of pituitary and serum prolactin which in turn stimulates MMTV production. However, it is possible that impairment of T-cell immunocompetence or Mϕ function is a major cofactor in the enhancement of mammary tumor frequency following DES exposure and accounts for the increased tumor frequency we have observed following DES exposure (*20*). This speculation is supported by the knowledge that a potent immunosuppressive drug such as cyclophosphamide enhances the frequency of mammary tumors in low MMTV expressor strains (BALB/c-CRGL) (Lopez, personal communication).

B(a)P failed to alter susceptibility of B6C3F1 mice to challenge with PYB6 tumor cells. This was consistent with the observation reported in this volume (*28*) and previously (*33-35*) that B(a)P primarily affects B-cell response. Immunity to virus and chemically induced tumors is primarily mediated by cell-mediated and not humoral immunity (*10, 36*). The previous reports of an association between depressed humoral antibody responses and increased tumor frequency in B(a)P-treated mice (*34*) probably represent separate nonassociated events based on the minimal alterations in immunological and host resistance parameters observed in our comprehensive study.

Finally, TPA induced a statistically increased frequency in the outgrowth of PYB6 tumors following challenge. This is consistent with preliminary data which indicates that the concentration of TPA that altered tumor outgrowth likewise affects T-cell mediated immunity.

In a search for a more rapid and quantitative

Table 2. Effect of chemical exposure on the growth of B16 melanoma in the lung following intravenous challenge.

B 16 melanoma challenge	Chemical treatment	Colonies/lung	$\bar{X}$ cpm of 3H-TdR incorporation ± SEM[a]	Change in ^{3}H-TdR over control, %[b]
–	None	0	239 ± 15	—
+	Corn Oil	8	465 ± 138	+95
+	CY (180 mg/kg)	>50	6359 ± 873[c]	+2550
+	DES (10 mg/kg)	0	296 ± 32	+23
+	TPA (400 μg/mouse)	>50	1808 ± 429[c]	+653

[a]Six mice per group were injected with 5×10^4 B16F10 melanoma cells by the intravenous route 3-5 days following the last exposure (day 0). All mice received 1 mg of FUDR on day 20 following at 1 hr by approximately 1×10^6 CPM of ^{125}IUdR (IP) and were sacrificed 18-20 hr later. The lungs were perfused with saline, placed in a counting tube and counted for 10 min in a Packard Biogamma Counter. The data are expressed as mean counts per minute ($\bar{x}$ CPM) of ^{3}H-TdR incorporation ± standard error of the mean (SEM) from six mice/group.

[b]% Change $= \dfrac{\bar{x}\text{ (cpm) of }^{125}\text{IUDR in the lungs of non-tumored mice}}{\bar{x}\text{ (cpm) of }^{125}\text{IUDR in the lungs of treated and tumored mice}} - 1 \times 100$

[c]Significantly different from mean CPM in control mice at $p < 0.01$ by Student's t-test.

method of assessing tumor resistance we have used an ^{125}IUdR (^{125}I-iododeoxyuridine) pulsing technique (*27*) to quantitate tumor mass in the lungs of chemical treated mice at 21 days following an intravenous challenge with the B16F10 melanoma line of Fidler (*37*). In this assay mice are challenged with 5×10^4 viable tumor cells at 2-3 days following their last dose of chemical. This model assumes that normal surveillance and immunological mechanisms operating systemically in the lungs of mice resist or destroy a large portion of the tumor cell inoculum. The remaining tumor cells which escape surveillance primarily develop foci in the lungs, appearing as a black mass of tumor which can easily be enumerated. Treated and tumored mice are then injected with approximately 1×10^6 cpm of ^{125}IUdR on day 20 following tumor cell injection and the lungs are removed on day 21, perfused with saline, and the amount of ^{125}IUdR incorporation determined by a gamma counter.

Table 2 summarizes preliminary studies of the lung tumor mass found in B6C3F1 mice following exposure to the standard immunosuppressive agent cyclophosphamide (CY, 180 mg/kg) and the chemicals DES (10 mg/kg) and TPA (200 μg) followed by challenge with B16F10 melanoma cells. As expected, an immunosuppressive dose of CY resulted in a 2500-fold increase in tumor mass as estimated by ^{125}IUdR and TPA exposure resulted in a 653-fold increase in tumor mass. These data roughly correlated with the number of colonies seen on the surface of the lungs (i.e., 8 versus > 50). Corn oil (i.e., carrier) or DES did not significantly increase lung tumor mass, in fact DES treated mice had a lower incorporation rate than controls. This apparent paradox produced by DES in this model may be due to the exquisite sensitivity of the B16F10 tumor line to nonspecifically activated macrophage cytotoxicity (*37*) and the potency of DES as a Mϕ activator (*38, 39*). Morahan et al. (*40*) have previously shown that low MW pyran copolymers will potentiate the tumorcidal activity of macrophages against the transplantable form of B16 melanoma. Our interpretation of increased susceptibility to B16F10 tumor cells in CY and TPA treated mice is consistent with the adverse effect of both CY (*28*) and TPA (*41*) on T-cell mediated immunity. B(a)P has not yet been examined in this model. This model may also be a useful procedure for screening chemicals suspected of immunopotentiatory effects.

Altered Susceptibility to Listeria Infections Following Chemical Exposure

The role of immunocompetent T-cells and macrophages in controlling the intracellular replication and destruction of the gram positive bacteria *Listeria monocytogenes* has been well documented (*42, 43*). Recently, Newborg and North (*44*) re-examined this model using nude mice and found that survival during listeriosis ultimately requires the generation of T-cell mediated immunity. In our studies, control and chemical treated mice were challenged with 1×10^6 viable Listeria which produced a mortality frequency of approximately 10-20% (LD_{10-20}) in normal B6C3F1 mice. Table 3 summarizes mortality following Listeria challenge in mice exposed to DES, B(a)P and TPA. DES significantly reduced resistance to *Listeria* at all doses of chemicals (i.e., 100% mortality). The mean latency to death was likewise reduced. In contrast, B(a)P and TPA did not significantly alter Listeria resistance.

The increased mortality we observed following DES was associated with a significant increase in the number of viable Listeria in the spleens and livers at 4 days (*20*), a time when T-cell immunity is

Table 3. Effect of chemical exposure on mortality to *Listeria monocytogenes* challenge.[a]

Chemical	Dose	No. deaths / No. inoculated	Mortality, %
DES	0	2/10	20
	1.0 mg/kg	10/10[b]	100
	10.0 mg/kg	10/10[b]	100
	40.0 mg/kg	10/10[b]	100
B(a)P	0	0/10	0
	50 mg/kg	1/10	10
	200 mg/kg	0/10	0
	400 mg/kg	2/10	20
TPA	0	4/23	17
	40 μg	0/10	0
	400 μg	0/10	0
	800 μg	0/10	0

[a]Animals were challenged with 1×10^6 viable bacteria and mortality followed in 14 days.
[b]Significantly different from control mice by chi-square analysis.

thought to be expressed. Bacterial numbers similar to control mice were observed at day 1 post-infection, a time when Mϕ are thought to exert their greatest effect. In a previous study, Heller (*45*) found that synthetic estrogens increased phagocytosis, but that enhanced phagocytosis did not result in enhanced protection against microbial infection. Thus, the altered resistance to *Listeria* observed appears to be related to the functional T-cell defect observed following DES and reported in the companion presentation (*46*). B(a)P was not expected to alter resistance to *Listeria* since antibodies have no defined role in controlling listerosis (*44*) and B(a)P primarily effects B-cell function (*34, 41*). In contrast, the lack of effect of TPA on Listeria resistance was unexpected since in preliminary studies TPA altered T-cell function and increased the susceptibility to PYB6 tumor cells. Thus, the T-cell dysfunction observed following TPA appears to be unique to tumor resistance. This observation awaits confirmation and definition through further studies.

Altered Hypersensitivity to Endotoxin Following Exposure

The detoxification of gram negative bacterial endotoxin is believed accomplished primarily by liver macrophages (*47*) and endotoxin challenge is thought to mimic the response to gram-negative bacteria. Increased sensitivity to endotoxin has been observed following the administration of known reticuloendothelial system stimulants such as glucan and zymozan or certain environmental chemicals (*11*). In the studies described here, B6C3F1 mice were challenged intravenously with 650 μg of *Escherichia coli* endotoxin 2-3 days following the last chemical dose and animal survival was observed for 48 hours. Table 4 summarizes mortality at 48 hr following challenge with endotoxin in mice exposed to DES, B(a)P and TPA. Mortality was significantly increased (44-90%) in DES treated mice. B(a)P treated mice likewise had increased mortality following endotoxin challenge (30-80%). TPA treated mice did not have increased hypersensitivity to endotoxin. The pattern of increased endotoxin sensitivity following chemical treatment appears to remain ill-defined correlating with Mϕ alterations in the case of DES. Significantly increased mortality to endotoxin following B(a)P exposure only at the high dose may reflect macrophage toxicity. Thus, hypersensitivity to endotoxin appears to offer a sensitivity indicator of chemical immunotoxicity.

Table 4. Effects of chemical exposure on endotoxin hypersensitivity.

Chemical	Mortality, %[a] Control	Low	Medium	High
DES	0	67[b]	90[b]	44
B(a)P	0	30	40	80[b]
TPA	0	10	0	10

[a]After 48 hr in mice challenged with 650 μg of *E. coli* endotoxin.
[b]Significantly different from control by chi-square analysis at $p < 0.05$.

Altered Expulsion of Adult Trichinella Following Chemical Exposure

The role of T-cell mediated immunity in the expulsion of adult *Trichinella spiralis* has been clearly shown by Larsh and associates (*48*). In this model mice are challenged with 200 larvae following chemical exposure and are then sacrificed on day 14 and the number of adult worms in the gut are enumerated. Table 5 summarizes adult worm counts following exposure to DES, B(a)P and TPA in B6C3F1 mice. DES treated mice had a significantly greater number of adult worms ($p < 0.01$) in their gut at day 14 than did control mice. These data demonstrate that DES treatment of naive mice prior to larvae challenge reduced the normal expulsion of adult worms (63%) and substantiated the role of T-cell immunocompetence in eradicating the host of adult worms. Similarly, nude mice have been shown to have reduced expulsion rates with adult worms lingering for up to 83 days (*49*). B(a)P

Table 5. Effect of chemical exposure on *Trichinella spiralis* expulsion.

Chemical treatment	Dose, mg/kg	Adults recovered[a]	% of control,[b]
DES	0	1.1 ± 0.8	0.7
	1.0	0.9 ± 0.5	0.6
	40	96.0 ± 14.7[c]	62.8
B(a)P	0	1.3 ± 0.7	1.4
	50	3.0 ± 1.0	3.3
	200	5.0 ± 0.7[d]	6.4
	400	49.8 ± 12.6[c]	54.2
TPA	0	4.8 ± 2.5	4.3
	40	7.5 ± 3.5	6.7
	400	1.5 ± 1.1	1.3
	800	5.8 ± 3.4	5.2

[a]Average adult counts ± SEM of 7 mice/group examined at 14 days post-infection.
[b]% Control = % of adult worm remaining at day 14 versus controls killed on day 7.
[c]Significantly different by Student's *t*-test at $p < 0.001$.
[d]Significantly different by Student's *t*-test at $p < 0.01$.

also reduced parasite expulsion (54%), at the two highest doses administered. This finding may suggest that humoral immunocompetence is also required for efficient expulsion. Finally, TPA had no effect on adult worm expulsion.

Conclusion

In conclusion, the studies described here demonstrate that adult exposure of female B6C3F1 mice to various chemicals can severely impair host resistance to syngeneic tumor cells, Listeria, endotoxin and *T. spiralis* challenge. These studies also point out the sensitivity of and significance of examining host resistance parameters when evaluating the immunotoxicity of environmental chemicals or drugs. The study of chemical toxicity might also provide a powerful tool for dissecting mechanisms of altered host resistance and immunocompetence. The data presented in this and the companion report (*28*) strongly suggest that exposure of adult mice to DES, B(a)P or TPA can result in thymus atrophy, impaired DHR responses, impaired T-cell responses *in vitro*, impaired AB production, and enhanced *in vitro* peritoneal macrophage activity as measured by phagocytosis and cytostasis of tumor target cells. The host resistance models described are strongly dependent on depressed T-lymphocyte immunocompetence if effects are to be observed, although a secondary specific defect in peritoneal Mϕ function (i.e., associated with DES) cannot be ruled out and will require further clarification through more specific *in vitro* examination of the intracellular killing capacity of macrophages from exposed mice. Finally, these assays appear quite sensitive for detecting chemical induced immune dysfunction.

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