Update: Immunotoxic and Immunosuppressive Effects of Food Chemicals (Focus on Diethylstilbestrol)

D. L. Archer*
B. G. Smith

Immunotoxicology

The recent growth of interest in the area of Immunotoxicology has been remarkable. There have been a few dedicated souls who have published in this general area for some time, but a focus for these studies was only recently provided by Vos (1977). Since then, communication between toxicologists and immunologists has begun in earnest, as evidenced by the following meetings at which Immunotoxicology was discussed.

1978 - New York Academy of Sciences' Science Week
Gordon Research Conference on Toxicology and Safety Evaluation
IV FDA Science Symposium

1979 - Society of Toxicology
European Society of Toxicology
Gordon Research Conference on Toxicology and Safety Evaluation
Technology Transfer Workshop; Litton Bionetics

The list is probably incomplete, and thus far, it appears that 1980 will be even a more "involved" year for Immunotoxicology.

The word "Immunotoxicology" may in fact over-legitimize this new area of concern and confer on it a title which implies that it is a permanent subspecialty of toxicology. In fact, applied immunologists and immunopharmacologists have been carrying out immunotoxicology studies of sorts for many years. An excellent commentary on the subject was presented by Moore (1979).

One problem facing the immunotoxicologist is presenting his data in an understandable way to non-immunologists. The field of immunology is one of the most rapidly growing areas of biology. There is a virtual glut of information to be found in countless journals on a monthly basis. The job of maintaining an adequate, up-to-date knowledge of this field is a full-time effort; trying to maintain an up-to-date knowledge of toxicology simultaneously would be nearly impossible. Joint efforts are therefore necessary. Additionally, immunologists have spawned an awesome vocabulary of immunologic "jargon" and acronyms to describe the numerous cells, cell-products, and phenomena which go towards making the immune response work. To the layman, and sometimes to immunologists, this seems an insurmountable obstacle to understanding. It would behoove the immunotoxicologists then, to present their findings in a more palatable and straightforward manner, e.g. if an animal consumes compound X at dose level Y or greater, he is 5 times more likely to contract condition Z.

The Immune System

The sheer complexity of the immune response is another obstacle to understanding for non-immunologists. The foundation of the system is its divisibility into two parts, the humoral immune system and cellular immune system. Humoral immunity is that type which involves serum and circulating, specific antibodies in conferring protection. Cellular immunity involves the destruction of invaders by cells of the immune system without antibody involvement. Some cell types are common to both systems and their development and functions are shown in Figure 1. Primitive undifferentiated stem cells mature to form precursor lymphocytes in the bone marrow. The precursor lymphocytes can follow one of two divergent maturational pathways. Under influence of the thymus, they mature to become so-called T cells; under influence of the fetal liver or gut-associated lymphoid tissues (GALT), they mature to become B cells (B stands for Bursa of Fabricius in chickens; the fetal liver and GALT are its equivalent in mammals). The T cells are mainly concerned with cell mediated immunity, regulation of the immune response, and the killing of tumor cells. B cells are mainly concerned with the production of specific antibodies, an aspect of humoral immunity. Another involved cell is the macrophage (MØ), generally regarded as an accessory cell for many immune reactions aside from its phagocytic chores. It should be stressed that this is the simplest scheme, and that there are numerous subpopulations of each cell type. The maturation of stem cells to T- or B-cells occurs during prenatal life; therefore, chemicals which affect the stem cells, or any of the organs involved in stem cell maturation, would have profound and long-lasting consequences. Indeed, the developing immune system is most susceptible to chemical insult. In neonatal and adult life, B and T cells are still generated, but at

*D. L. Archer and B. G. Smith, Division of Microbiology, Food and Drug Administation, 1090 Tusculun Avenue, Cincinnati, OH 45226

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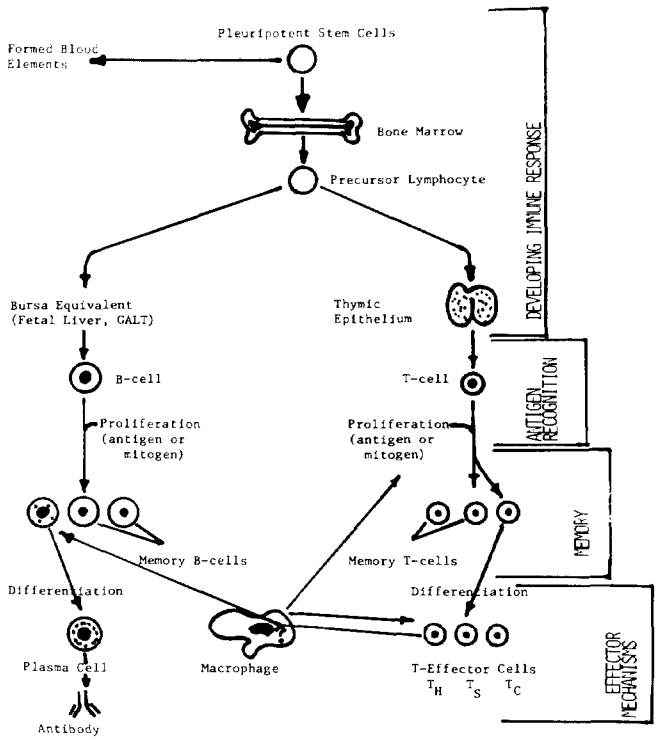

Figure 1. The Immune Response

a decreasing rate with age. Most neonatal and adult immune phenomena involve further maturation of these cell types due to encounters with antigens (viruses, bacteria, toxins, etc.) and the resultant formation of cells to deal with the invader. This stage could be termed the "antigen-recognition stage" in terms of the effect of chemical insult. On further or repeated encounter with antigen, memory cells are formed, this stage being termed "memory." Lastly would be the "effector" stage, during which antigen-specific, mature cells produce their products which are suited to deal with the invader. Each of these stages, developmental, antigen-recognition, memory, and effector, may exhibit differential sensitivities to chemicals.

The Problem

There is a tendency to study the effects of chemicals on the immune response by looking at isolated phenomena and individual cell types. This is not the fault of the investigators, but is due to the complexity of the system. No one test is suitable for examining the entire spectrum of immune responses. Some tests, however, have been weighted heavily, most notably, mitogen assays (or lymphoproliferative tests). Mitogens are substances which switch on lymphocytes and induce DNA synthesis and cell division. There are mitogens which are specific for T cells, such as concanavalin A and phytohaemagglutinin, and for B cells, such as bacterial lipopolysaccharide. Loss of the ability of splenic (or peripheral blood) T cells from chemical-exposed animals to divide in the presence of mitogen in cell culture has been presumptive evidence for a defect in cell mediated immunity. We have long felt that this type of test, although valuable, was overly utilized in terms of conclusions drawn from the data. Our reasons for this were as follows: 1.) Cells may be washed free of chemical during preparation for *in vitro* mitogen exposure; therefore, chemical and cell stimulus (mitogen) are not present concurrently, and 2.) alternatively, *in vitro* culture places demands on cells which might not be encountered were those cells maintained *in vivo*, where repair or compensatory mechanisms may maintain them in a functional state. We thought, therefore, that an *in vivo* mitogen assay would be superior to the methods now used, in that mitogen and suspect chemical would be present simultaneously in the animal. The afore-mentioned mitogens, concanavalin A and phytohaemagglutinin, are undesirable for injection because of their ability to agglutinate bood cells; this may result in the death of the animal prior to activation of the splenic T cells. We turned to another type of T-cell mitogen, staphylococcal enterotoxin A (SEA), which was shown by Smith and Johnson (1975) to be T-cell specific, and which does not agglutinate blood cells. Intravenous injection of up to 100 μg SEA into adult female C57B1/6 mice caused no mortality (data not shown).

The Approach

To test the ability of SEA to activate T cells *in vivo*, the following experiments were carried out. Groups of six adult female C57B1/6 mice were given intravenous injections of 10 μg SEA in normal saline or normal saline alone via the retroorbital plexus. Thymus and spleens, both of which contain SEA-reactive T cells, were removed at 24, 48, and 72 hours after injection. Cells from individual organs were dispersed by mincing with forceps and counted in a Coulter counter. Cells (2×10^5 in 200 μl volumes) were cultured for 4 hours in 96 well culture plates using RPMI 1640 medium with 10% fetal calf serum and 0.5 μCi tritiated thymidine (for specific labelling of DNA). Cells were harvested on filter paper using a multiple well harvester and activity incorporated into DNA quantitated in a liquid scintillation counter. A summary of the data is presented in Figure 2. The data are presented as the percent difference of SEA-injected animals from saline controls at each time point. The data show that spleen cells are activated to DNA synthesis by SEA at 24 and 48 hours post injection; DNA synthesis rate returns to normal by 72 hours. In contrast, thymus cells show no activation at 24 and 48 hours, but are activated at 72 hours after injection. Spleen cells were chosen for subsequent experiments and examined for DNA synthesis rates at 48 hours post-injection only.

To determine whether the observed activation was specifically due to T cells, the following experiments were carried out: 1.) 24 hours prior to SEA injection, mice were injected subcutaneously with 0.25 ml antithymocyte serum (ATS), and 2.) congenitally athymic nude mice, which lack functionally mature T cells but have normal B cells and MØ, were injected with SEA. The results of these experiments are also shown in Figure 2. ATS pretreatment, which specifically lyses T cells, significantly reduced the amount of thymidine incorporation into DNA (closed triangle compared with open triangle). Furthermore, spleen cells from athymic nude mice

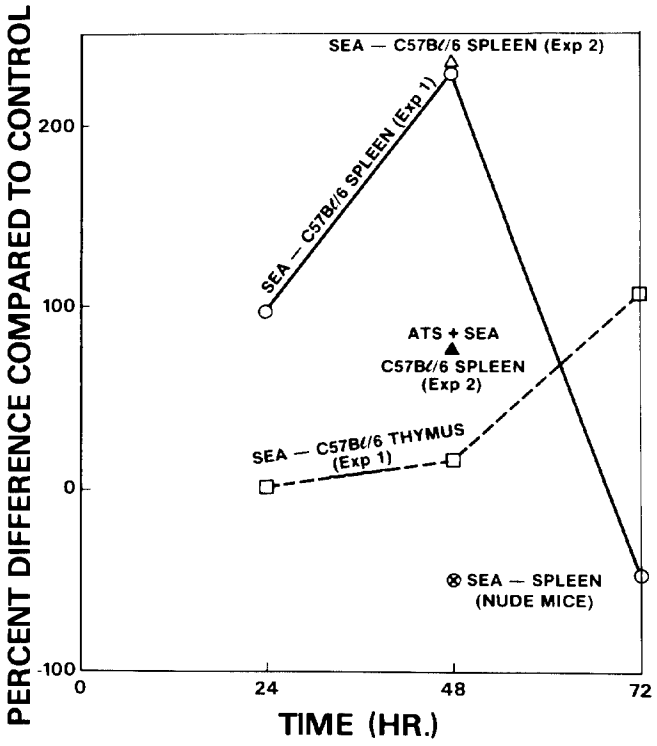

Figure 2.

were not activated by SEA (Ⓧ). These facts strongly suggest that SEA specifically activates T cells *in vivo*.

The Application: Diethylstilbestrol

Confident that we now had an *in vivo* method for studying T-cell activation, we attempted to apply the method to the evaluation of an environmentally important immunotoxic substance. We chose diethylstilbestrol (DES) because it has known effects on various aspects of immunity and it is unquestionably of timely significance to the food industry and the Food and Drug Administration. A brief review of DES's biological activities should be given. DES is a synthetic nonsteroidal compound possessing estrogenic activity. It has been used as a growth promoting agent in cattle and sheep, in anti-abortion therapy, for treatment of gonadal dysgenesis and as a contraceptive drug for women. Young women, exposed to DES in utero, demonstrate an increased incidence of clear-cell adenocarcinoma of the vagina. The adverse health effects attributed to DES led to the issuance of a regulation by the Department of Agriculture on April 22, 1980, that feedlots suspected of using DES implants file sworn affidavits that all implants in their cattle have been surgically removed (removal to be accomplished by a USDA accredited veterinarian) and that cattle have been held for 61 days after implant removal to permit DES excretion (41 day hold if liver and kidney discarded).

As previously stated, DES has been shown to exert a variety of effects on the immune system; some of these are summarized in Table 1. The immune response is known to be responsible for tumor surveillance. Depression of immunologic capability, then, could predispose an individual to tumor formation. Consistent with this are the observations that DES suppresses natural killer cell activity (Kalland, 1980) and decreases cell mediated immunity (Luster *et al.*, 1980) and delayed hypersensitivity responses (Kalland and Forsberg, 1978), and thus could adversely affect killer (cytotoxic) T cell function.

Table 1. Known Effects of DES on the Murine Immune Response

↑ macrophage phagocytosis
↑ null cells
↑ activation of suppressor macrophages
↓ natural killer cell activity
↓ resistance to tumors, bacteria, parasites, endotoxin
↓ cell mediated immunity
↓ delayed hypersensitivity
↓ lymphoproliferative response to mitogens
↓ bone marrow stem cell proliferation
↓ % splenic T cells
↓ helper T cells in spleen

↑ = increase in function
↓ = decrease in function

Duplicate groups of six C57B1/6 female mice, 6-8 weeks old, were given daily injections of either 0.1 ml corn oil subcutaneously (s.c.) or 160 μg DES s.c. in 0.1 ml corn oil (8 mg DES/kg body weight) for 5 consecutive days. The mice were rested for two days, then injected intravenously with SEA as previously described. Splenectomies were performed and rates of DNA synthesis determined at 48 hr post-injection as previously described. Concomitant with the dosing of these mice, parallel experiments were carried out such that our *in vivo* method could be directly compared with *in vitro* mitogen-induced proliferation. In these experiments, spleen cells from untreated or DES-treated mice were prepared and cultured as previously detailed, but tritiated thymidine was not added at the start of the culture period. Instead, SEA and concanavalin A, both T-cell mitogens, were added to the 200 μl cell cultures (of 2×10^5 cells/culture) at 0.5 μg/well and 1.0 μg/well, respectively. Cells were cultured for 44 hr in a 5% CO_2 atmosphere at 37°C. Tritiated thymidine (0.5 μCi/well) was then added for the final 4 hr of incubation. Cells were harvested and counted as previously described. (This is the normal way mitogen assays, or lymphoproliferative tests, are carried out.)

Results

The data from the parallel experiments are summarized in Table 2. The results of the standard, *in vitro* analysis of mitogenic activation of spleen cells from untreated and DES-treated mice are consistent with published values (Luster *et al.*, 1980). DES treatment of animals resulted in a uniform depression of DNA synthesis rates of approximately 30% (for controls, SEA and ConA). Although these depressions were not statistically significant, they were consistent and, therefore, indicate a general trend towards depression of DNA synthesis. The data with the newly developed *in vivo* system was

Table 2. Effects of DES on the *In Vitro* and *In Vivo* Mouse Lymphoproliferative Response to Mitogen[a]

Treatment	In Vitro	In Vivo
Control	100[b]	100
DES	72	102
SEA	1346	205
DES + SEA	906	400
ConA[c]	2231	ND[d]
DES + ConA	1544	ND

[a]expressed as percent of control counts per minute.
[b]means not connected by a common line are significantly different at $\alpha = 0.05$
[c]ConA = concanavalin A
[d]ND = not done

surprising. DES-treated mice not exposed to SEA had similar rates of DNA synthesis to non-DES-treated controls. SEA injection of normal (corn oil only) mice resulted in a significant ($\alpha = 0.05$) stimulation of DNA synthesis in spleen cells (205% of control). SEA injection of DES-treated mice resulted in a significantly enhanced ($\alpha = 0.05$) stimulation of DNA synthesis (400% of controls). This pattern has been observed in $B_6C_3F_1$ mice as well, so is not peculiar to C57B1/6 mice only (unpublished observation).

Discussion

At first glance, the data from the standard mitogen assay and the new *in vivo* activation assay would appear to conflict. The standard assay showed that DES-pretreatment of animals led to a 30% reduction in the ability of their splenic T cells to respond to mitogens. It should be noted, however, that the same 30% depression of DNA synthesis was observed for DES-treated, non-mitogenized controls; this situation usually indicates toxicity. A comparison of the relative viabilities of 48 hr-cultured spleen cells from DES-treated and untreated mice showed no significant loss of viability (as assessed by trypan blue dye exclusion) in cultures of DES-treated cells. However, those cells, while viable by this criterion, were morphologically altered (personal observation). It is possible that a mitogen-responsive subpopulation of cells was rendered unable to divide in cell culture because of subtle damage or leakage, but that this population could function normally *in vivo* in the compatible microenvironment of an intact spleen.

Although other explanations are possible, the data are compatible with a model in which a subpopulation of T cells that function as a natural feedback control to other T cell proliferation is altered or removed in the spleens of DES-treated animals. Without this feedback population, other T cells are permitted to divide at a more rapid rate. A DES-induced shift in T-cell subpopulations has indeed been shown by Kalland (1980). The hypothesis of a DES-induced loss of feedback control *in vivo* is supported by other data (to be presented elsewhere) which show a loss of interferon production in DES-treated animals concomitant with the enhanced T-cell proliferation. This type of interferon has been shown to quantitatively reflect suppressor T cell activity (Johnson *et al.*, 1977). Loss of interferon producting capability could, in part, explain the increased incidence of tumors in DES-treated animals, as interferons are known to activate or enhance the acitivity of natural killer (NK) cells (Djeu *et al.*, 1979). NK cell activity has been shown to be depressed in DES-treated animals (Kalland, 1980). The connection between DES's ability to alter the immune response and its ability to predispose animals to tumors is far from established, but certainly warrants further investigation.

Discussion

N. G. Marriott, Virginia Polytechnic Institute and State University: You showed the effects of diethylstilbestrol in a slide, but did not attempt to quantify concentrations. Later you mentioned something about a 5X dose of diethylstilbestrol—can you give a further explanation?

D. Archer: That slide was a compilation of data from approximately 20 different papers. The lowest dose used was five micrograms per mouse, which is about 250 micrograms per kilogram. In order to extrapolate that to humans, it must be multiplied by 50 or 60 which results in an amount above the therapeutic dose used in cases of prostatic carcinoma, where the maximum dose a human would get could be one milogram per day.

M. J. Marchello, North Dakota State University: Dr. Archer, if the results you described are in fact greatly in excess of therapeutic doses, do you anticipate trying to do the work with therapeutic doses so that more realistic answers could be obtained? It bothers me when we see a lot of research work, especially in this type of area where when such large over-doses are used, are the effects really of the chemical, or are they from an oversaturated system.

D. Archer: That's exactly the point I tried to make. In our testing situation, we give the animal enough DES to last a human being a life time. My laboratory is not suitable for the low dose/long term testing, but it is being done right now in two places. One is in Bergen, Norway and the other is right at National Institute of Environmental Health Sciences. Low level/long term studies are underway for both prenatal and neonatal periods. The adult is practically immune to the effects. The neonatal is the most critical time period.

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