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THE ROLE OF HOMEOSTATIC IMBALANCE IN THE REPORTED IMMUNOMODULATION OF T-2 TOXIN

by

Michael Jay Taylor

A dissertation submitted in partial fulfillment

of requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
1988

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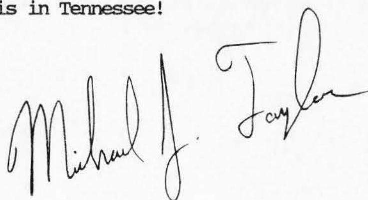
I owe much to many. My family has made the following contribution possible through personal provision and sacrifice. The support and tutelage of Dr. Raghubir P. Sharma have provided me with a foundation. Dr. Ross A. Smart has helped me to visualize pathological problems and to conceptualize their role in untoward effects on the immune system. Dr. Reed P. Warren has provided me with immunological insightfulness. Dr. Donald V. Sisson, thanks for your patience and various factorial decisions. Dr. Jon Y. Takemoto, your contributions are within. Dr. Roger Coulombe has provided various resources. I am indebted to the College of Agriculture for the provision of my support. During my residence I have had the opportunity to meet many graduate students from many countries, thanks to all. I also appreciate the technical and clerical support of associated personnel. To Marge, I still travel and give talks; to Jay, I am glad you were born in Utah; to Florence, I am a toxicologist; and to Dr. Shupe, Lynchburg is in Tennessee!



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LIST OF ACRONYMS USED IN TEXT

ACTH = adrenocorticotropic hormone

ATA = alimentary toxic aleukia

BCG = bacillus Calmette-Guerin

C3 = complement factor 3

CH_{50} = fifty percent complement mediated lysis of antibody coated red blood cells

Con A = concanavalin A

DAS = diacetoxyscirpenol

DNA = deoxyribonucleic acid

DNP-BSA = dinitrophenyl-bovine serum albumin

DNP-Ficoll = dinitrophenyl-Ficoll

DNP-OVA = dinitrophenyl-ovalbumin

DON = deoxynivalenol

DTH = delayed-type hypersensitivity

HA = hemagglutinin

$[^3H]$-TdR = $[^3H]$-thymidine

$[^3H]$-T2 = $[^3H]$-T-2 toxin

ID_{50} = fifty percent inhibition dose

Ig^+, Ig^- = immunoglobulin positive, negative

IgA = immunoglobulin A

IgE = immunoglobulin E

IgG = immunoglobulin G

IgM = immunoglobulin M

im = intramuscular

LIST OF ACRONYMS USED IN TEXT (Continued)

ip = intraperitoneal

iv = intravascular

LD_{50} = fifty pecent lethal dose

LPS = lipopolysaccharide

PFC = plaque-forming cell

PFU = plaque-forming unit

PHA = phytohaemagglutinin

po = per os

ppm = parts per million

PVP = polyvinylpyrrolidone

PWM = pokeweed mitogen

α-SRBC = anti-sheep red blood cell antibody

sc = subcutaneously

SRBC = sheep red blood cell

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ABSTRACT

The Role of Homeostatic Imbalance in the
Reported Immunomodulation of T-2 Toxin

by

Michael Jay Taylor, Doctor of Philosophy
Utah State University, 1988

Major Professor: Dr. R.P. Sharma
Department: Toxicology

T-2 toxin (T-2), produced by the genus Fusarium, is a cytotoxic trichothecene mycotoxin, a feed contaminant, and has been shown to be immunomodulatory. It is suspected that T-2-associated immunomodulation is mediated partly through the hypothalamic-pituitary-adrenal axis. The presence of endotoxin, a bacterial product capable of activating the hypothalamic-pituitary-adrenal axis as well as the levels of several hormones, also associated with activation of the hypothalamic-pituitary-adrenal axis, were determined in both vehicle- and toxin-treated animals. Endotoxemia was evident twenty-four hours after a single oral exposure to T-2. Blood levels of adrenocorticotropic hormone and corticosterone, parameters of the stress response, also increased twenty-four hours after T-2 exposure. Hypothalamic norepinephrine and serum corticosterone levels increased in a dose-related manner after two weeks of T-2 exposure. An increased corticosteroid level was associated with thymic involution leading potentially to decreased T-dependent antibody

response, a known effect of T-2. The effects of exposure to T-2 on the development of both T-dependent and T-independent antibody response were determined in nonoperated, sham-operated and adrenalectomized mice. T-2 decreased the antibody response to a T-dependent antigen and increased a T-independent response. The effects of T-2 were partially nullified by adrenalectomy. These results provide a further confirmation of the postulate that the hypothalamic-pituitary-adrenal axis plays an important role in T-2 toxin-immunomodulation. In vitro studies were undertaken to investigate the direct effects of T-2 on various populations of lymphatic cells. Exposure to T-2 after twenty-four hours caused an increase in the uptake of ^{3}H-thymidine by mouse splenic cells. Pokeweed mitogen stimulation also increased in this system; the response to lipopolysaccharide increased to a lesser extent. However, T-cell responses to phytohaemagglutinin and concanavalin A (Con A) decreased. Thymic cells were also sensitive to T-2. The possibility of pharmacological activity of T-2 with thymocytes was investigated. Both specific and nonspecific cell associations were observed. The association of T-2 with thymocytes was altered in the presence of dexamethasone, a synthetic corticosteroid. T-2 was shown to have both indirect as well as direct activities on the immune system. Endocrine dysfunction resulting from chronic stress and possible pharmacologic activity of T-2 provide the impetus for further investigations.

(142 pages)

CHAPTER I

INTRODUCTION

Many environmental and industrial chemicals have been demonstrated to affect the immune system (Dean et al., 1982, 1985; Sharma and Reddy, 1987). Chemical effects often have both toxicologic and pharmacologic ramifications. Ultimately, many of these chemicals have been appropriately defined as immunomodulators. Trichothecene mycotoxins, demonstrably immunomodulatory compounds, have both stimulatory and inhibitory effects (Taylor et al., 1987b).

T-2 toxin (Fig. I-1) is one of many trichothecene mycotoxins, secondary fungal metabolites produced primarily by members of the genus _Fusarium_. Trichothecene mycotoxins are known collectively as the most

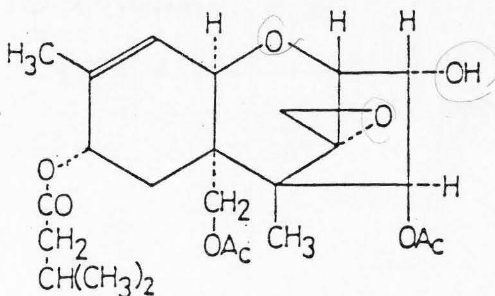


FIG. I-1. Structure of T-2 toxin.

potent naturally occurring small molecules to interfere with protein synthesis (McLaughlin et al., 1977). Alimentary Toxic Aleukia (ATA), the human mycotoxicosis associated with trichothecene mycotoxins, affects primarily the alimentary tract mucosa and leucocytes. The pathological characteristics of the toxicosis have been described in detail (Joffe, 1978; Mayer, 1953). Victims are generally afflicted with a state of hemorrhagic diathesis; sepsis is common, particularly septic angina (septic angina is a synonym for ATA). Petechial hemorrhages of the skin of the trunk and intestinal bleeding often accompany ATA. Interestingly, endotoxin has been suggested as a component of the coagulopathy known as disseminated intravascular coagulation (DIC), as gram-negative sepsis is regularly accompanied by DIC (Osterud, 1985). Petechial hemorrhages occur frequently with DIC. Bone marrow necrosis, leucocytosis, and leucopenia--symptoms of trichothecene toxicosis--have also been attributed to endotoxin (Yoshida et al., 1980).

Endotoxins are cell-wall components of gram-negative bacteria, abundant within the intestinal lumen, and initiators of many biological activities (Table I-1). Damage to the epithelium of the gastrointestinal mucosa, resulting in dysfunction of its absorptive-barrier function, may predispose an animal to the effects of endotoxin. The immunomodulatory activities of endotoxin include nonspecific-polyclonnal B-cell activation, a T-independent process. Endotoxin is also reported to have adjuvant-like qualities. T-2 toxin exposure decreases T-dependent and increases T-independent antibody responses (Rosenstein et al., 1979). Thus, the immunomodulatory effects of T-2 appear similar to effects also attributed to endotoxin.

TABLE I-1

BIOLOGICAL ACTIVITIES OF ENDOTOXIN

NONTOXIC	TOXIC
Immunological	*Pyrogenicity
*B-lymphocyte mitogenicity	*Lethality
Adjuvanticity	Shwartzman phenomenon
*Polyclonal B-cell activation	*Bone-marrow necrosis
*Thymic independent antigen	*Leucopenia
Macrophage activation	*Leucocytosis
Enhanced phagocytosis	*Hypotension
Enhanced nonspecific resistance to infection	*Abortion
Induction of: prostaglandin synthesis, interferon, colony stimulating factor, plasminogen activator	Tumor necrosis
Complement activation	
Hagemann factor activation	

Kabir et al. (1978).

*Indicates that the characteristic has also been associated with trichothecene toxicoses.

Figure I-2 illustrates a proposed immunomodulatory scenario following T-2 insult. The primary toxic event is disruption of gut mucosal integrity, followed by homeostatic imbalance (i.e., increased levels of corticosteroid and endotoxin), resulting in immunomodulation. In general, chemicals exert their effects only after entering the body. The alimentary tract is a primary route of entry. The alimentary tract mucosa provides a semi-permeable barrier separating the organism from its environment. The barrier, normally quite effective, can be severely compromised if its integrity is damaged. The sequelae, following mucosal damage, often include sepsis. Terao (1983) reported that within 10 minutes after the injection (ip) of LD_{50} doses of trichothecenes, almost all undifferentiated cells of intestinal crypts lost free polysomes in

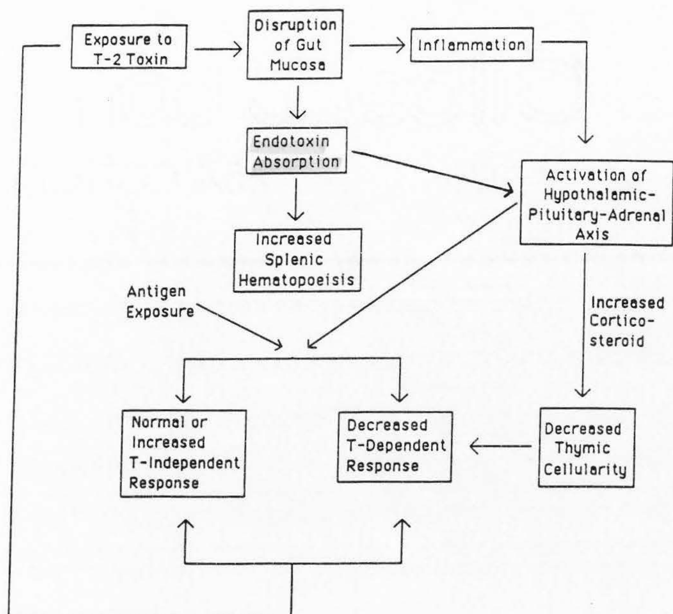


FIG. I-2. T-2 toxin-induced immunomodulation by corticosteroid and endotoxin imbalances.

their cytoplasm. Karyorrhexis and necrosis followed in the lower half of crypts. T-2 was detectable within the duodenal epithelium of isolated villi in mice exposed orally to 11 mg/kg (Lee et al., 1984). The presence of T-2 was associated with desquamation of villous tips. The immunomodulatory activities of T-2 maybe linked to increased endotoxin exposure resulting from mucosal damage.

Inflammation is a typical host response to damaged tissue. Corticosteroid hormones, produced in the adrenal cortex, are endogenous anti-inflammatory compounds. The trichothecene mycotoxins induce inflammatory responses. T-2 treatment increased plasma cortisol and the size of the adrenal glands in swine (Rafai and Tuboly, 1982) and cats (Lutsky et al., 1978). Corticosteroid hormones diminish the cellularity of the thymic cortex (reviewed by Claman, 1972) and hence the availability of T cells. Thymic cortical depletion was also a characteristic of T-2 toxicosis (Rosenstein et al., 1979). A decrease in T cells may restrict an organism's ability to respond effectively to T-dependent antigens, a phenomenon associated with T-2 exposure (discussed earlier).

An increase in serum corticosteroids may reflect an involvement of the hypothalamic-pituitary-adrenal axis. Thus, neurotransmitters may also be involved in the response to T-2. Lorenzana et al. (1985) reported increased serum norepinephrine levels in T-2 treated swine. Both norepinephrine and serotonin have been implicated as endogenous substances capable of reducing the immune response (Sanders and Munson, 1985; Boranic et al., 1984).

In summarizing, the reported immunomodulatory activities of T-2 may be a consequence of the toxin's effect on alimentary tract mucosa and more directly associated with endotoxin and corticosteroid hormone imbalances. The role of homeostasis in the assessment of immunomodulatory compounds may have implications on the design of such investigations.

Specific Aims

1. Are the immunomodulatory effects of T-2 toxin related to endotoxin and/or corticosteroid levels?
2. Is antibody production altered by T-2 toxin alone or mediated by the activity of the adrenal gland?
3. Does T-2 toxin have immunomodulatory effects on murine splenocytes and thymocytes *in vitro*?
4. Do corticosteroids influence the binding of T-2 toxin to mouse thymic cells?

CHAPTER II

LITERATURE REVIEW

Trichothecene Mycotoxicoses

The effect of trichothecene mycotoxins on the immune system of animals has gained much attention recently. Animal models to date include monkey, cat, pig, cattle, sheep, guinea pig, rabbit, rat, mouse, chicken, duck, and turkey. Research efforts have been directed toward understanding both antibody production and cell-mediated immunity following exposure to the toxins. The trichothecene mycotoxins are a large group; however, only a few of its members have been investigated, specifically, for their respective immunotoxic potentials. Much of the information concerning their immunotoxic effects has been gleaned from studies that have included observations of peripheral white blood cell counts and/or histopathologic changes in lymphoid organs and tissues. T-2, fusarenon-X, diacetoxyscirpenol (DAS, anguidine), and deoxynivalenol (DON, vomitoxin) have been investigated as immunotoxic compounds. Assessment of mitogenic responses, T-dependent and independent antibody production, delayed-type hypersensitivity responses, graft rejection, characterization of cell phenotypes, and the effects of toxin exposure on bacterial and viral infection have been investigated and discussed in the following pages. A unifying theme has been pursued; it appears that the trichothecene mycotoxins affect a suppressive function within the immune

network. The specific cell type(s) or functions affected by trichothecene mycotoxins have yet to be definitely ascertained.

Reviews to date have focused primarily on results obtained using the mouse as a model (Otokawa, 1983; Ueno, 1980). Immunologically, the mouse is an appropriate selection. However, the toxicosis induced by trichothecenes has, in fact, affected both man and agricultural animals. Consequently, in this review an attempt was made to summarize effects on both typical laboratory animals as well as non-typical laboratory animal species.

Alimentary Toxic Aleukia and its Immunotoxic Nature

Summaries of clinical observations from persons afflicted with Alimentary Toxic Aleukia (ATA) have been compiled by Mayer (1953) and Joffe (1978). Their summaries, in turn, have made use of the extensive Russian literature.

The name ATA indicates that the target of trichothecene toxicity is the leucopoietic system. In fact, a leucopenic stage (2nd stage) has been described for the syndrome. In the first stage of the disease, the number of peripheral leucocytes either does not change or increases slightly. As the 2nd stage begins there is a sharp decrease in the absolute number of leucocytes and a relative decrease in the percentage of granulocytes. Throughout much of the second stage the leucocytes show toxic granulation and vacuolization within the cytoplasm. During the 3rd stage of ATA a general atrophy of the bone marrow occurs. In the 3rd stage necrotic changes are apparent. It has been suggested that the

necrotic changes occur due to impairment of leucocyte-phagocyte and reticuloendothelial functions. Regional lymphnodes are often swollen. Histologically, the necrotic stage of ATA is characterized by lymphoid depletion and hyperplasia of the reticuloendothelium. The entire lymphoid and reticuloendothelial systems are affected, including the spleen and liver.

Janovskij's tenet (cited by Mayer, 1953) proposed that the origin of alimentary toxic aleukia was of an immunological nature and proposed the following progression of an allergic response: 1) local action of the compound, 2) incubation or period of sensitization, 3) anaphylactic changes, followed by 4) reconvalescence. Perhaps allergic reactions should be studied in greater detail in animals following repeated dermal exposure to the trichothecene mycotoxins. The ability of trichothecenes to induce erythema after topical application has been used as a determinant of trichothecene-contamination. The cutaneous response to both T-2 and DAS in ethyl acetate has been studied morphologically and quantitatively using both rats and rabbits (Hayes and Schiefer, 1979). The cutaneous responses, described as being inflammatory reactions, were characterized by accumulations of neutrophils at the luminal margins of venules. Neutrophils were observed throughout the dermis by 12 h and the outer layer of the epidermis by 24 h. The neutrophils were concentrated in and below the epidermis by 48 h; few were observed by 72 h or later.

Although considerable evidence suggests an inflammatory cutaneous response to topically applied trichothecenes, recent information regarding the inhibition of acute phase reaction by parenterally administered T-2 indicates that the toxin may also lessen some

nonspecific process of inflammation (Dyck et al., 1985). The acute phase response is a dramatic but incompletely understood physiologic reaction to tissue injury and its concomitant inflammation. This response is characterized by a rapid increase in hepatic synthesis of several plasma proteins, such as coagulation and complement components. Products of injured tissue, as well as biochemical and cellular constituents of the inflammatory process, are possible mediators of this response, such as leukocyte endogenous pyrogen and interleukin 1. The biological role of the acute phase response is still unclear, but many workers believe that it is a nonspecific and immediate participant in inflammation and tissue repair. Although the biological role of some acute phase reactants can be inferred from their known in vivo functions (i.e., fibrogen, C3), the roles of the most dramatic acute phase reactants, C-reactive protein and serum amyloid-A component in man and serum amyloid-P component in the mouse, have been only partially defined.

No acute phase response was observed in mice treated with T-2 (0.01 to 2.0 mg/kg) either subcutaneously (sc) or intraperitoneally (ip) based on the measurement of the two murine acute phase reactants, serum amyloid-P component and plasma fibronectin (Dyck et al., 1985). T-2 (0.17 to 1.50 mg/kg, ip) was demonstrated to block the acute phase response to sc-injected $AgNO_3$ in a dose-related fashion. In addition, if T-2 (2.0 mg/kg, ip) and $AgNO_3$ (sc) together were given to the animals, half of them died after 48 h, and the survivors were extremely ill. Mice given the same dose of T-2 along with subcutaneous injection of buffer, however, appeared well. Since the acute phase is an immediate physiologic reaction to tissue injury and may be a nonspecific

participant in the tissue repair, its abrogation by T-2 may contribute to the toxicity of this mycotoxin.

Immunotoxic Effects

Trichothecene-Induced Immunodysfunction in Man

Relatively little information is available concerning the immunotoxicity of trichothecene mycotoxins and the human immune system. Much information to date has been provided by clinical trials to determine the efficacy of DAS as a therapeutic anti-cancer agent (Goodwin et al., 1978, 1983). To date, the use of DAS has been for its cytostatic properties and not as a modulator of the immune system. Myelosuppression has been reported in patients exposed to DAS at a dose of 4.5 mg/m^2 administered iv (Push) given on a five-day schedule. Stimulation of peripheral lymphocytes with phytohaemagglutinin (PHA), a T-cell mitogen, following DAS exposure was evaluated; however, results were not described (Goodwin et al., 1978). Cooray (1984) reported decreased responses of peripheral lymphocytes to PHA following in vitro exposure to T-2 or DAS. The lymphocytes were first exposed to PHA for a 24-h period, after which the toxins were added and incubation continued for another 48 h. Diacetoxyscirpenol and T-2 (both concentrations were 3.0 ng/ml) reduced [^{3}H]-thymidine ([^{3}H]-TdR) incorporation by 80 and 90 percent, respectively. The incorporation of [^{3}H]-TdR was used as an index of DNA synthesis and hence a measurement of cell proliferation. Diacetoxyscirpenol or T-2 alone reduced [^{3}H]-TdR incorporation (cells were not exposed to PHA); their respective ID_{50} concentrations were 1.5

and 2.7 ng/ml. It is worth noting that total inhibition of [^{3}H]-TdR incorporation was observed with both DAS and T-2 at 8.0 ng/ml.

Yarom et al. (1984) also studied the effects of T-2 on human leucocytes in vitro; viability, chemotaxis, chemiluminescence, and phagocytosis were monitored. The authors reported a difference in the susceptibilities of mononuclear cells (lymphocytes, monocytes) and polymorphonuclear cells to T-2 toxicity. A dose of 300 ug/10^{6} cells caused ultrastructural damage to approximately 40 percent of the polymorphonuclear cells, while the mononuclear cells showed no structural damage. Chemotaxis of peripheral leucocytes was decreased by a dose of 3 ug/10^{6} cells; the decreasing response was dose-related. T-2 (1-20 ug/10^{6} cells) was able to reduce the chemiluminescence of leucocytes stimulated with opsonized streptococci. A statistically significant reduction in phagocytic activity of polymorphonuclear cells was demonstrated for cells exposed to T-2.

Both T-2 and several of its metabolites, HT-2, 3'-OH T-2, 3'-OH HT-2, T-2 triol and T-2 tetraol, inhibited the mitogen-induced blastogenesis of human peripheral lymphocytes (Forsell et al., 1985). The cells were exposed in vitro to the compounds. Leukoagglutinin, concanavalin (Con A), and pokeweed mitogen (PWM) were the selected mitogens. Hydroxylation of T-2 to 3'-OH HT-2 dramatically decreased [^{3}H]-TdR uptake; further hydroxylation to the triol and tetraol resulted in even less [^{3}H]-TdR uptake.

The incorporation of [^{3}H]-TdR into the DNA of PHA-stimulated peripheral blood lymphocytes was inhibited by both DON and 3-acetyl-DON (Atkinson and Miller, 1984). Deoxynivalenol was more effective than was

3-acetyl-DON. The ID_{50} values were 220 and 1060 ng/ml for DON and 3-acetyl-DON, respectively.

Animal Models Use to
Assess Trichothecene Immunotoxicity

Monkey. Rhesus monkeys treated with 0.1 mg/kg/day for fifteen days by stomach tube developed leukopenia (Rukmini et al., 1980). When greater amounts of toxin were given to the animals a decrease in polymorphonuclear cells was observed without noticeable changes in lymphocyte percentages. Spleens and lymph nodes showed the presence of atrophic follicles.

Jagadeesan et al. (1982) administered 100 ug/kg body weight T-2 po daily for 4-5 weeks. Total peripheral leucocyte counts were reduced after 4 weeks of treatment; total leucocyte counts approached normal values following cessation of toxin treatment. Histopathologic examination revealed cortical depletion of lymphocytes from the lymph nodes of autopsied animals. The bactericidal activity of peripheral neutrophils, assessed *in vitro* by their ability to phagocytize *E. coli*, was decreased following *in vivo* exposure to T-2. Rosette positive cells (T cells) also decreased. T-cell function investigated in a PHA-stimulated system was decreased in animals that had been treated with T-2. Serum immunoglobulin levels (total IgG and IgM) and hemolytic complement (CH_{50}) were also assessed. Immunoglobulin levels decreased, IgG to a greater extent than IgM. The complement factor did not change. Most immunological parameters, depressed by toxin treatment, increased to near normal values following termination of treatment.

Cat. The cat has served as a reliable model for clinical symptoms of ATA. Pancytopenia, bone marrow aplasia, and severe alterations of lymphatic tissues have been observed in cats treated with T-2 (Lutsky et al., 1978; Lutsky and Mor, 1981a, 1981b). Cats treated with 0.1 or 0.08 mg/kg body weight T-2 po once every 48 h exhibited a slight leucocytosis followed by persistent leucopenia. A peripheral lymphocytosis was also observed and accompanied by neutropenia. Lymphopenia later developed and neutropenia continued. All cats treated with T-2 succumbed by day 40 of T-2 treatment. The neutrophil population was observed to undergo morphological alterations, including increased size and altered nuclear morphology. Neutrophils from animals in terminal stages of T-2 mycotoxicosis displayed nuclear changes of pyknosis and karyorrhexis. Eosinophils were increased; cells of the myeloid series decreased in maturation. Lymphnodes were usually enlarged. This phenomenon was most prominent in the mesenteric lymphnodes. A loss of typical architectural patterns within lymph follicles and hyperplasia of the reticuloendothelial cells were observed. The reticuloendothelial cells were observed to replace cells of lymphoid compartments. A depletion of splenic lymphocytes from germinal centers was seen. Sinusoidosis was evident around the lymph follicles.

Swine. Pigs developed leucopenia after consuming grain that had caused ATA in people (Forgacs and Carll, 1962). Massive lymphocytic necrosis characterized by pyknotic nuclei and karyorrhexis was observed in Peyer's patches of the ileum, lymphoid nodules of the cecum, lymphoid follicles of the spleen, and germinal centers of the mesenteric lymph nodes of pigs that died acutely after receiving a single iv injection of

T-2 at doses ranging from 1.2 to 2.5 mg/kg (Weaver et al., 1978a). However, pigs that survived a single iv injection of T-2 at doses ranging from 0.96 to 1.5 mg/kg did not have any detectable morphological changes in these lymphoid organs and tissues. In the same study, there were no effects on bone marrow or leucocyte counts. Similarly, when DAS was administered iv to pigs in an LD_{50} study, there were no effects seen in bone marrow of survivors (Weaver et al., 1978b). Although bone marrow was not examined from pigs which died, necrosis of germinal centers of mesenteric lymph nodes and splenic white pulp was consistently present. In contrast, neither T-2 at 1, 2, 4 or 8 ppm nor DAS at 2, 4, 8 or 9 ppm in 19 percent protein rations fed to young pigs for eight weeks produced effects on the leucocyte counts or morphology of lymphoid organs or tissues (Weaver et al., 1978a, 1978b, 1981). Although mild leucopenia developed in one pig that received T-2, the typical lesions of lymphoid organs and tissues of piglets, dosed orally with purified T-2 or DAS (0.1 mg/kg) or equivalent doses of crude extracts of F. tricinctum for periods of 14 to 36 days, did not develop (Patterson et al., 1978).

Widespread necrosis of B-cell regions of lymphoid organs and tissues, including germinal centers of lymph nodes, palatine tonsil, spleen, Peyer's patches of the ileum, and both gut and bronchial associated lymphoid tissues, was seen in animals that received 2.4 mg/kg T-2 po (Beasley, 1983). Although necrosis of the T-dependent regions was less prominent in secondary lymphoid organs and tissues, a moderate to severe multifocal lymphoid necrosis was evident in the thymus. Lymphocytic necrosis could be observed in secondary lymphoid organs and tissues as well as the thymus as early as 1 or 2 h after iv

administration of T-2. Cellular necrosis of the bone marrow was noted in pigs given lethal or potentially lethal doses of T-2, but these lesions were inconsistent even in animals entering later stages of acute toxicosis.

In a preliminary clinical pathology study, pigs that received an iv or po lethal or sublethal dose of T-2 developed leucocytosis initially, largely attributable to neutrophilia, followed by lymphopenia (Beasley, 1983). Lorenzana et al. (1985) reported a similar response in an acute study that pigs had received a single lethal (4.8 mg/kg) or sublethal (0.6 mg/kg) dose of T-2 iv. The hematology was characterized by initial leucocytosis followed by leukopenia, due primarily to absolute neutropenia and secondarily to a lymphopenia.

Rafai and Tuboly (1982) demonstrated that after 21 days of feeding 5 ppm T-2 (average daily T-2 intake was 1.27 mg) to weanling pigs, leucocyte counts were significantly decreased. Antigen-induced blast transformation in the T-2 treated group, as measured by [^{3}H]-TdR incorporation and expressed as stimulation index, was approximately 41.7 to 45.6% of control. Immunofluorescent detectable IgG$^+$ cells were significantly decreased in the T-2 treated group. Although the percentage of rosette-forming cells in both T-2 treated and control groups increased, the T-2 treated groups were significantly lower than the control group. Toxin-neutralizing titers to <u>Clostridium</u> bacterin were significantly lower in the T-2 treated animals. The relative weights of submaxillary lymph nodes did not differ significantly. However, the relative weights of thymus and spleen from T-2 treated pigs were significantly reduced. Interestingly, the authors reported that plasma

cortisol concentrations were significantly increased in T-2 treated animals throughout the study, relative to control animals. Additionally, the relative adrenal weights of T-2 treated animals were significantly increased. Although the direct effect of T-2 might play a role, the authors suggested that increased adrenocortical activity was attributable to reduced feed consumption. It is known that trichothecene mycotoxins inhibit protein synthesis (Ueno and Shimada, 1974). The elevated plasma cortisol level in T-2 treated animals may enhance protein degradation via gluconeogenesis. The authors speculated that this dual effect may adversely affect protein turnover in growing pigs, thereby suppressing both cell-mediated and humoral immune responses.

In a sequential immunotoxicity study, a single dose of 15 mg/kg T-2 in 0.75 ml absolute dimethylsulfoxide was applied to a 10 x 15 cm^2 clipped area on the back of pigs immunized sc with 10^9 SRBC on the same day and again 21 days later (Pang et al., 1987a). Mitogen-induced blastogenic responses of enriched peripheral blood lymphocytes and hemagglutinin (HA) titers to SRBC were evaluated. Significantly lower blastogenic responses to PHA, Con A, and PWM were found in the T-2 treated group. These depressed responses occurred mainly between days 3 and 5, as well as between days 20 and 28 after dermal application. In contrast, the blastogenic response to LPS was slightly but significantly higher in T-2 treated animals on day 14. Although mean HA titers for T-2 treated animals were approximately 2-fold greater than control values between days 10 and 24, these differences were not significant.

Recently, effects of T-2 on systemic and local immunity of swine following inhalation exposure were studied (Pang et al., 1987b). In one

study, animals were dosed with 8 mg/kg of nebulized T-2 in absolute ethanol. Animals were subsequently immunized sc with 10^9 SRBC and challenged 21 days later. No differences in blastogenic responses to various mitogens (PHA, Con A, PWM, LPS) were observed between T-2 treated and control groups; however, significantly lower HA titres were found in T-2 treated animals between days 3 and 7 after primary immunization. In a separate study, pigs were dosed with 9 mg/kg nebulized T-2 in absolute ethanol, and both systemic and local pulmonary immunity were evaluated. Effects on systemic immunity were measured by mitogen-induced blastogenic responses performed on enriched peripheral blood lymphocytes. Effects on local pulmonary immunity were measured by bacterial phagocytosis of alveolar macrophages and mitogen-induced blastogenic responses of enriched pulmonary lymphocytes. Alveolar macrophages and enriched pulmonary lymphocytes were obtained by broncho-alveolar lavage. A few pigs died or were moribund 8 to 10 h after inhalation of T-2. Other pigs which survived exposure were killed on days 1, 3 or 7. The capacity of alveolar macrophages to phagocytize bacteria was significantly decreased in the T-2 treated animals 8 to 10 h or 1 day after exposure. No significant effects were noted on days 3 and 7. The mitogen-(PHA, Con A, or PWM) induced blastogenic responses of enriched pulmonary lymphocytes were lower in the T-2 treated animals at all time points, although the differences were not significant.

Cattle. Recently the effects of T-2 on cellular immune responses of calves were studied (Mann et al., 1984). When treated orally (by capsule) with either 0.3 mg/kg/day for 56 days or 0.5 mg/kg/day for 28 days, the animal at the lower dose displayed reduced neutrophil function

as quantitated via nitroblue tetrazolium reduction. The numbers of peripheral B cells, identified as immunoglobulin positive (Ig^+) cells, were reduced. The numbers of T cells, identified as peanut agglutinin positive cells, however, were slightly elevated above control values on days 7, 35 and 42. In animals exposed to T-2 at 0.5 mg/kg/day, the numbers of Ig^+ cells initially fell below control values, then increased between days 7 and 19 and decreased thereafter. T-cell numbers did not vary greatly between treated and control animals.

The response to PHA by both unseparated peripheral blood mononuclear cells and a T-cell enriched (nylon wool nonadherent) population was decreased after the animals were treated with T-2 at 0.5 mg/kg/day for 19 days (Mann et al., 1984). Similarly, PHA responses of lymphocytes from calves exposed to T-2 at 0.6 mg/kg/day for a total of 43 days were decreased on days 1, 8 and 29 (Buening et al., 1982). In addition, the lymphocyte responses to Con A and PWM were also decreased on day 29. When calves were immunized with Anaplasma vaccine on days 1 and 21, lymphocyte responses to Anaplasma antigen in vitro (used similarly to mitogens) did not differ between treated animals and controls. In addition, neutrophil function, as assessed by random migration in agarose, nitroblue tetrazolium reduction, or glucose uptake, was unchanged during exposure to T-2; however, chemotaxis was depressed.

In vitro treatment of bovine lymphocytes with varying concentrations of T-2 and either PHA, PWM, or Con A demonstrated the suppressive effects of the toxin on $[^3H]$-TdR incorporation (Mann et al., 1984). Cell responses to Con A were inhibited 50 percent by T-2 at 2.0 ng/ml. Nylon wool separated cells, both adherent (B-cell enriched) and nonadherent (T-

cell enriched), were more sensitive to the effects of in vitro T-2 treatment than the unseparated cells. A 50 percent reduction of the lymphoblastic response to mitogens occurred in both populations by T-2 in concentrations as low as 1.4 ng/ml.

Mann et al. (1982) observed reductions of total serum globulin of calves treated orally with T-2 at 0.6 mg/kg/day for 43 days. IgM, IgA, and C3 concentrations were decreased, but the IgG concentration did not change.

These studies indicated that a persistent exposure of calves to T-2 po at doses of 0.3 to 0.6 mg/kg could result in adverse effects on both humoral and cellular immunity.

Sheep. Leucopenia was evident on day 7 for sheep administered 0.6 mg/kg/day T-2 orally (Friend et al., 1983a). Recovery from depression in leucocyte counts was apparent by day 21. Lymphopenia was also observed on days 7 and 14. Several microscopic changes in lymphoid tissue cellularity were recorded, including decreased cellularity of splenic white pulp, Germinal centers of lymphoid follicles were hypocellular while periarteriolar lymphoid sheaths were less affected; cortical areas of mesenteric lymphnodes were depleted (depletion was not evident in medullary regions), and lymphocytes from all areas of the mesenteric lymphnodes were markedly depleted with numerous pyknotic cells. An increased myeloid:erythroid ratio was observed. Responses to the B-cell mitogen, LPS, were reduced in treated animals on days 7, 14 and 21. Concanavalin A responses, reduced on day 7, were comparable to control values on days 14 and 21. The effects on T-cell function appeared

transitory. However, the effect on B-cell function was persistent throughout the study.

Guinea Pig. Guinea pigs treated with T-2 (2.5 or 5.0 mg/kg, po) developed necrotic lesions of lymphoid tissue (DeNicola et al., 1978). Necrosis was observed in the cortical follicles of mesenteric lymph nodes, Peyer's patches of the ileum, periobronchiolar lymphoid follicles, and palpebral lymphoid follicles. The necrosis within mesenteric lymphnodes was further characterized by pyknosis, karyorrhexis, and karyolysis. Necrosis was less evident in the thymus and minimal in the spleen. At lower doses (0.5 and 0.75 mg/kg, ip, daily) T-2 produced no observable microscopic lesions. However, moderate leucopenia was recorded by day 33 and both lymphopenia and neutrophilia were observed by day 21. A higher daily dose (0.9 mg/kg) again produced moderate leucopenia; however, lymphopenia was less severe and neutrophilia was not observed. The myeloid:erythroid ratio, as well as the number of lymphocytes within the bone marrow, were reduced.

When guinea pigs were dosed with DAS at 0.6 to 1.6 mg/kg daily for 30 days, no effects on hematology were detected and no lesions were found (Kriegler, 1981). However, guinea pigs dying from single oral doses of from 1 to 8 mg/kg in an LD_{50} study had necrosis of lymphoid organs. Dying animals, and to a lesser extent survivors, had necrosis of round cells in bone marrow.

Treatment with T-2 decreased the anti-DNP-BSA (dinitrophenol-bovine serum albumin) response in animals treated daily for 5 days with 0.5 mg/kg of the toxin (Hiromichi et al., 1982). A similar treatment regime with fusarenon-X (0.75 mg/kg) did not affect the anti-DNP-BSA response.

The animals were treated with either toxin prior to injection of the antigen. It was observed that T-2 was considerably more effective in the in vitro suppression of mitogenic responses of splenic cells to either LPS or Con A.

Rabbits. After rabbits had received a single iv dose of T-2 at 0.5 mg/kg, there was a rapid and significant decrease in total white blood cells (Gentry and Cooper, 1981). The decline was greatest at 66 percent of normal on the third day and then returned toward normal. Differential counts were not performed. However, during and after daily administration of T-2 (2 mg/kg, po) for 4 consecutive days, there were no changes in white blood cell counts, total serum protein, or albumin. Neither po nor iv administration of T-2 produced any change in rectal temperature of the rabbits.

Rat. In-depth studies of T-2 induced alterations in the morphology and functional capacity of rat alveolar macrophages were published recently (Gerberick and Sorenson, 1983; Gerberick et al., 1984). The alveolar macrophages were isolated by tracheal lavage; toxin treatment was then performed in vitro. Total exposure time was 20 min. Effective concentrations of T-2 which reduced cell viability, cell number, and viability index by 50% were 8.93, 0.33, and 0.89 uM, respectively. The viability index reflected the number of viable cells as a percentage of control. Mean cell volume decreased in a dose-related fashion; 1 uM T-2 treated cells were significantly different from controls. As observed with cell volume, chromium release from pre--loaded cells increased in a dose-related manner in the presence of T-2. Scanning electron microscopy revealed several alterations of alveolar macrophage surfaces

following a 20 h exposure to T-2. Most cells completely lost their surface processes and developed bleb-like structures and an appearance of being broken open.

Leucine incorporation ceased immediately in cultures containing 0.10 uM T-2 and terminated after a 2 h incubation in cultures containing 0.01 uM T-2. The toxin effectively reduced phagocytosis of serum-opsonized yeast cells. Concentrations of 0.01 and 0.05 uM reduced the phagocytic activity of alveolar macrophages by approximately 25 and 85 percent, respectively. It was determined that the binding of yeast cells to the surface of the macrophage was not impaired. The effect of T-2 on the activation of alveolar macrophages by endotoxin, lipopolysaccharide (LPS), or supernatants from Con A-or PHA-stimulated splenic cell cultures was assessed by the uptake of $[^{14}C]$-glucosamine by macrophages. This parameter of macrophage function was decreased by 0.01 to 0.10 uM T-2.

Yarom et al. (1984) investigated the effects of T-2 on the cellularity of peripheral blood and also of the bone marrow in animals exposed to 0.5 mg/kg/day ip for one, two, or three days. Peripheral leucocyte counts decreased daily. Differential counts indicated an initial granulocytophilia on day 1 followed by granulocytopenia on days 2 and 3. Lymphocytes increased on days 2 and 3. Differential counts for the bone marrow revealed a decrease in myeloid cells and an increase in lymphoid cells on day 1, 2 and 3. The total number of nucleated cells within the bone marrow decreased drastically on days 1, 2 and 3. Intramuscular injection of <u>Staphylococcus aureus</u> was followed typically by massive infiltration of the area by polymorphonuclear granulocytes and a later immigration of mononuclear macrophages. In rats given 5 daily

intramuscular doses of T-2 (0.5 mg/kg) there was much local edema and some myofiber necrosis but the cellular elements were few in number.

Saito et al. (1980) reported on the incidence of tumors found in animals following chronic exposure to Fusarenon-X. The toxin was incorporated into diet at 7 and 3.5 ppm and the animals were treated for 1 to 2 years. Treated animals were often afflicted with pulmonary infections. Chronic bronchopneumonia with acute exacerbation and abscess formation was the major causes of death in the 105 ppm treatment group. One case of leukemia was present in a toxin-treated animal; no cases were observed in controls. The authors noted no histologic alterations of the thymus, spleen, or bone marrow. Atrophy of these organs was apparent in animals that succumbed to pneumonia but may have been a secondary phenomenon due to exhaustion of cellularity as a result of chronic infection.

Both DON and 3-acetyl-DON caused a dose-dependent decrease in ^{3}H-TdR uptake in PHA-stimulated peripheral blood lymphocytes (Atkinson and Miller, 1984). Deoxynivalenol was more effective than 3-acetyl-DON. The ID_{50} values for ^{3}H-TdR incorporation were 90 and 450 ng/ml for DON and 3-acetyl-DON, respectively.

Mouse. The mouse model has been used more than any other in the study of the immunotoxicity of the trichothecene mycotoxins. Not only in the quantity but also in the quality of research accomplished to date, attempts to discern the mechanism by which the trichothecenes exert their effects on immunological function have been most successful in the mouse. Therefore, the following discussion attempts to clearly elucidate the impact of trichothecene mycotoxins on the murine immune system at two

distinct levels. The structural and the functional components of the system have been discussed in turn and a union of the two sought with discussion of in vitro cellular reconstruction experiments.

Hayes et al. (1980) treated mice with 20 ppm T-2, incorporated into feed, for 6 weeks. Hematological parameters were evaluated after one, two, three, four and six weeks. Peripheral leucocyte counts were reduced after 1 week and remained lower than controls for the entirety of the treatment period. Neutrophilia was apparent with a concomitant reduction in the percentage of peripheral eosinophils. The spleen weights of treated mice initially decreased in size and then increased and surpassed control values by 4 weeks. The thymus and Peyer's patches decreased in size and became atrophic. The white pulp of the spleen diminished and after 3 weeks was not visible. The red pulp atrophied, turning pale then tan and greatly reduced in size by days 7 and 14. As stated earlier, splenomegaly was observed by day 28 due to the proliferation of a grayish-red homogeneous tissue throughout the red pulp. By day 41, this was observed in 3 out of 4 mice. Also by day 41 the red pulp had resumed a normal dark color. Microscopically, cortical depletion of lymphocytes from the thymus was observable on day 7 and there was an infiltration of neutrophils and eosinophils into the medulla and some areas of the depleted cortex. The follicles in lymph nodes and spleen virtually disappeared. Thymic-dependent lymphoid populations, including periarteriolar sheaths in the spleen, paracortical regions of lymph nodes, and intraepithelial lymphocytes of the small intestine, were depleted. B-cell dependent populations in the intestinal lamina propria, medullary cords of lymph nodes, and splenic follicles also decreased in

all mice consuming T-2. Neutrophilic myelopoiesis appeared hyperplastic by days 21-28.

Other investigators have observed decreases in the weights of both the spleen (Hayes et al., 1980,; Friend et al., 1983a, 1983b; Kanai and Kondo, 1984; Ueno, 1984; Rosenstein et al., 1979, 1981; Lafont et al., 1977) and/or thymus of animals exposed to T-2 or DAS (Rosenstein et al., 1979, 1981; Lafarge-Frayssinet et al., 1979; Fromentin et al., 1980). The effects on the weights of the thymus and spleen were reversible and included hypertrophy of the spleen (Lafarge-Frayssinet et al., 1979). The effect on the weight of the thymus appeared greater than the effect on that of the spleen for both DAS and T-2 (Ueno, 1984; Rosenstein et al., 1979). Peripheral leucocyte and lymphocyte counts increased following a single ip injection of T-2; the increases were not accompanied by changes in polymorphonuclear cells (Lafont et al., 1977). The increases occurred by 3 h and were followed by decreases in both leucocytes and lymphocytes. Decreased leucocyte counts have been reported for animals receiving 3.0 mg/kg T-2 ip (Ueno, 1984). Additional information regarding histopathologic alterations of lymphoid tissues following short-term acute treatment of animals with Fusarium extracts has been documented by Lafarge-Frayssinet et al. (1979). They reported atrophy of T-dependent areas (periarteriolar sheaths) before B-dependent areas (follicles) within the spleen. Rapid depletion of cortical areas with proliferation in the medulla was observed for the thymus. Rosenstein et al. (1979) also reported cortical depletion of thymic lymphocytes; however, the medulla remained proliferative. Lafarge-Frayssinet et al. (1981) found the lymphoid tissues to be more

sensitive than the liver to the adverse effects of T-2 on DNA damage assessed via alkaline elution.

Radiomimetic effects of Fusarium nivale extracts were described by Saito et al. (1969). The authors reported a loss of reaction against stimulation of the skin but did not describe the phenomenon. Necrosis and karyorrhexis of nuclei were seen in the germinal center of lymphoid follicles and the thymic cortex. In the bone marrow, atrophy of the pulp with dilatation of the sinus, followed by karyorrhexis of the hematopoietic cells, was also observed. Fraction A-III caused an increase in leucocytes at 3 h. The percentage of neutrophils increased at this time also. Immature cells were present at both 24 and 96 h.

Both B and T cells have been enumerated in lymphoid tissues of animals treated with trichothecenes, both T-2 and fuasarenon-X. T cells bearing the antigenic phenotype Thy-1.2 decreased in the spleens of mice treated with 0.75 mg/kg T-2 ip daily for 7 days; they were reduced by approximately 50 percent (Rosenstein et al., 1981). T-cell numbers within the spleen failed to increase following injection of sheep red blood cells, as normally occurs in mice treated with T-2. Ig^+ (immunoglobulin positive) cells (B cells) within the spleen were not affected (Otokawa et al., 1979). Masuda et al. (1982a) reported a decrease in splenic T cells in mice treated with 50 ug/day fusarenon-X ip for 7 days; no changes in B cells were observed. Interestingly, the total number of cells within the spleen increased by approximately 50 percent. No significant changes were observed in splenic B- or T-cell numbers (though T cells were lower) from mice treated with 5, 10, 25 or 50 g Fusarenon-X ip daily for 7 days. However, as stated above the

number of cells residing in the spleen did increase (Masuda et al., 1982b).

Mitogens have often been used to evaluate the mitogenic responsiveness of lymphocytes isolated from animals treated with toxin or lymphocytes treated directly with a toxin in culture. Responses to Con A were clearly depressed in mice fed a diet supplemented with 20 ppm T-2 toxin for 1-4 weeks (Friend et al., 1983a). The responses to Con A, however, were comparable to control values after six weeks of toxin treatment. A similar phenomenon was observed for the responses to LPS. Taylor et al. (1985) observed a decreased response to PHA after two weeks treatment with 2.5 mg/kg T-2 po every third day. T-cell responses were comparable to control values following 4 weeks. In the same study the PWM response increased at both 2 and 4 weeks for animals treated with 2.5 mg/kg T-2. PWM is a T-dependent B-cell mitogen, hence stimulation of B cells by PWM is regulated by T cells. Treatment of mice with crude Fusarium extracts on days 0 and 1 resulted in an increased LPS response associated with a decreased PHA response (Lafarge-Frayssinet et al., 1979). The initial increase in the LPS response on day 3 was followed by a decreased response on day 10 and an increased response on day 19. The PHA response continued to decrease, reaching its minimum on day 10 and was normal by day 19. Similar results were obtained with lymphocytes isolated from mice treated with crude Fusarium extracts and stimulated in vitro with PHA (Rosenstein et al., 1981). When lymphocytes were exposed to T-2 in vitro and simultaneously exposed to PHA, 0.1 ng/ml toxin caused an increased uptake of [^{3}H]-TdR followed by a decrease with increasing toxin concentrations (Rosenstein and Lafarge-Frayssinet, 1983). Con A-

stimulated lymphocytes exposed to T-2 $\geq$ 12.5 ng/ml exhibited a decreased proliferative response (Gyongyossy-Issa and Khachatourians, 1985). Stimulation of both LPS and PHA responses was observed for splenic cells treated with 0.05-1.0 ng/ml T-2 toxin; inhibition occurred with greater concentrations (Lafarge-Frayssinet et al., 1979). Diacetoxyscirpenol inhibition was less severe; however, no stimulation was observed. In the same study the response of thymic cells exposed to T-2 and PHA in culture resulted in a greatly enhanced response when < 2.0 ng/ml toxin was used; greater concentrations resulted in a depressed response. Results obtained in our laboratory (Taylor et al., 1987a) suggest that an inverse, time-dependent relationship exists regarding T-cell and B-cell mitogen responsive cell populations isolated from the spleen. Decreased PWM and LPS responses were correlated with increased PHA and Con A responses and visa- versa, depending on the time of toxin exposure, in the presence of mitogen. Twenty-four pretreatment of splenic cells with fusarenon-X (0.001-10 ug/ml) prior to mitogen addition resulted in greater inhibition of T-cell responses to both PHA and Con A than was observed for B-cell responses to LPS (Masuda et al., 1982a). A slight stimulation of all mitogen responses was observed by treatment with 10^{-3} ug/ml fusarenon-X. In vivo exposure to fusarenon-X resulted in a slight enhancement of both PHA and Con A responses of animals treated with 25 ug/day and a clear decrease in LPS responsiveness.

Rosenstein et al. (1979, 1981) have studied the effects of both T-2 and DAS on T-dependent and independent antibody production. T-dependent responses require the participation of T cells; while T-independent responses do not. Both trichothecenes were able to suppress production of anti-sheep red blood cell (α-SRBC) antibodies. A dose > 0.75 mg/kg T-2 given ip daily completely suppressed antibody production. A dose of 2.0 mg/kg DAS was required to completely stop antibody production. Both toxins exerted their influences in a dose-related manner. The effective range for T-2 was much narrower than that observed for DAS. T-2 also suppressed the number of plaque forming cells per spleen. The evolution of antibody production toward SRBC, depressed immediately following toxin exposure, attained a level comparable to controls 12 days post-toxin treatment. If T-2 (0.75 mg/kg) were administered during the development of an α-SRBC response, the ensuing response failed to attain normal levels. Intermittent exposure to 2.5 mg/kg T-2 toxin (po) caused a depression in the number of α-SRBC plaque-forming cells (PFC) (Taylor et al., 1985). T-independent antibody responses to both DNP-Ficoll and polyvinylpyrrolidone (PVP) were increased above control values in animals treated with T-2; DAS caused an increased DNP-Ficoll response (Rosenstein et al., 1981). Again, as was observed earlier, the α-SRBC response was depressed by T-2 treatment. Elevated α-SRBC titers have been reported following T-2 treatment (Otokawa et al., 1979). SRBC-sensitized animals received a single ip injection (3 mg/kg) of T-2 two days after antigen sensitization and elevated titers were observed. α-SRBC titers were also elevated in mice dosed with 3.0 mg/kg T-2 toxin (ip) two days before or the same day as SRBC sensitization (Masuko et al., 1977). The titer

levels were measured 8 or 15 days after SRBC sensitization. The α-SRBC responses in mice treated with DAS ($\geq$0.75 mg/kg) po for 5 weeks were lower than control values; the PFC response was also depressed (Tryphonas et al., 1984). When fusarenon-X was given to animals (50 ug per day for 7 days) prior to antigenic challenge with the T-independent antigen DNP-OVA (ovalbumin), the IgE- and IgG_1-class immunoglobulin titers were depressed. The IgE-class antibody response appeared to be elevated above control values by lower doses of fusarenon-X at 14 and 21 days after immunization. The effect of fusarenon-X on antibody response diminished with increasing temporal separation of antigen sensitization and subsequent toxin exposure.

Overall, the complexity of the effects of trichothecenes on antibody production remains difficult to interpret. The dose frequency of exposure and time of exposure appear to play an important role. Although T-dependent antibody production often tends to decrease and T-independent antibody production often increases as a response to a repeated lower dose of trichothecenes, a single explanation cannot be assuredly provided. Possible mechanisms for the reduction in T-dependent antibody production include a direct toxic effect on B cells or helper T cells or an enhanced function of a particular population of suppressor T cells. In addition, the function of macrophages, such as presenting antigens to T cells and secreting T-cell-activating factor (interleukin 1), may be adversely affected.

The differing response (the increase) in antibody production by T-independent B cells may be explained in part by the fact that these are

cells of a distinct population with differing susceptibility to trichothecenes from those of the T-dependent B cells.

At sufficient doses, however, toxic effects may occur in populations of B cells and perhaps T cells as well. Because of the interplay between these cells in vivo, identifying the specific site(s) of action for any one effect is not a simple matter.

T-2 was observed to have an enhancing effect on the development of a delayed-type hypersensitivity (DTH) reaction, which is a T-cell mediated event (Masuko et al., 1977). The animals were first sensitized to SRBC and subsequently challenged on day 7 or 14 with SRBC in the foot-pad to elicit swelling, measured at 24 h, typical of the DTH reaction. If T-2 was given (3.0 mg/kg, ip) 2 or 3 days after the first injection of SRBC, then the DTH responses on days 7 and 14 were increased above control values. Support of the above phenomenon was given by Otokawa et al. (1979). The authors looked at the effects of T-2 in mice made tolerant to subsequent SRBC response by supra-optimal antigen sensitization. Such mice normally do not respond to challenge with SRBC. However, if mice were injected with T-2 toxin 1 or 2 days after a tolerizing dose of SRBC, a subsequent DTH reaction occurred. In effect, the mechanism whereby tolerance manifests itself was blocked. Repeated treatment with T-2 ($\geq$0.1 mg/kg, po, every third day) was observed to decrease a DTH response (Taylor et al., 1985).

Animals injected with 0.75 mg/kg T-2 for 7 days prior to receiving allografts and treated with toxin after graft had an increased time to tissue rejection as compared to control rejection times (Rosenstein et al., 1979). Graft rejection is typically a T-cell function.

Kanai and Kondo (1984) reported a decreased resistance to infection in mice treated with T-2. Animals were dosed orally with T-2 (0.1 mg) both before and after infection with either a kanamycin-resistant strain of tubercle bacilli (H37vR-km) or Mycobacterium bovis. Tubercle bacilli counts per spleen were increased above controls in animals treated with T-2. Toxin was given one day before infection, continued at one-day intervals for seven days, and followed by daily injection for 5 more days. The spleen weights of infected mice not treated with T-2 increased as was expected. However, the spleen weights of T-2 treated infected mice changed very little, eventually decreasing in weight. The effect of T-2 on the infection of vaccinated animals was also assessed. Toxin treatment impaired the immunity provided by vaccination, as evidenced by bacilli counts for the spleen and lung when a milder exposure schedule was established (0.1 mg at two-day intervals for a total of six doses); decreased survival times were observed for fatally infected mice. The average survival period for mice infected with M. bovis decreased from 35 to 19 days.

Friend et al. (1983a, 1983b) did not observe reactivation of a latent Herpes Simplex Virus-1 (HSV-1, KOS strain) in mice treated with T-2. Mice were infected with HSV-1 (6.0×10^7 PFU/ml) via application to scarified lips. Human anti-HSV-1 antibody was given to the infected animals at 3, 48 and 95 hours post-infection. The antibody was given in an effort to minimize mouse immunity to HSV-1 but still allow development of latency. Ten weeks after HSV-1 infection T-2 treatment was begun (5, 10 or 20 ppm).

Diacetoxyscirpenol treatment resulted in increased mortality of mice treated with Candida albicans (Fromentin et al., 1980). The mice were treated with either 1.12 or 2.25 mg/kg DAS ip. Inoculation of C. albicans was done on day 0 with DAS injections given on days 3, 4, 7, 8 and 9. Deaths at both dose levels were greater than in controls.

Otokawa et al. (1979) used spleen cells from mice injected with 10^9 SRBC (iv) as a source of DTH suppressor cells. The suppressor cells, collected 4 days after SRBC injection, were transferred (iv) to syngeneic recipients pretreated with cyclophosphamide. When the suppressor cells were obtained from mice that had been treated with 3 or 4 mg/kg T-2, the DTH response of recipients was decreased but was significantly less reduced than that observed in animals that had received cells from control animals. This experiment demonstrated the negative effect of T-2 on the generation of suppressor cells. Rosenstein et al. (1981), in a similar experiment, evaluated the effects of spleen cells from T-2 treated animals on the generation of -SRBC plaque forming cells. Animals were treated with 0.75 mg/kg/day ip for 7 days and their splenic cells injected into syngeneic recipients. Splenic cell recipients were immunized with SRBC and the PFC response enumerated 5 days later. The result of this experiment indicated that the PFC response was reduced in recipients following injection of splenic cells from animals that had been treated with T-2. Treatment of mice with T-2 alone was enough to increase a suppressor-type cell activity within the spleen.

The results of the preceding two experiments may appear contradictory. In the first T-2 decreased the activity of suppressor cells and in the latter suppressor cell activity was enhanced. The

suppressor cells involved in DTH responses have been reported to be a distinct population of cells (Whistler and Stobo, 1978). In a recent review, Ozer (1985) discusses the concept of positive and negative regulatory cell functions being affected by cyclophosphamide.

Masuda et al. (1982a, 1982b) separated membrane immunoglobulin positive (Ig^+) and negative (Ig^-) cells from the spleens of animals that had been treated with 50 ug of fusarenon-X ip daily for 7 days. Culturing various combinations of Ig^+ and Ig^- cells from fusarenon-X treated and non-treated animals revealed that Ig^- fusarenon-X treated cells decreased the number of intracytoplasmic Ig^+ cells (plasma cells) formed in the presence of PWM or LPS. (Membrane Ig^+ cells are transformed into intracytoplasmic Ig^+ cells.) It was determined that the suppressive Ig^- cells were best characterized as non-lymphocytic cells. This observation was supported by the finding that the addition of fusarenon-X treated T cells did not disrupt the formation of intracytoplasmic Ig^+ cells. However, the addition of fusarenon-X treated non-lymphocytic cells depressed the formation of intracytoplasmic Ig^+ cells. The non-lymphocytic cells were said to be composed of both granular (neutrophil) and non-granular (macrophage) cells.

Poultry. The negative effects of T-2 on tissue components of the avian immune system have been recognized for several years. Wyatt et al. (1973) reported on the toxicity of T-2 to broiler chickens. In their report, a decreased weight for the bursa of Fabricius and spleen were observed when birds were fed diets containing 8 ppm or 4 ppm T-2, respectively. Similar effects were not observed in poults maintained on a 10 ppm T-2 diet for 4 weeks (Richard et al., 1978). The authors did

not record decreases in bursa of Fabricius or spleen weights, although the thymus was reduced in size for birds exposed to T-2. Additionally, microscopic evaluation of thymic tissue revealed cortical depletion of lymphocytes; accumulation of macrophages was observed in the depleted areas. The medullary regions of the thymus showed loss of large lymphocytes and proliferation of epithelial and reticular cells. In the same study, birds (both chicks and poults) were immunized with Pasteurella multocida on day 11 and their antibody titers evaluated at the end of the 4-week treatment. The authors reported no differences in titres between treated and control animals. However, the mean and maximum titre values of poults treated with T-2 were greater than the control values. Electrophoresed proteins-total protein, albumin, total globulin, and α-, β-, and γ-globulin levels were significantly greater in the serum of treated poults.

Lymphoid necrosis and depletion of lymphocytes from the spleen, bursa of Fabricius, cecal tonsil, thymus, and ectopic lymphoid foci were reported in chickens fed diets containing $\geq$ 50 ppm T-2 or DAS for a total of days (Hoerr et al., 1982a). Daily dosing of chickens for 14 days with T-2 (1.5, 2.0, 2.5, or 3.0 mg/kg body weight) or DAS (2.5, 3.0, or 3.5 mg/kg body weight) produced similar histopathologic changes as reported above (Hoerr et al., 1982b). The authors summarized that T-2 was more detrimental than DAS to the lymphoid tissues of chickens. Reduction in splenic and bursa of Fabricius weights was related to toxin doses.

The effects of the trichothecene mycotoxins, T-2 and DAS, have been reported as acute and transient in chickens given a single dose of either toxin (Hoerr et al., 1981). Necrosis of lymphoid tissue and bone marrow

was observable 1 hour after a single treatment of T-2 (2.5 mg/kg body weight) and was followed by rapid cell depletion. Complete restoration of typical lymphoid tissue cellularity was seen 72 h after dosing. Again, as stated above, the authors concluded that T-2 was more potent in the disruption of lymphoid tissues.

Boonchuvit et al. (1975) infected T-2 treated chickens with pathogenic bacteria and observed increased mortality as compared to birds not treated with T-2. Neither factor alone caused mortality, but in combination there was a significant mortality in all groups. The birds were administered T-2 (16 ug/g feed) for 3 weeks. The increased mortality was not associated with differences in specific anti-bacterial antibody titer levels. T-2 alone decreased the weights of spleens and bursas. The spleens of infected birds treated with T-2 increased in weight. This phenomenon was not observed in the bursa of Fabricius.

Mallard ducks exhibited changes in lymphoid tissues similar to those discussed for poultry: generalized atrophy of all lymphoid tissue (Hayes and Wobeser, 1983). The birds were fed diets containing 20-30 ppm T-2 for 2-3 weeks. Cortical depletion of lymphocytes was seen in thymic tissues. The bursas of Fabricius were atrophic, exhibiting marked depletion of follicular lymphoblasts and mature lymphocytes. Lymphocytes in splenic white pulp were also depleted.

A detailed microscopic investigation of trichothecene mycotoxin-induced cellular changes was conducted by Terao et al. (1978). The effects of T-2, fusarenon-X, and nivalenol on the bursa of Fabricius dissected from day old chicks were studied independently. The toxins, 5 mg/kg body weight, were injected into the residual yolk sac. Histologic

changes were monitored over time using both light and transmission electron-microscopy techniques. Both fusarenon-X and nivalenol were less potent than T-2. Within 15 min after injecting T-2 pinocytotic vesicles, follicle-associated deck epithelial cells increased in size and number. Considerable cellular disruption was observable after 30-60 min in the central portion of deck epithelium. Large cytoplasmic autophagic vacuoles containing cell debris were seen. By 30 min lipid droplets were noted in the cytoplasm of lymphoid cells adjacent to degenerated deck epithelial cells. No pathological changes were observed in the reticular epithelial cells juxtaposed with affected lymphoid cells. Macrophages containing cell remnants were occasionally seen. The disk-like formations on deck epithelium completely disappeared by 6 h after injection of T-2. The authors noted the relative insensitivity of the surface epithelium of the bursa of Fabricius. Additionally, the authors calculated that trichothecene toxins are 40 times more toxic than cyclophosphamide to bursal lymphoid cells.

Anti-trichothecene Mycotoxin Antibodies

It is doubtful that anti-trichothecene antibodies are produced in vivo as a result of exposure to the powerful cytotoxic agents. Their capacity to inhibit macromolecular synthesis is well documented. Chu et al. (1982) discussed and demonstrated carrier facilitation of normally non-immunogenic mycotoxin haptens. Indeed, Chu et al. (1979) have produced an anti-T-2 antibody using hemisuccinate as a carrier. Hunter et al. (1985) demonstrated the toxicity of conjugated T-2-bovine serum albumin on human B lymphoblastoid cells. The authors selected a

concentration of the hapten-carrier used in standard immunizing regimes. T-2 concentrations as low as 10 ng/ml caused profound inhibition of protein synthesis. The utility of the anti-T-2 antibody as a tool for the detection of T-2 has been demonstrated (Peters et al., 1982; Fontelo et al., 1983).

CHAPTER III

RELATIONSHIP OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS WITH THE IMMUNOMODULATORY ACTIVITY OF T-2 TOXIN, A TRICHOTHECENE MYCOTOXIN

Introduction

T-2 toxin (4,15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-trichothec-9-en-3-ol) is a trichothecene mycotoxin produced by several Fusarium species. Trichothecene mycotoxins, known to be immunomodulatory compounds, have both stimulatory and inhibitory effects on the immune system (Taylor et al., 1987b). Naturally occurring trichothecene mycotoxins and their derivatives are currently being assessed in phase II anti-cancer trials in the United States. Their success, however, has not been remarkable (Schoental, 1985).

Trichothecene mycotoxins have been categorized as irritants by many researchers. A common detection assay for trichothecenes has been the elicitation of a dermal inflammatory response (Hayes and Schiefer, 1979). Gastritis has been a common observation following trichothecene intoxication. Schoental et al. (1979) observed the development of various gastrointestinal tract lesions following T-2 exposure. The lesions consisted of hyperkaratosis and hyperplasia of the squamous epithelium of the esophagus and stomach with ulceration and submucosal edema.

It is possible that much of the immunomodulatory activity attributed to T-2 may, to some extent, be associated with an upset in homeostasis. T-2 gastric mucosal damage may predispose an animal to increased levels of endotoxin. Endotoxin has pronounced effects on both the immune and hematopoetic system (Kabir et al., 1978) and elicits a stress response in laboratory animals (Carroll et al., 1969). Fig. I-2 (page 4) presents a proposed hypothesis describing several events that may be associated with the immunomodulatory activity of T-2. Oral exposure to T-2 may damage the gastrointestinal tract mucosa predisposing an animal to the ensuing events of inflammation (e.g., an increase in corticosteroids), as well as increased levels of systemic endotoxin. Continual intermittant insult by T-2 may maintain increased corticosteroid production. Corticosteroids are known to cause thymic involution (Claman, 1972) leading, over time, to a potential imbalance in the T-cell pool ultimately manifested in the modulation of immune response. As indicated in Fig. I-2, T-2 may exert a direct effect on immune response as suggested by the results of several in vitro studies (Taylor et al., 1987a; Forsell et al., 1985; DiNinno et al., 1985; Gyongyossy-Issa and Khachatourians, 1985). The present investigation was designed to evaluate the proposed hypothesis that immunomodulation after T-2 exposure may be associated with activation of the hypothalamic-pituitary-adrenal axis.

Methods

Male CD-1 mice (virus antigen free), mean weight 20 g, were purchased from Charles River Laboratories (Wilmington, MA) and housed in the AAALAC-accredited Laboratory Animal Research Center at Utah State

University. The animals were randomly sorted and housed 5-6 per cage on corncob bedding and allowed ad libitum access to feed (Wayne Lab Blocks, Chicago, IL) and water. The environmental conditions were a 12 h light:dark cycle, ambient temperature of 22 $\pm 1^{o}C$, and 50% humidity. The animals were handled frequently, as body weight was recorded and animals treated every other day. Animals were euthanized by carbon dioxide asphyxiation, an approved method for the euthanasia of small animals (National Institutes of Health, 1985).

T-2 Toxin Preparation and Administration

T-2 (Romer Labs, Washington, MO) of 95%+ purity was dissolved in ethanol and suspended in corn oil, producing a stock solution of T-2 in a 4% ethanol:corn oil suspension. The stock was aliquoted and stored at $-20^{o}C$ until needed. Upon thawing, the stock was diluted in 4% ethanol:corn oil. The dose volume was 0.05 ml/10g body weight; T-2 dose levels have been indicated in the Results section. Animals receiving more than one exposure to T-2 were dosed every other day. The appropriate vehicle was administered to control animals. Dose volumes were adjusted with body weight changes.

Blood Collection, Preparation, and Storage

Following the sacrifice of animals, blood samples were taken from the inferior vena cava. An aliquot was transferred to appropriate media for assaying blood-borne bacteria. Blood for endotoxin analysis was allowed to clot in endotoxin-decontamined (as described below) glass tubes and the serum removed and stored at $-80^{o}C$ until analyzed. Plasma for adrenal corticotropic hormone (ACTH) determinations was prepared

using EDTA and stored at -80°C. Serum was used for measurement of corticosterone collected from clotted blood and stored at -80°C.

Histopathology

After gross examination, selected organs, i.e. the spleen, thymus, liver, adrenal gland, stomach, intestine, and cecum, were dissected and fixed in 10% formalin phosphate buffered solution. The tissues were later blocked, sectioned, and stained with Hemotoxylin and Eosin for routine pathological examination. The stomachs were also stained with either Brown and Brenn or Kossa's (Luna, 1968) to discern the presence of gram positive and negative bacteria or calcium deposition, respectively.

Blood Culture

Blood aliquots were incubated in brain heart infusion broth (BHI, Difco, Detroit, MI) for 18 h (37°C). After the initial incubation, blood-agar plates (5% defibrinated sheep red blood cells in trypticase soy agar, BBL, Cockeysville, MD) were inoculated with the initial BHI cultures. The plates were again incubated (37°C, 24 h) and observed for the presence of bacterial colonies. If colonies were detected they were isolated, stained with Gram stain, and evaluated.

Corticosterone and Plasma
Adrenocorticotropic Hormone (ACTH)

Corticosterone, the major gluocorticoid in the mouse (Gorbman et al., 1982), was measured using a commercial radioimmunoassay kit (radioassay Systems Laboratories, Inc., Carson, CA). The assay was linear over the concentration range 0.05-2.0 ng/ml corticosterone. There

was a 6.1% cross reactivity with deoxycorticosterone; however, there was essentially no cross reactivity with other endogenous corticoids. Adrenocorticotropic hormone, an anterior pituitary trophic hormone which stimulates adrenal hormone production, was also measured using a radioimmunoassay kit (Radioassay Systems Laboratories, Inc.).

Serum Endotoxin

The presence of serum endotoxin was determined for using the *Limulus* amebocyte lysate test kit (Cape Cod, Inc., Woods Hole, MA). The microtechnique described by Melvaer and Fystro (1982) was used. All glassware were decontaminated by heating to 250°C for 3 h. Serum samples were diluted 1:10 in pyrogen-free water. The diluted samples were immersed into a waterbath (97°C) for 60 s. Following heating, a 20 ul aliquot of sample and an equal volume of Pyrotell® were mixed in the individual well rings of microtest tissue plate lids (Falcon Plastics, Oxnard, CA). The lids were then incubated for 60 min at 37°C in a humidified chamber. Endotoxin was determined to be present by the formation of a gel.

Hypothalamic Catecholamines and Indolamines

The hypothalamus was dissected (Glowinski and Iverson, 1966) and associated catecholamines and metabolites analyzed according to reported methods (Mayer and Shoup, 1983) utilized in this laboratory (Coulombe and Sharma, 1985). The following neurotransmitters and metabolites were measured: dopamine (DA), norepinephrine (NE), homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxymandelic acid (VMA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA).

Statistical Analysis

Data were analyzed utilizing either one- or two-way ANOVA designs (Dixon and Massey, 1969). The analysis of variance for corticosterone data was based upon $\log_{10}$ transformation. An F statistic was chosen to test the hypothesis of equality among group means. Individual means were compared using the least significant difference (LSD) test.

Results

Acute Response to T-2 Toxin

Fig. III-1 presents the observed relationships between endotoxemia, corticosterone, and ACTH in mice 24 h after T-2 treatment. Endotoxemia was evident 24 h after a single acute oral exposure to T-2 (2.5-20 mg/kg body weight). The apparent increases in adrenocorticotropic hormone levels, the pituitary signal for increased adrenal steroid production, did not differ significantly from control values. The level of corticosterone increased significantly, paralleling the increased incidence of endotoxemia in T-2 treated mice.

Subchronic Toxicity Evaluation of T-2 Toxin

The weight of mice exposed to $\leq$2.5 mg/kg body weight T-2 was similar to that of control animals following 1, 2 or 4 week exposure (Table III-1). Liver weights increased following exposure to 2.5 mg/kg T-2 after 1, 2 and 4 weeks. Thymus weights decreased after 1 and 2 weeks exposure to 2.5 mg/kg T-2. The weight of the spleen increased after 2- and 4-week

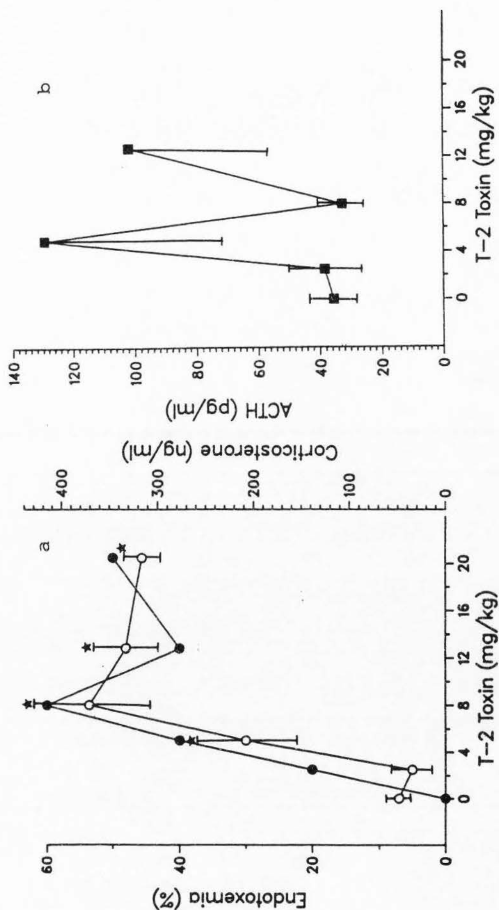


FIG. III-1. (a) Relationship between endotoxemia (●), corticosterone (○), (b) and ACTH 24 h after a single oral exposure to T-2 toxin. Endotoxemia is expressed as the percent animals with detectable endotoxin. Corticosterone and ACTH levels are indicated by mean ± S.E.M., n=5. Values marked with an asterisk (*) differ significantly from appropriate control ($p<0.01$).

TABLE III-1

BODY AND ORGAN WEIGHTS OF ANIMALS EXPOSED TO T-2 TOXIN FOR 1, 2 OR 4 WEEKS[a]

		Body Weight (g)		g Organ/100g Body Weight			
Time (wk)	T-2 Toxin (mg/kg)	Initial	Final	Liver	Adrenal	Thymus	Spleen
1	0	27.4±1.4	29.5±0.7	6.22±0.32	0.034±0.008	0.17±0.02	0.29±0.06
	0.1	27.4±2.1	28.9±2.3	6.05±0.40	0.028±0.004	0.17±0.04	0.33±0.06
	0.5	28.1±1.1	29.9±0.9	6.23±0.49	0.023±0.004	0.16±0.04	0.34±0.04
	2.5	27.3±1.3	29.1±1.9	6.96±0.42*	0.030±0.005	0.12±0.04*	0.37±0.06
2	0	28.9±1.0	31.4±2.8	6.12±0.46	0.020±0.004	0.13±0.02	0.31±0.03
	0.1	27.6±2.3	32.5±0.4	6.28±0.52	0.020±0.003	0.14±0.02	0.33±0.06
	0.5	29.2±1.8	32.9±5.3	6.06±0.33	0.019±0.002	0.13±0.02	0.34±0.04
	2.5	26.8±3.0	29.4±4.8	6.82±0.49*	0.021±0.003	0.10±0.05*	0.52±0.18*
4	0	27.4±3.1	33.3±5.5	5.69±0.43	0.021±0.007	0.12±0.02	0.29±0.05
	0.1	27.6±2.3	31.9±3.6	5.81±0.48	0.022±0.004	0.11±0.02	0.32±0.03
	0.5	27.4±2.7	33.4±3.8	5.74±0.38	0.021±0.001	0.12±0.02	0.33±0.05
	2.5	28.8±1.7	32.8±2.3	6.47±0.38*	0.019±0.001	0.10±0.03	0.44±0.14*

[a]mean ± S.E.M., n=6

*Values differ significantly from appropriate control ($p<0.05$)

exposure to 2.5 mg/kg T-2. The weight of the adrenal gland was not influenced by T-2.

Histopathology of Select Organs

After a 2-week exposure to 2.5 mg/kg T-2, the mucosal lining of the gastric cardia was proliferative and hyperkaratotic. The lesions were observed grossly (Fig. III-2) and appeared to originate in the proximal portion of the cardia as the lesions diminished toward the limiting ridge (i.e., that zone demarking the nonglandular cardiac from the glandular pyloric regions). The pronounced proliferative and hyperkaratotic response, seen in the cardiac region of T-2 treated mice, is demonstrated in Fig. III-3. Ulcerated areas juxtaposed with the aforementioned gastric pathologies were observed (Fig. III-3). The ulcerated areas which were partially calcified were located in the proximal portion of the cardia. An influx of mononuclear cells into the ulcerated areas was observed. The glandular area of the stomach appeared resiliant to T-2 as did the duodenum and cecum. The gastric pathology was not evident after one week, nor did exposure to < 2.5 mg/kg T-2 cause such change. Staining the forestomach with Brown and Brenn (gram specific) indicated the presence of bacteria. Fig. III-4 illustrates the increased number of bacteria (both gram negative and positive) within the ulcerated areas of T-2 treated animals. Cortical depletion was observed in the thymus of mice treated with 2.5 mg/kg T-2 (Fig. III-5). The change in cellularity was apparent after 1- and 2-weeks exposure to T-2. No histopathologic lesions were observed in either the cortical or medullary regions of the

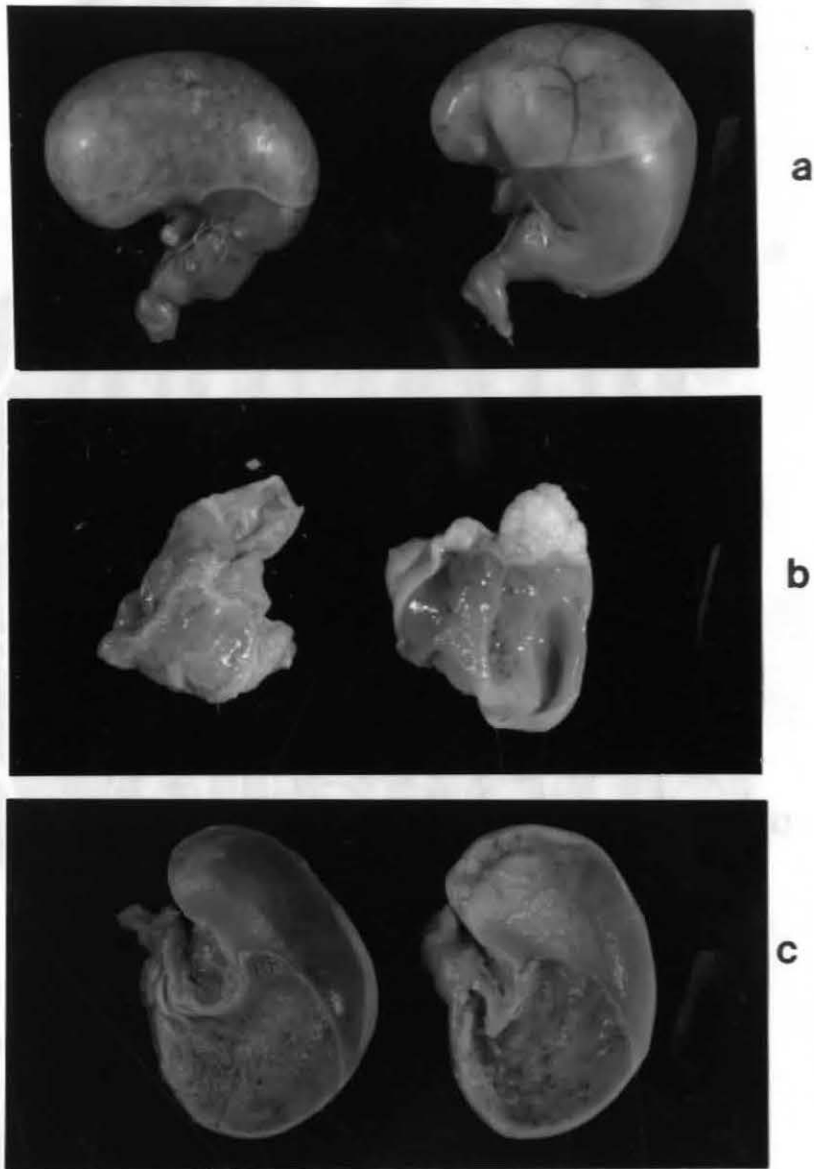


FIG. III-2. Stomachs from vehicle-(left) and T-2 toxin-(right) treated mice (a). Note the affected proximal region of the cardia from T-2 toxin-treated animal. Epithelial lesion seen in fresh (b) and formalin fixed (c) specimens.

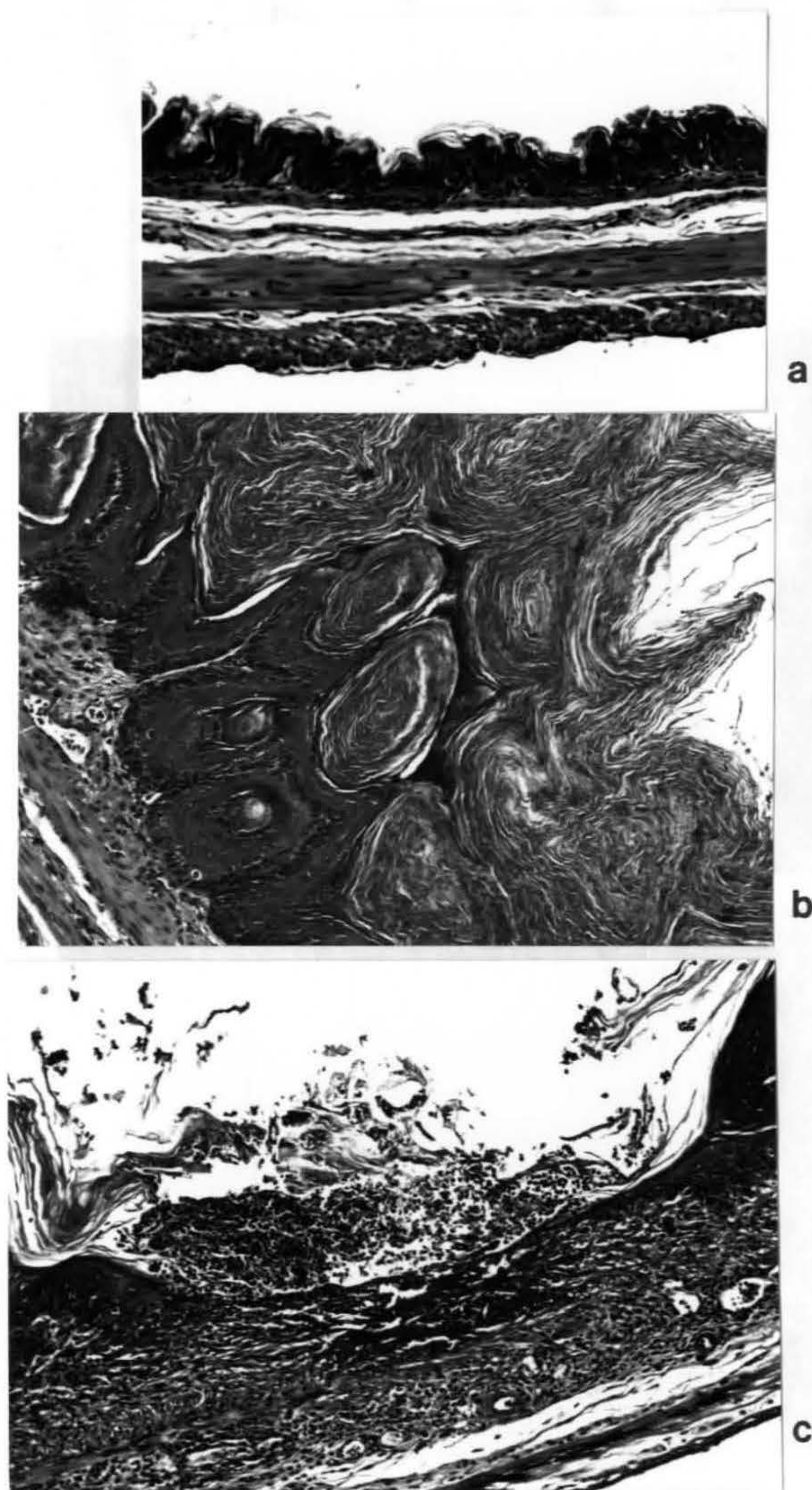


FIG. III-3. Gastric mucosa from vehicle-(a) and T-2 toxin-(b) treated mice. Note epithelial proliferation and hyperkeratinization in T-2 toxin-treated animal. The ulcer is from the proximal region of the cardia (c). H&E, x 31.25.

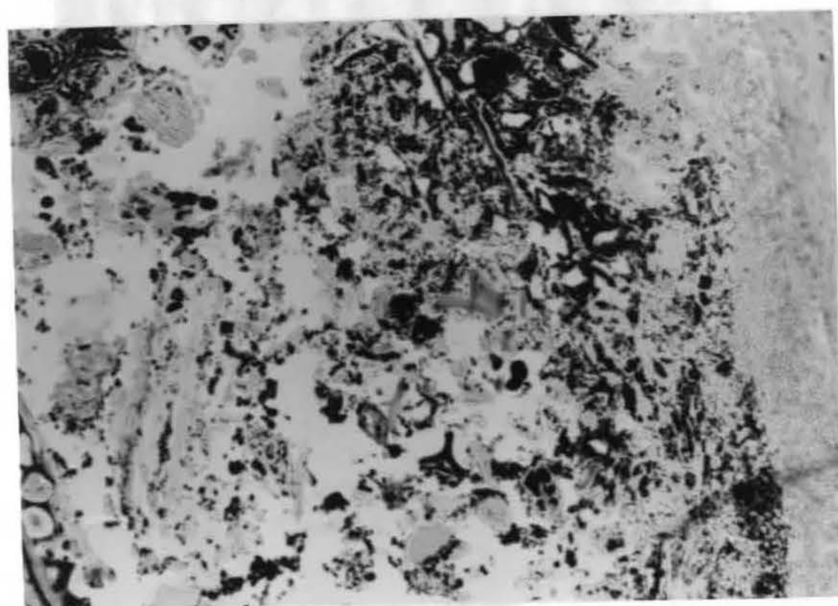


FIG. III-4. Gastric bacteria associated with the mucosa of vehicle-(a) and T-2 toxin-(b) treated mice. The section, stained with Brown and Brenn, from a toxin-treated animal, was from an ulcerated area. x 62.5.

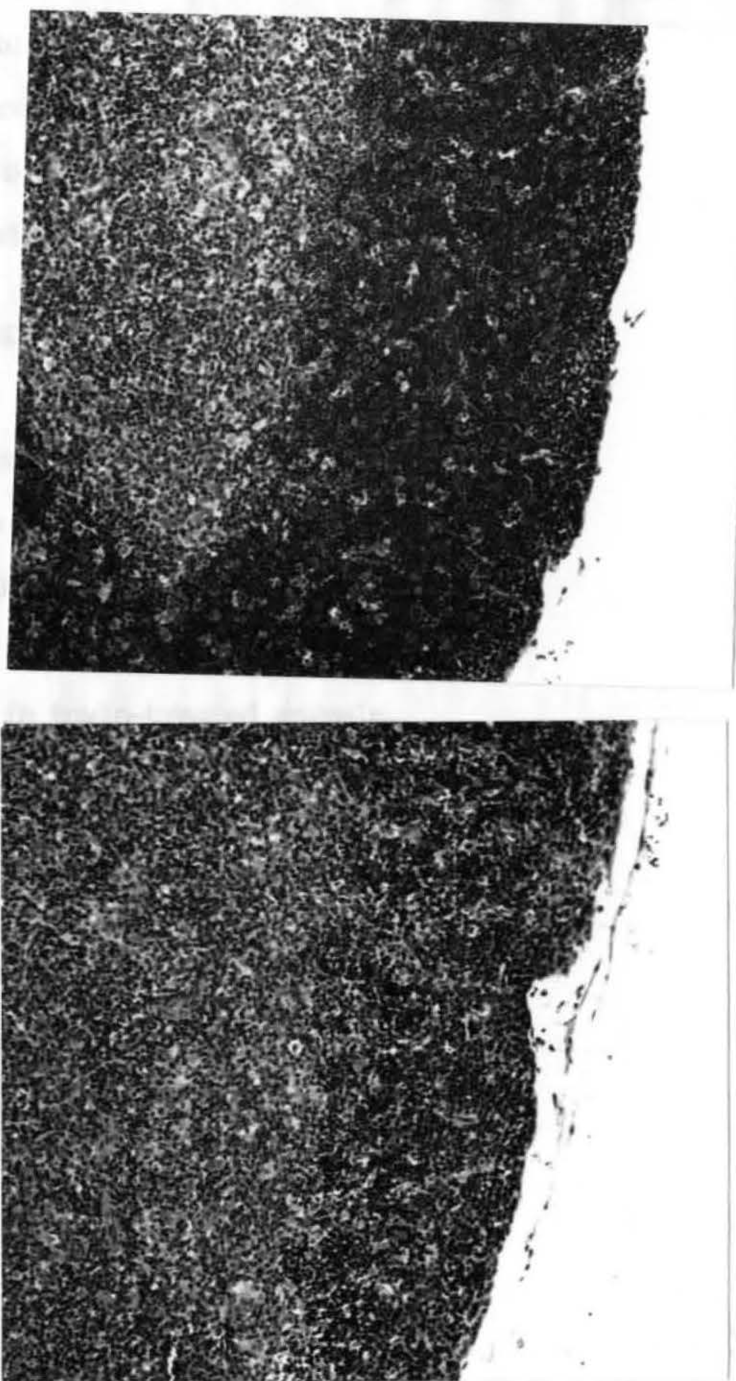


FIG. III-5. Thymus from vehicle-(a) and T-2 toxin-(b) treated mice. Note lymphoid depletion of cortical area in T-2 toxin-treated animal. H& E x 31.25.

adrenal glands. The splenomegaly, observed in animals exposed to 2.5 mg/kg T-2, was associated with red pulp changes (Fig. III-6). There were no observable differences in white pulp areas. The red pulp changes included proliferation of myeloid components, including erythroid and granulocytic precursors. No histopathological changes were detected in the enlarged livers of animals exposed to 2.5 mg/kg T-2.

Blood Bacteria Culture

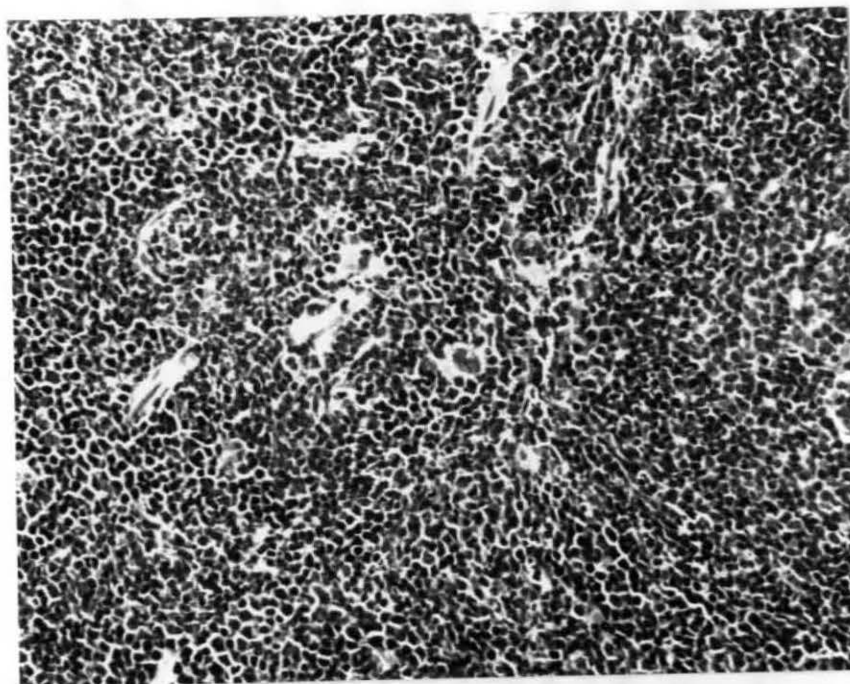
While blood-borne bacteria were occasionally detected, no relationship was discerned between their presence and toxin exposure. However, as indicated in Table III-2, endotoxin, a cell-wall component of gram negative bacteria, was detected in the serum of animals which received 2.5 mg/kg T-2 for 1 week. Endotoxin was not detectable after 2 or 4 weeks in toxin-treated animals.

Hypothalamic Neurotransmitter and Metabolite Levels

Hypothalamic norepinephrine levels were elevated after 2 weeks of repeated exposure to T-2 (Fig. III-7). The increase was dose-dependent and indicated a statistical significance at 2.5 mg/kg T-2. No significant changes were noted in other transmitter or metabolite levels (Table III-3).

Serum Corticosterone

After two weeks, serum corticosterone levels in toxin-treated animals increased with increasing levels of T-2 exposure (Fig. III-8). After four weeks treatment with T-2, serum cortiocosterone levels remained elevated.



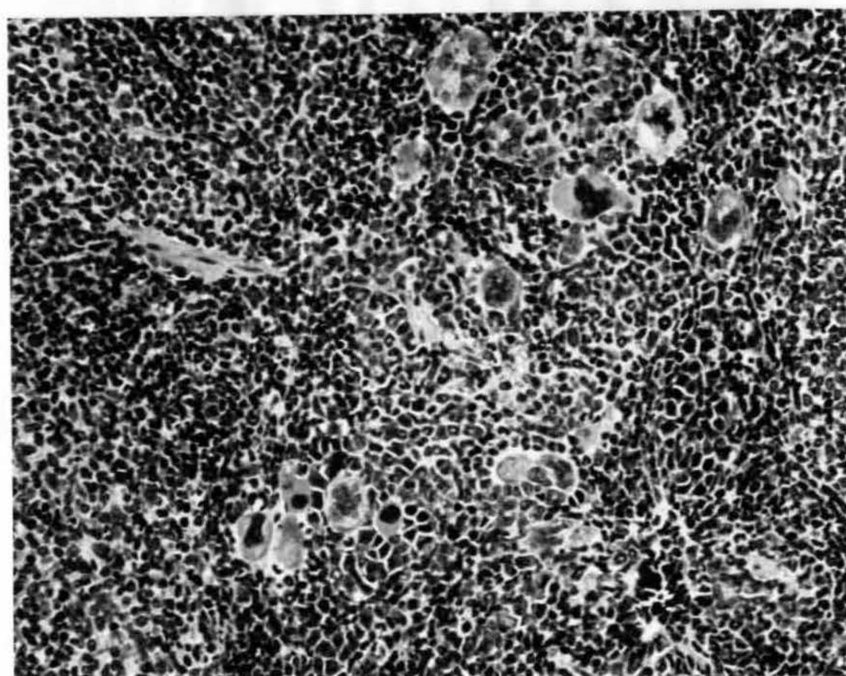


FIG. III-6. Spleen from vehicle-(a) and T-2 toxin-treated (b) mice. Note proliferation of red pulp myeloid elements in T-2 toxin-treated animal. H&E x 62.5.

TABLE III-2

ENDOTOXEMIA IN T-2 TOXIN-TREATED ANIMALS[a]

	T-2 Toxin mg/kg			
Time (wk)	0	0.1	0.5	2.5
1	1/5	0/5	0/5	4/5
2	0/5	0/5	0/5	0/5
4	0/5	0/5	0/5	0/5

[a]The data are expressed as the number of endotoxin positive animals per group, n=5

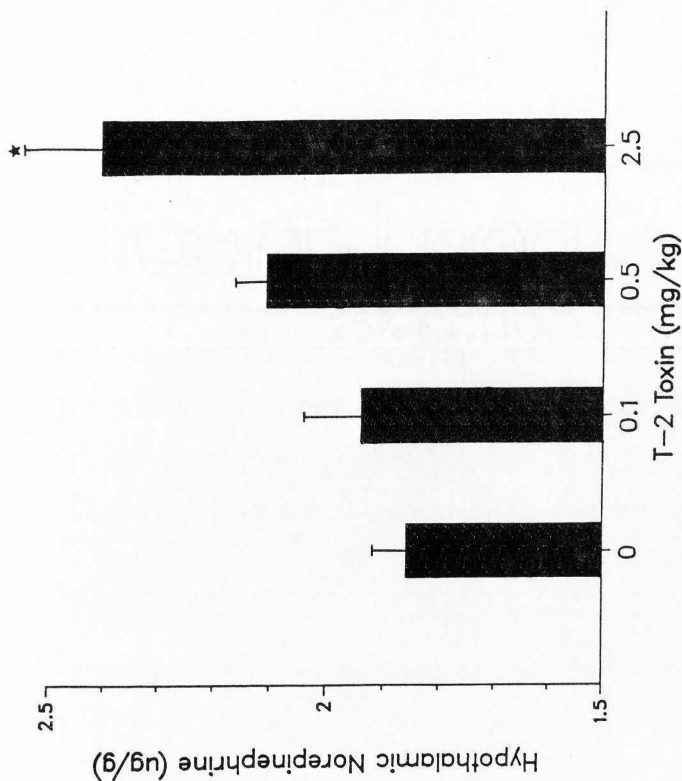


FIG. III-7. Hypothalamic norepinephrine levels. Norepinephrine was determined following two-week exposure to T-2 toxin. Values indicate mean ± S.E.M., asterisk (*) indicates significant difference from control values ($p<0.025$).

TABLE III-3

LEVELS OF VARIOUS BIOGENIC AMINES AND THEIR MAJOR METABOLITES IN THE HYPOTHALAMUS FOLLOWING TWO-WEEK EXPOSURE TO T-2 TOXIN[a,b]

T-2 Toxin mg/kg	DA	DOPAC	HVA	VMA	5-HT	5-HIAA
0	0.46±0.06	0.19±0.05	0.13±0.06	0.15±0.03	0.94±0.18	0.75±0.18
0.1	0.51±0.10	0.20±0.01	0.17±0.60	0.12±0.02	1.21±0.12	0.69±0.04
0.5	0.58±0.10	0.20±0.03	0.19±0.03	0.11±0.02	1.29±0.07	0.65±0.07
2.5	0.53±0.12	0.22±0.06	0.20±0.07	0.12±0.04	1.37±0.17	0.85±0.08

[a]Values are expressed as ug/g wet weight brain tissue, mean ± S.E.M., n=5

[b]See text for explanation of abbreviations

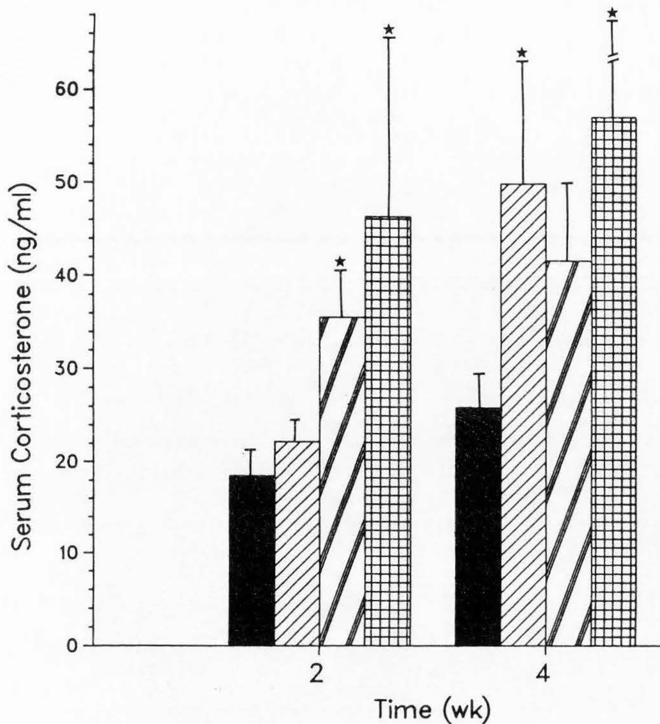


FIG. III-8. Serum corticosterone levels. Serum corticosterone was determined after two and four weeks exposure to T-2 toxin 0 (▬), 0.1 (▨), 0.5 (▨), and 2.5 (⊞) mg/kg body weight. Corticosterone levels are represented by mean ± S.E.M., n=5. Values marked with asterisks (*) differ significantly from appropriate control ($p<0.10$).

Discussion

The present investigation was designed to elucidate potential relationships between T-2 immunomodulation and parameters typically associated with stress. Activation of the hypothalamic-pituitary-adrenal axis is a hallmark of the stress response (Makara et al., 1980). T-2 has previously been reported to elevate serum cortisol (Rafai and Tuboly, 1982) and circulating norepinephrine and epinephrine levels (Lorenzana et al., 1985) in swine. A series of events concomitantly occurring during stress include gastritis, thymic involution, and adrenal cortical hyperfunction (Selye, 1936).

Endotoxin is capable of eliciting a stress response (Carroll et al., 1969). The parallel increase in corticosterone and the incidence of endotoxemia twenty-four hours after exposure to T-2, suggest that the stress response described herein is associated with systemic endotoxemia. Glucocorticoid hormones (e.g. corticosterone) protect animals against bacterial endotoxins (Agarwal and Yoshida, 1983) and dexamethasone, a synthetic analog of cortisol, had a protective effect against endotoxin (Lazar and Agarwal, 1986). Tremel et al. (1985) also reported that dexamethasone significantly reduced lethality from T-2. The cellular changes that were observed in the red pulp areas of the spleen were similar to those described after the injection of endotoxin into mice (McNeill, 1970). Endotoxemia may therefore be a factor in the observed stress-like response.

In the present investigation, both gastric and thymic changes occurred when animals were treated with repeated doses of 2.5 mg/kg T-2.

Prolonged intermittent exposure to 2.5 mg/kg T-2 caused disruption of the non-glandular gastric mucosa with ensuing inflammation. Hormone levels, serum corticosterone and hypothalamic norepinephrine increased. Thymic cortical depletion was observed; murine thymocytes were particularly sensitive to the thymolytic effects of corticosteroids (Claman, 1972). The prolonged stress-like response in mice may depend on irritation of the gastic mucosa; T-2 has been described as an irritant (Hayes and Schiefer, 1979).

As indicated in Fig. I-2 (page 4), T-2 directly affects immunocytes in in vitro assay conditions. Several studies have indicated that T-2 is cytotoxic to lymphocytes in culture (DiNinno et al., 1985; Forsell et al., 1985; and Gyongyossy-Issa and Khachatourians, 1985). Taylor et al. (1987a) demonstrated that T-2 is also immunomodulatory in culture. Toxicologic concentrations of T-2 either stimulated or decreased cell activity depending upon time of toxin exposure or mitogen used.

In an earlier study (Taylor et al., 1985) it was observed that exposure to 2.5 mg/kg T-2 po every third day reduced the response of animals to the particulate T-dependent antigen, sheep red blood cells. T-dependent responses require the cooperation of T-cells (thymus derived) in the developing immune response. Thymic atrophy may predispose an organism to reduced T-dependent responses. T-cell mediated responses are, in general, affected by exposure to T-2. Rosenstein et al. (1979, 1981) reported decreased T-dependent and increased T-independent responses in mice exposed to T-2. Decreased levels of hypothalamic norepinephrine have been associated with increased response to SRBC (Besedovsky et al., 1979a). In vitro α-SRBC antibody production was

inhibited by norepinephrine (Besedovsky et al., 1979b). In the present investigation T-2 treatment was associated with increased hypothalamic norepinephrine.

The manifestation of reduced immune response during stress has been documented (Kelley, 1980; Monjan and Collector, 1977; Keller et al., 1981), though many voids exist regarding specific interactions. It is apparent from the results reported herein that treatment of animals with T-2 was associated with changes in several parameters of the hypothalamic-pituitary-adrenal axis. Activation of the hypothalamic-pituitary-adrenal axis may have been initiated by the development of endotoxemia following T-2 treatment. The role of chemically-induced stress is particularly important in the assessment of both immunopharmacologic and immunotoxicologic effects of chemicals.

CHAPTER IV

IMMUNOMODULATORY ACTIVITY OF T-2 TOXIN IN RELATION TO ADRENAL CORTICOSTEROID ACTIVITY

Introduction

The effects of T-2 on the immune systems of animals have been reviewed (Otokawa, 1983; Taylor et al., 1987a). Thymic involution, characterized by depletion of cells in cortical area, has been associated with T-2 exposure. In general, T-cell activities are sensitive indicators of T-2 immunotoxicity. Prolonged graft survival (Rosenstein et al., 1979), delayed-type hypersensitivity responses either increased (Masuko et al., 1977; Otokawa et al., 1979) or decreased (Taylor et al., 1985), enhanced resistance to listeriosis (Corrier and Ziprin, 1986), and decreased resistance to mycobacterial infection (Kanai and Kondo, 1984) have been reported for mice treated with T-2.

T-2 has been demonstrated to modulate antibody production. Several factors influence the outcome of antibody production of T-2 treated animals. Such factors include the temporal relationship of T-2 treatment and antigen challenge, dose of T-2, the antigen (i.e., T-dependent or independent), and the animal model. Production of α-sheep red blood cell antibody by mice was depressed by daily (Rosenstein et al., 1981) or intermittent (Taylor et al., 1985) T-2 treatment prior to and following antigen challenge. If mice were injected with T-2 2 days

after SRBC challenge, then higher α-SRBC titers were observed (Masuko et al., 1977). Rosenstein et al. (1981) reported increased responses to the T-dependent antigens, dinitrophenylaminoethylcarbomylmethyl-Ficoll or polyvinylpyrrolidone, as a result of T-2 treatment.

Preliminary studies suggested that endocrine imbalance, subsequent to T-2 exposure may be a mitigating factor in the immunomodulatory activity of T-2. Other researchers have indicated either endocrine pathology (Thurman et al., 1986) or hormonal imbalances (Lorenzana et al., 1985; Rafai and Tuboly, 1982) after T-2 treatment. It had been reported that adrenalectomy did not influence the immune response of trichothecene treated mice (Rosenstein et al., 1979). However, as indicated above, involvement of the adrenal gland in the immunomodulatory activity of T-2 appeared likely.

The present investigation was designed to determine whether or not adrenal gland function was associated with the known immunomodulatory effects of T-2. Nonoperated, sham-operated, and adrenalectomized mice were utilized. The responses to both the T-dependent (i.e. SRBC) and T-independent (i.e., DNP-Ficoll) antigens were evaluated. Additionally, we treated animals with metyrapone, a pharmacologic antagonist of corticosterone production (Temple and Liddle, 1970), in an effort to determine the role of corticosterone. Corticosterone is the major glucocorticoid in rodents (Gorbman et al., 1982).

Methods

Animals

Nonoperated, sham-operated, and adrenalectomized male CD-1 mice (virus antigen free), mean weight 20g, were procured from Charles River Laboratories (Wilmington, MA) and housed in the AAALAC-accredited Laboratory Animal Research Center at Utah State University. A minimum of 2 weeks were allowed for recovery from operative procedures. The animals were randomly sorted and housed 5 per cage on corncob bedding and allowed _ad libitum_ access to feed (Wayne Lab Blocks, Chicago, IL) and water; adrenalectomized animals were given 0.85% saline in drinking water. The following environmental conditions were maintained: a 12 hour light:dark cycle, ambient temperature of $22\pm1^{\circ}C$, and 50% humidity. Carbon dioxide asphyxiation was used for euthanasia an approved method for the euthanasia of small animals (National Institutes of Health, 1985). Body temperatures were measured using a small animal rectal probe after an acute exposure to T-2 (Yellow Springs Instrument Co., Yellow Springs, OH).

Toxin Preparation and Administration

T-2, (purchased from Romer Labs, Washington, MO; purity 95%+) was dissolved in ethanol and suspended in corn oil producing a stock solution of T-2 in a 4% ethanol:corn oil suspension. The stock was aliquoted and stored at $-20^{\circ}C$ until needed. The dose volume was 0.05 ml/10g body weight. The 4% ethanol:corn oil suspension was administered to control animals. Dose volumes were adjusted with body weight changes.

Experimental Design

The treatment of animals used to determine the effects of adrenalectomy on T-2 immunomodulation was as follows. The animals were dosed po every other day for 6 days followed by a 2 day break, after which the dose was reduced to 1.25 mg/kg body weight and dosing continued on alternate days for an additional 7 days. The dose was adjusted because of mortality in adrenalectomized animals. The total treatment period was 15 days. In order to maintain equal numbers of animals per treatment, additional adrenalecomized, T-2 treated mice were utilized.

To determine the role of corticosterone in T-2-immunomodulation animals were treated twice daily (po) with 30 mg/kg Metyrapone (Sigma Chemical Co., St. Louis, MO). Metyrapone blocks the hydroxylation of 11-deoxycorticosterone to corticosterone by inhibiting 11β-hydroxylase (Temple and Liddle, 1970). T-2 treatment (2.5 mg/kg) on alternate days, began the same day as did Metyrapone treatment and continued every other day for 15 days.

Antibody Producing Cells-
α-Sheep Red Blood Cell
(α-SRBC) Antibody Producing Cells

Mice were injected ip with 0.25 ml of a 10% (v/v) suspension of defibrinated SRBC preparation. The SRBCs were suspended in sterile physiological saline solution (PSS). The day of antigen sensitization coincided with T-2 treatment on the eleventh day of treatment. Four days later, the time corresponding to peak α-SRBC antibody production, the animals were sacrificed and splenic α-SRBC antibody producing cells were

assayed. The procedure used, involving microchambers for assaying plaque-forming cells, has been described by Cunningham and Szenberg (1968).

<u>α-DNP-Ficoll Antibody Producing Cells</u>

Mice were injected iv with 10 ug of dinitrophenylaminoethylcarbomyl-methyl-Ficoll (DNP-Ficoll, Bio-search Research Chemicals, San Rafael, CA). The DNP-Ficoll was dissolved in PSS, sterilized by filtration, and injected in 100 ul aliquots into a tail vein. Injection of DNP-Ficoll did not coincide with T-2 treatment and was on the tenth day of treatment. The number of α-DNP-Ficoll antibody producing cells was determined 5 days later, a time corresponding to peak α-DNP-Ficoll antibody activity. The procedure used was essentially as has been described for determination of α-SRBC antibody-producing cells; however, the indicator cells were labeled with 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma Chemical Co.) as follows. Sodium-TNBS (64.8 mg) was dissolved in phosphate buffered saline (0.01 M, pH 7.4) and 3 ml of packed SRBC added drop-wise. The mixture was stirred in the dark for 10 min. The TNBS-modified cells were washed 3 times with PSS prior to their use. The immunogenic determinants of the hapten DNP elicited an antibody that was cross-reactive with the determinants of TNB.

<u>Serum -SRBC Antibody Titer</u>

An indirect ELISA method was used to detect α-SRBC titers (Taylor et al., 1985; Voller et al., 1979). Plates were initially coated with sheep red blood cell ghosts 5 ug/ml protein solubilized in 0.2% sodium dodecyl sulfate. Serum samples were diluted 1:200, 1:150 and 1:100 in PSS. Peroxidase-labeled goat-anti-mouse-immunoglobulin (Hyclone Systems, Inc.,

Logan, UT) diluted 1:1000 was used to detect α-SRBC. Results have been expressed as Δ.O.D. at 490 nm, t=15 min. Non-sensitized mouse sera was used as a negative control.

Serum Corticosterone

Serum corticosterone measurements were made using a radioimmunoassay kit developed by Radioassay Systems Laboratories, Inc. (Carson, CA). The assay is linear over the concentration range 0.05-2.0 ng/ml corticosterone. There is a 6.1% cross reactivity with deoxycorticosterone; however, there is no cross reactivity with other endogenous corticoids.

Statistical Analysis

Data were analyzed utilizing various factorial designs (Dixon and Massey, 1969). The analysis of variance for corticosterone data was based upon $\log_{10}$ transformation. An F statistic (p values indicated in the footnotes of tables and figures) was chosen to test the hypothesis of equality among group means. Individual means were compared using the least significant difference (LSD) test.

Results

Hypothermia, evidenced by a dramatic and significant decrease in body temperature, developed rapidly in T-2 treated adrenalectomized animals (Fig. IV-1). Body temperatures decreased (nonsignificant) in T-2 treated non-operated and sham-operated animals also; however, the onset of hypothermia was delayed. A 50 percent mortality was observed for T-2 treated adrenalectomized animals. Mortalities occurred within the

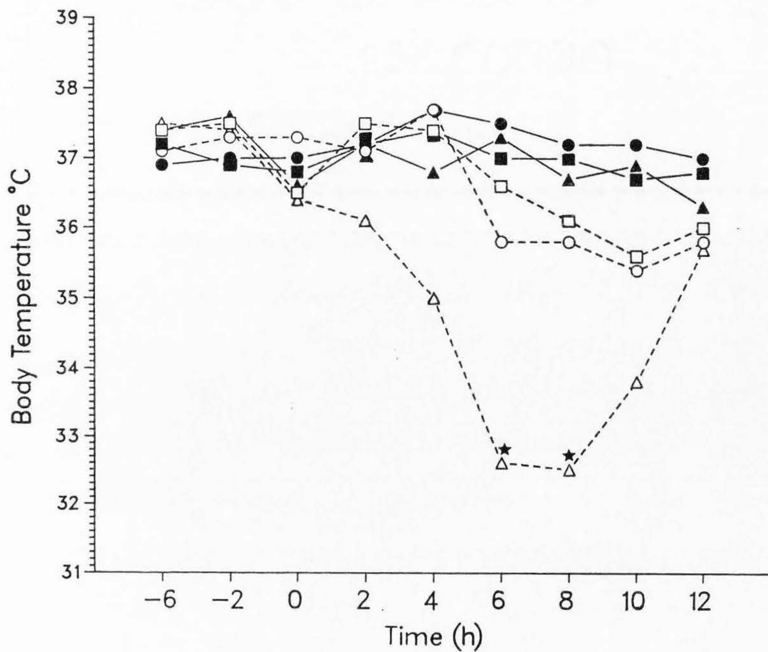


FIG. IV-1. The effect of T-2 toxin on body temperature. Nonoperated (●, ○) sham (■, □), and adrenalectomized (▲, △) mice were treated with vehicle (solid) or 2.5 mg/kg T-2 toxin (open). Values represent mean, n=4. Asterisks (*) indicate significant difference from appropriate control (p<0.05).

first 12 h of treatment. Mortality occurred in T-2 treated nonoperated and sham-operated groups; however, post-morteum examination indicated deaths occurred from improper handling.

Neither T-2 exposure nor surgical manipulation affected body weight gain, expressed as percent change, during the 2-week treatment period (Table IV-1). Both liver and spleen weights increased with T-2 treatment but were unaffected by surgery. The increased spleen weight of T-2 treated adrenalectomized mice was not significantly different from vehicle-treated control. No significant changes in thymus weights were observed. Circulating levels of corticosterone following 2-week treatment with T-2 are illustrated in Fig. IV-2. Adrenalectomy did not completely abolish the presence of corticosterone; however, the level of the hormone was reduced by approximately 50 percent ($p<0.10$) compared to nonoperated control. Corticosterone levels were greater in T-2 treated nonoperated and sham-operated groups than in respective controls. Such an increase in corticosterone was not observed for T-2 treated adrenalectomized animals (Fig. IV-2).

The effects of T-2 treatment on the splenic content of α-SRBC antibody-producing cells is presented in Fig. IV-3. Treatment with T-2 reduced the concentration of plaque-forming cells in nonoperated and sham-operated groups. There was no difference in the α-SRBC responses of vehicle or T-2 treated adrenalectomized animals. The relative spleen weights, number of splenic lymphocytes, and the total number of α-SRBC antibody-producing cells per spleen have been compared in Table IV-2. Spleen weights were not affected by surgery or toxin. The number of

TABLE IV-1

COMPARISONS[a] OF BODY[b] AND ORGAN[c] WEIGHTS OF SURGICALLY ALTERED, T-2 TOXIN-TREATED ANIMALS

TREATMENT	BODY WEIGHT	LIVER	SPLEEN	THYMUS
No Surgery	112.6±1.4	7.30±0.10	0.39±0.04	0.19±0.02
No Surgery + T-2	106.8±1.1	8.16±0.37**	0.53±0.14*	0.17±0.02
Sham	106.2±1.7	6.94±0.22	0.39±0.08	0.23±0.03
Sham + T-2	110.4±2.9	8.32±0.66***	0.63±0.17*	0.21±0.02
Adrenalectomy	111.6±1.8	6.84±0.26	0.36±0.03	0.19±0.03
Adrenalectomy + T-2	109.5±1.9	7.62±0.28*	0.48±0.04	0.17±0.01

[a] Values are mean±S.E.M., n=4
[b] Percent change as compared to first day of treatment
[c] g/100 g body weight
* Indicates significant difference from appropriate control (*, $p<0.05$; **, $p<0.025$; ***, $p<0.01$)

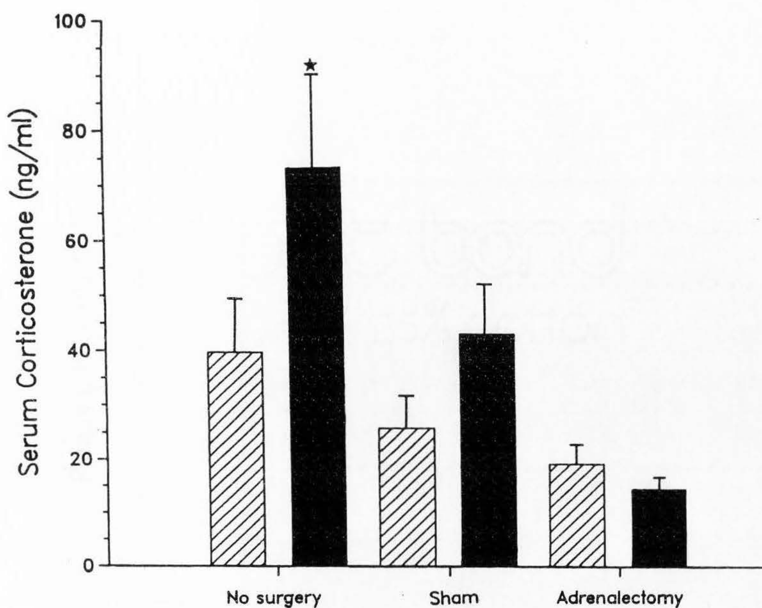


FIG. IV-2. Serum corticosterone levels of mice after two-week treatment with vehicle (▨) or 2.5 mg/kg T-2 toxin (■). Values represent mean ±S.E.M., n=4. Asterisks (*) indicate significant difference from appropriate control (*, $p<0.10$; **, $p<0.05$)

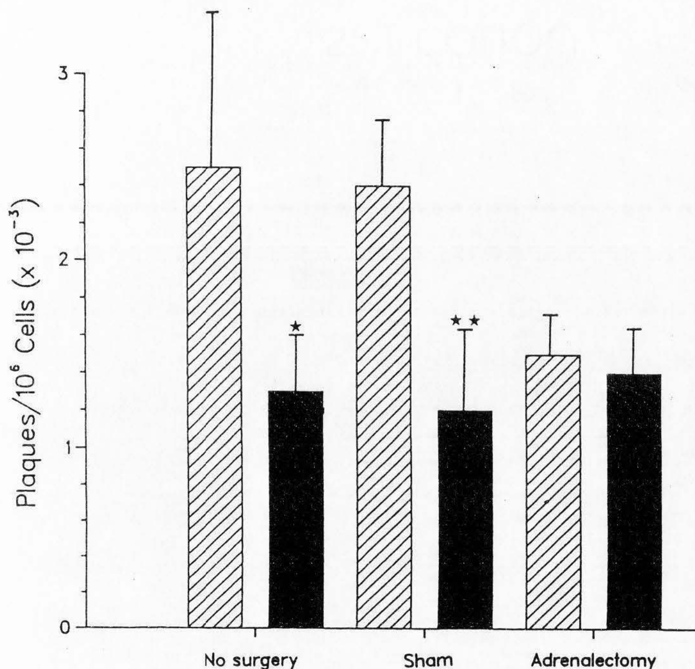


FIG. IV-3. Antibody response to sheep red blood cell challenge after two-week treatment with vehicle (▨) or 2.5 mg/kg T-2 toxin (■). Values represent mean ± S.E.M., n=5. Asterisks (*) indicate significant difference from appropriate control (*, $p<0.10$; **, $p<0.025$).

splenic lymphocytes increased in T-2 treated, sham-operated animals as compared to appropriate control group. No significant changes in the total number of plaques per spleen occurred following the T-2 treatment. The number of plaques per spleen in vehicle-treated adrenalectomized animals was comparable to those of the vehicle-treated nonoperated and sham-operated groups. Serum α-SRBC antibody titer has been compared in Fig. IV-4. The level of antibody decreased (nonsignificant) following T-2 treatment.

The concentrations of α-DNP-Ficoll antibody-producing cells increased in T-2 treated nonoperated and sham-operated groups (Fig. IV-5). As we observed for the α-SRBC response, there was no difference in the concentrations of α-DNP-Ficoll antibody-producing cells obtained from either vehicle- or T-2 treated adrenalectomized animals. The concentration of α-DNP-Ficoll antibody-producing cells was unaffected by adrenalectomy. Table IV-2 compares the relative spleen weights, numbers of splenic lymphocytes, and the total number of α-DNP-Ficoll antibody-producing cells per spleen. Both spleen weight and the number of splenic lymphocytes increased in T-2 treated groups. The number of splenic lymphocytes was the highest in the T-2 treated adrenalectomized group. The total number of plaques per spleen increased following T-2 treatment regardless of the presence or absence of the adrenal gland.

The levels of circulating corticosterone in animals which were sensitized with either SRBC or DNP-Ficoll have been contrasted in Fig. IV-6. Corticosterone levels in T-2 treated, SRBC-challenged groups were lower than in their respective control groups. Adrenalectomy reduced the

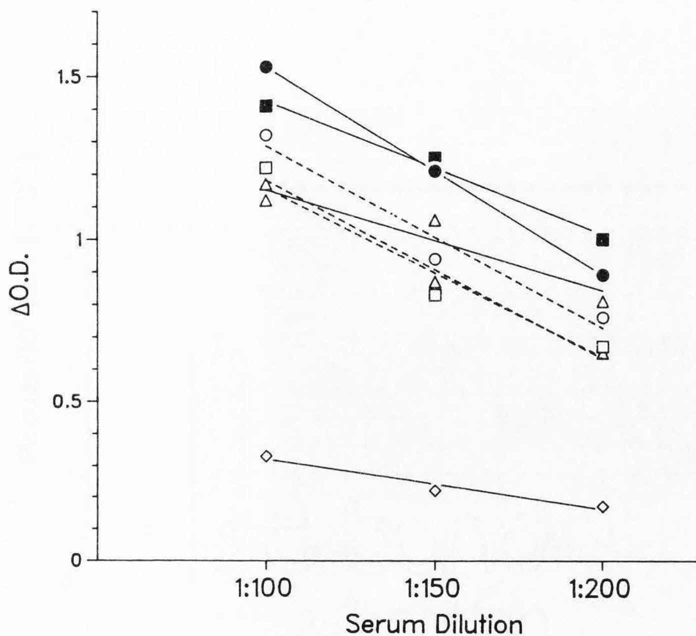


FIG. IV-4. α-Sheep red blood cell antibody titer in nonoperated (●,○) sham-operated (■,□), and adrenalectomized (△,△) mice treated with vehicle (solid) or 2.5 mg/kg T-2 toxin (open). Values represent mean, n=5. Negative control titer (◆).

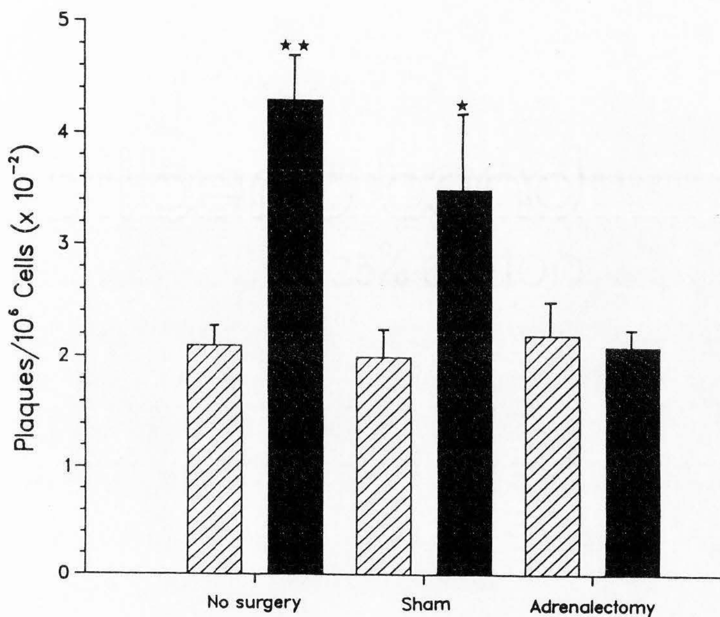


FIG. IV-5. Antibody responses to DNP-Ficoll challenge after two-week treatment with vehicle (▨) or 2.5 mg/kg T-2 toxin (■). Values represent mean ± S.E.M., n=5. Asterisks (*) indicate significant difference from appropriate control (*, $p<0.05$; **, $p<0.01$).

TABLE IV-2

COMPARISONS[a] OF SPLEEN WEIGHTS[b], SPLENIC LYMPHOCYTES[c] AND ANTIBODY-PRODUCING CELLS[d] IN SURGICALLY ALTERED T-2 TOXIN-TREATED, ANTIGEN-CHALLENGED ANIMALS

Treatment	SRBC[e]			DNP-Ficoll[f]		
	Spleen Weight	Splenic Lymphocytes	Plaques/ Spleen	Spleen Weight	Splenic Lymphocytes	Plaques/ Spleen
No Surgery	0.35±0.04	3.17±0.71	7.18±1.95	0.26±0.02	2.08±0.43	0.44±0.12
No Surgery + T-2	0.42±0.04	3.96±0.42	5.66±1.41	0.37±0.02***	3.28±0.30**	1.41±0.27****
Sham	0.39±0.01	2.47±0.24	5.74±0.74	0.32±0.02	2.63±0.30	0.50±0.09
Sham + T-2	0.48±0.06	5.03±1.01****	4.87±0.90	0.40±0.02***	3.74±0.36**	1.19±0.22****
Adrenalectomy	0.51±0.05	4.28±0.42	7.46±1.48	0.33±0.03	2.75±0.17	0.58±0.10
Adrenalectomy + T-2	0.48±0.03	4.95±0.54	6.35±0.84	0.42±0.03****	4.86±0.72*	0.99±0.16*

[a] Values are mean ± S.E.M., n=5
[b] g/100 g body weight
[c] $(x10^{-8})$
[d] Expressed as plaques per spleen $(x10^{-5})$
[e] Sheep red blood cells, see text for antigen preparation
[f] Dinitrophenylaminoethylcarbomyl methyl-Ficoll, see text for antigen preparation
* Indicates significant difference from appropriate control
(*, p<0.10; **, p<0.05; ***, p<0.025; ****, p<0.01).

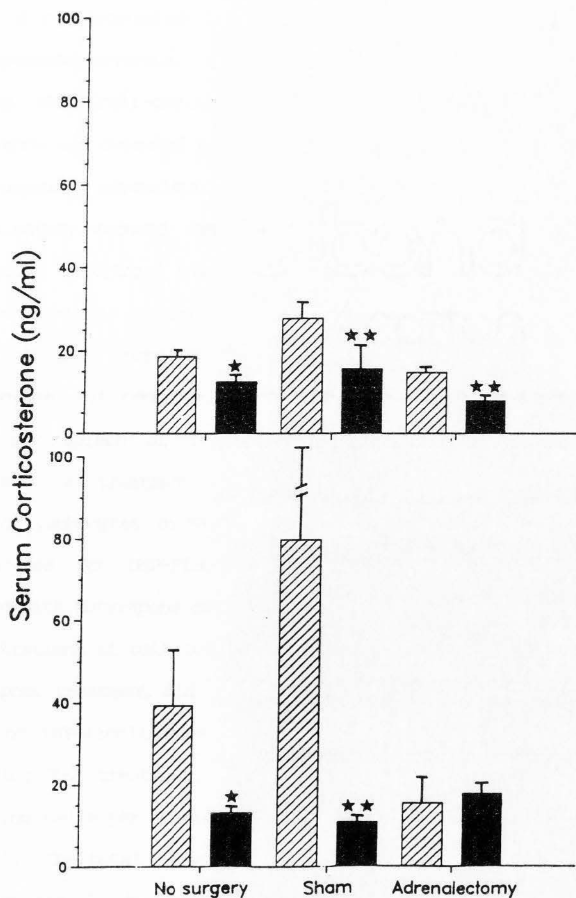


FIG. IV-6. Serum corticosterone levels of antigen challenged mice; (a) sheep red blood cells, (b) DNP-Ficoll. Mice received vehicle (■) or 2.5 mg/kg T-2 toxin (▨) for two weeks. Values represent mean ± S.E.M., n=5. Asterisks (*) indicate significant difference from appropriate control (*, $p<0.05$; **, $p<0.01$).

level of corticosterone in vehicle-treated mice ($p<0.01$) as compared to sham-operated controls. The levels of corticosterone were reduced in T-2 treated, DNP-Ficoll-challenged nonoperated and sham-operated groups. No difference was observed between the corticosterone levels of vehicle- and T-2 treated adrenalectomized animals challenged with DNP-Ficoll. Adrenalectomy reduced the level of corticosterone in vehicle-treated, DNP-Ficoll challenged mice as compared to nonoperated ($p<0.05$) and sham-operated ($p<0.01$) controls.

Table IV-3 compares the relative spleen weights, number of splenic lymphocytes and concentration and total number of antibody-producing cells per spleen of T-2 and Metyrapone-treated antigen-challenged animals. No treatment effects were observed for spleen weights or splenic lymphocytes in SRBC challenged animals. The numbers of splenic lymphocytes for DNP-Ficoll challenged animals decreased in animals treated with Metyrapone or T-2. Metyrapone treatment alone increased the concentrations of both α-SRBC and α-DNP-Ficoll antibody-producing cells. Metyrapone treatment did not alter the effects of T-2; the responses to SRBC and DNP-Ficoll were still decreased and increased, respectively, following T-2 treatment. The total number of α-DNP-Ficoll antibody-producing cells per spleen increased for animals treated with Metyrapone and T-2. The total number of α-SRBC antibody-producing cells per spleen was decreased by T-2 treatment regardless of Metyrapone treatment. The α-SRBC titer (Fig. IV-7) reflects the differences reported for antibody-producing cells. Circulating levels of corticosterone were unaffected by the various treatments (data not shown).

TABLE IV-3

COMPARISONS[a] OF SPLEEN WEIGHTS[b], SPLENIC LYMPHOCYTES[c] AND ANTIBODY-PRODUCING CELLS[d] IN METYRAPONE AND T-2 TOXIN-TREATED, ANTIGEN-CHALLENGED ANIMALS

Treatment	SRBC[e]				DNP-Ficoll[f]			
	Spleen Weight	Splenic Lymphocytes	Plaques/ 10^6 cells	Plaques/ Spleen	Spleen Weight	Splenic Lymphocytes	Plaques/ 10^6 cells	Plaques/ Spleen
No Metyrapone No T-2	0.47±0.02	2.67±0.41	0.91±0.26	2.55±0.94	0.32±0.03	2.01±0.32	3.67±0.25	7.42±1.30
Metyrapone	0.42±0.02	1.88±0.21	1.64±0.31***	3.20±0.90	0.27±0.01	1.45±0.14**	6.45±0.70***	9.53±1.38
T-2	0.51±0.08	2.08±0.38	0.25±0.02*	0.53±0.12*	0.34±0.04	1.08±0.18***	8.64±1.09***	9.67±2.27
Metyrapone + T-2	0.45±0.04	1.96±0.28	0.22±0.08*	0.36±0.11*	0.40±0.03	2.11±0.16	7.90±0.77***	16.45±1.30***

[a] Values are mean ± S.E.M., n=5
[b] g/100 g body weight
[c] ($\times 10^{-8}$)
[d] Expressed as plaques/10^{-6} cells ($\times 10^{-3}$) and as plaques/spleen ($\times 10^{-5}$)
[e] Sheep red blood cells, see text for antigen preparation
[f] Dinitrophenylaminoethylcarbomyl methyl-Ficoll, see text for antigen preparation
* Indicates significant difference from appropriate control
(*, $p<0.10$; **, $p<0.05$; ***, $p<0.01$)

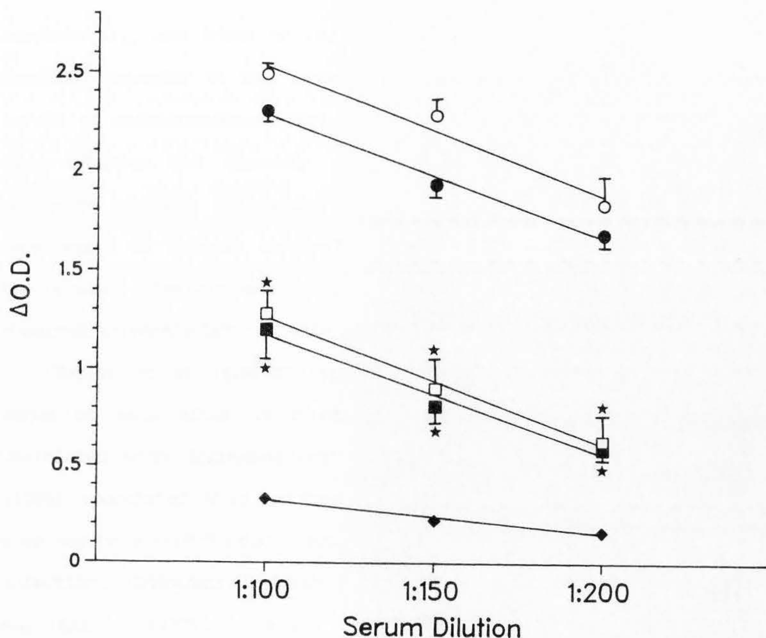


FIG. IV-7. α-Sheep red blood cell antibody titer of Metyrapone treated animals. Animal treatments were: vehicle (●), vehicle and Metyrapone (○),2.5 mg/kg T-2 toxin (■), or T-2 toxin and Metyrapone (□). Values represent mean ± S.E.M., n=5. Asterisks (*) indicate significant difference from control. ($p<0.01$). Negative control titer (◆). See text for Metyrapone treatment.

Discussion

The endocrine system is a regulatory component of the immune system (Ader, 1981; Besedovsky et al., 1977, 1983). Glucocorticoid and catecholamine hormones, products on the adrenal cortex and medulla, respectively, are known to influence the immune response. Single and repeated exposure to T-2 leads to and maintains increased circulating levels of corticosterone (Taylor et al., 1987c). Presently, it is not clear whether T-2 directly affects adrenal tissue or elicits its endocrine response indirectly. Thurman et al. (1986) reported the development of adrenal cortical parenchymal cell necrosis beginning in the X-zone interface and extending peripherally. The phenomenon was observed in female but not male mice.

Taylor et al. (1987b) reported the presence of endotoxin in the serum of mice after 24 h of T-2 treatment. The endotoxemia was correlated with increased corticosterone levels. Corrier and Ziprin (1986) speculated that endotoxin from enteric bacteria of T-2 treated mice may be a contributing factor in T-2 enhanced resistance to Listeria infection. Endotoxin is known to increase glucocorticohormones (Agarwal and Lazar, 1977). Rafai and Tuboly (1982) observed heightened adrenocortical activity in swine fed T-2. The current investigation demonstrated the development of a hypothermic response in T-2 treated animals. The rodent hypothermic response is a characteristic symptom of endotoxemia (van Miert and Ferns, 1968). In the present investigation the adrenal glands were important for animal survival following acute T-2

treatment. Dexamethasone, a synthetic glucocorticoid, reduced acute lethality in T-2 treated rats (Tremel et al., 1985).

In the present study circulating levels of corticosterone increased following T-2 treatment. Adrenalectomy reduced and prevented an increase in corticosterone subsequent to T-2 exposure. Satellite adrenal tissue, partial adrenal regeneration, or cross reactivity of the antibody used in the RIA corticosterone detection system may account for detection of the hormone in adrenalectomized animals. Corticosteroids act directly upon thymocytes, thus altering relative T-cell availabilities. Murine thymocytes are sensitive to the lytic effects of corticosteroids (Claman, 1972). Depletion of cortical thymocytes has often been associated with T-2 exposure. A significant reduction in thymic mass following T-2 exposure did not occur in the present investigation. We suspect that dose reduction, in an effort to maintain adrenalectomized animals, was accountable for the lack of thymic involution. Persistent elevation in circulating corticosterone levels may alter T-cell recruitment from the thymus, consequently leading to imbalance of regulatory (or effector) T cells and their products.

Increased corticosterone levels were associated with decreased T-dependent and increased T-independent responses. Corticosteroid hormones are speculated to have a regulatory role in the development of the antibody response (Besedovsky and Sorkin, 1977). Decreased corticosterone levels were observed in T-2 treated, antigen-challenged animals. The association of immune response with corticosterone levels may be exemplified by the altered antibody responses of T-2 treated nonoperated and sham-operated groups, both of which, independent of

antigen used, had lower corticosterone levels than did vehicle-treated controls. The lack of absolute dependence of the DNP-Ficoll response on corticosterone is illustrated by the low level of corticosterone in DNP-Ficoll challenged vehicle-treated adrenalectomized animals, which had an immune response comparable to vehicle-treated, nonoperated and sham-operated animals.

Metyrapone treatment did not elicit parallel effects when compared to adrenalectomy. The results obtained for T-2 or T-2 and Metyrapone treated animals were similar, in that the responses to SRBC or DNP-Ficoll either decreased or increased, respectively. Metyrapone treatment alone did not affect the corticosterone levels of antigen-challenged mice. The failure of Metyrapone to affect circulating levels of corticosterone may account for the dissimilarity in results obtained following adrenalectomy vs. Metyrapone treatment.

These results support the earlier findings of an association of the adrenal gland with the immunomodulatory activity of T-2. At present we suspect that the major glucocorticohormone of rodents (i.e., corticosterone) is the adrenal product associated with the toxin's immunoactivity. However, adrenomedullary catecholamines may also be contributing factors. The immunopharmacologic contributions of the adrenal hormones in xenobiotic immunotoxicity need greater consideration in future investigations.

CHAPTER V

DOSE AND TIME RELATED EFFECTS OF T-2 TOXIN ON MITOGENIC RESPONSES OF MURINE SPLENIC CELLS *IN VITRO*.

Introduction

T-2 toxin (4, 15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy thrichothec-9-en-3-ol), a trichothecene mycotoxin, has posed hazards to both human and animal health (Joffe, 1978; Ueno, 1980). Taylor et al. (1987b) have summarized the effects of trichothecene mycotoxins on lymphoid tissues and the hemopoietic system. Gross and microscopic findings indicated that T-2 induces atrophy of bone marrow and depletes lymphoid cells from cortical areas of the thymus and T-dependent and independent areas of the spleen. The cytotoxic effects of the trichothecene mycotoxins have been attributed to observed inhibition of macromolecular synthesis (McLaughlin et al., 1977; Ueno, et al., 1973).

Specific immunotoxic effects of trichothecene mycotoxins have been reviewed (Otokawa, 1983; Taylor et al., 1987b). Increased delayed-type hypersensitivity (DTH) responses and reversal of DTH tolerance have been demonstrated in T-2 treated mice (Masuko et al., 1977; Otokawa, et al., 1979). T-2 toxicosis also decreased resistance to mycobacterial and viral infections (Kanai and Kondo, 1984; Friend et al., 1983b). Rosenstein et al. (1979) reported an increased time to rejection of

allografts from mice treated with T-2. In all of the above immunological phenomena, T cells either affect or regulate immune response.

This study investigated the effects of T-2 exposure time, timing of exposure, and concentration on mitogenic responsiveness of splenocytes in vitro. We chose an experimental design such that the effects of T-2 on early and late transformational events could be observed on immunocompetent cells.

Methods

Media

RPMI 1640 (Gibco, Grand Island, NY) was the standard medium used in all experiments. The growth medium was supplemented with 5% heat-inactivated and filtered fetal bovine serum (Hyclone Labs, Logan, UT), 100 units penicillin, and 100 ug streptomycin per ml (Gibco). The medium was buffered with sodium bicarbonate and the final pH was adjusted to 7.4.

Lymphocyte Cultures

Spleens were aseptically excised from male NFS/N mice (21 g body wt.). Tissues were immediately immersed into ice-cold RPMI 1640. Spleens from several animals were pooled for each experiment, teased apart, and aspirated through hypodermic needles of decreasing diameters (18, 21, and 25 gauge) to prepare a single-cell suspension. Connective fascia and cell clumps were separated during settling. Erythrocytes and dead cells were removed from splenic cell preparations by centrifugal separation through Ficoll-Hypaque (Parish et al., 1974). After separation, cells were washed twice in RPMI 1640 and resuspended to a

final concentration of 2.0 x 10^7 viable cells per ml. Cell viability was assessed using the trypan blue exclusion technique of Phillips (1973). Cells (10^6) were seeded into wells of microtest tissue culture plates (Falcon Plastics, Oxnard, CA) and incubated for 48 h at 37°C in 5% CO_2 with or without mitogen as indicated below. Six hours prior to collection of cells, 0.5 uCi of [^{3}H]-thymidine ([^{3}H]-TdR, specific activity 2 Ci/mmol, New England Nuclear, Boston, MA) in 50 ul medium was added to each well. Cells were harvested onto glass microfiber filters and [^{3}H] activity determined via liquid scintillation counting (Model 2660, Packard Instrument Co., Drowners Grove, IL). Mean [^{3}H]-TdR uptake, expressed as DPM/10^6 cells, was based upon quadruplicate well counts.

Mitogen Preparation

The following mitogens were utilized: lipopolysaccharide from E. coli (Sigma Chemical Co., St. Louis, MO), pokeweed mitogen (Gibco), purified phytohaemagglutinin (Wellcome Diagnostics, Beckenham, England), and concanavalin A type IV (Sigma Chemical Co.). Three concentrations of each mitogen were selected based upon previous titration profiles in splenic cell systems. Stock solutions of each mitogen were prepared in PSS, aliquoted, and stored at -20°C until needed. Stock concentrations and dilutions of each were: LPS 200 ug/ml x $2^{-3,-6,-8}$, PWM 100 ul/ml x $2^{-4,-6,-8}$, PHA 50 ug/ml x $2^{-2,-4,\ -5}$, and Con A 100 ug/ml x $2^{-6,-7,-8}$. The greatest concentration of each mitogen produced maximal stimulation of the cells. Mitogens were added in quadruplicate at the initiation of lymphocyte cultures in 50 ul aliquots.

T-2 Toxin Preparation

T-2 (purity 95%+, Myco-Lab Co., Washington, MO) was dissolved in dimethylsulfoxide (DMSO) and diluted in PSS to produce a working stock of T-2 in 1% DMSO and stored at -20°C. Cultured cells were exposed, in quadruplicate, to 10^{-8}-10^{-12}M T-2 prepared by serial dilution of the working stock into medium. Maximal DMSO concentration, 0.3×10^{-4} percent, did not affect the uptake of ^{3}H-TdR by cultured cells. Stoek et al. (1985) reported that Con A stimulation of murine splenic cells was not affected by DMSO up to a concentration of 0.1%.

24 and 48 Hour T-2 Toxin Exposure Experiments

Incubating cells were exposed to the toxin either at the initiation of cultures (48 h exposure) or 24 h after initiation of cultures (24 h exposure). Similar experiments were also conducted in the presence of mitogens. Adding T-2 at the initiation of cultures or after 24 h of mitogen stimulation differentiated the effects of T-2 on early and late transformational events. Well volumes for 48 h exposures were 150 ul; well volumes for 24 h exposures were initially 150 ul and subsequently increased to 200 ul by the addition of T-2. Cell viability was assessed by [^{3}H]-TdR incorporation and trypan blue exclusion. All experiments were repeated three times and the results of representative experiments have been depicted.

T-2 Toxin Pre-treatment

Aliquots of cells (1 ml, 2×10^{7} cells) were incubated in media with or without T-2 (10^{-10} or 10^{-9}M) at 0 or 37°C for 15, 30, 60, or 90 min. The cultures were shaken continuously. All cultures were incubated

for 90 min. T-2 additions were made at 0, 30, 60, or 75 min to facilitate 90, 60, 30 or 15 min exposure periods, respectively. Following incubation, ice-cold medium was added to each culture and the cells pelleted at 4°C. The cells were resuspended and washed repeatedly, after which they were again suspended in 1 ml of medium. Aliquots (50 ul, 10^6 cells) were seeded into culture plates. Mitogens were added in 50 ul aliquots in the following concentrations, LPS 200 ug/ x 2^{-3}, PWM 100 ul/ml x 2^{-4}, PHA 50 ug/ml x 2^{-2}, and Con A 100 ug/ml x 2^{-6}. An additional 50 ul of medium was added to each well and the cultures incubated and pulsed with [^{3}H]-TdR as described earlier. [^{3}H]-TdR additions were done in triplicate.

Statistical Analysis

Data were analyzed utilizing a one-way ANOVA design (Dixon and Massey, 1969). Normal distribution and equal variances were assumed. An F statistic was chosen to test the hypothesis of equality of group means. Individual means were compared using the least significant difference (LSD) test.

Results

Uptake of [^{3}H]-TdR into splenic cells following 24 or 48 h exposure to T-2 has been illustrated in Fig. V-1. [^{3}H]-TdR uptake increased in cultures exposed to the toxin for 24 h. The increase occurred in cells exposed to 10^{-11}-10^{-10}M T-2. T-2 concentrations of 10^{-10}M or greater decreased [^{3}H]-TdR uptake after 48 h exposure. The number of viable cells following 24 or 48 h exposure to T-2 has been presented in Fig. V-

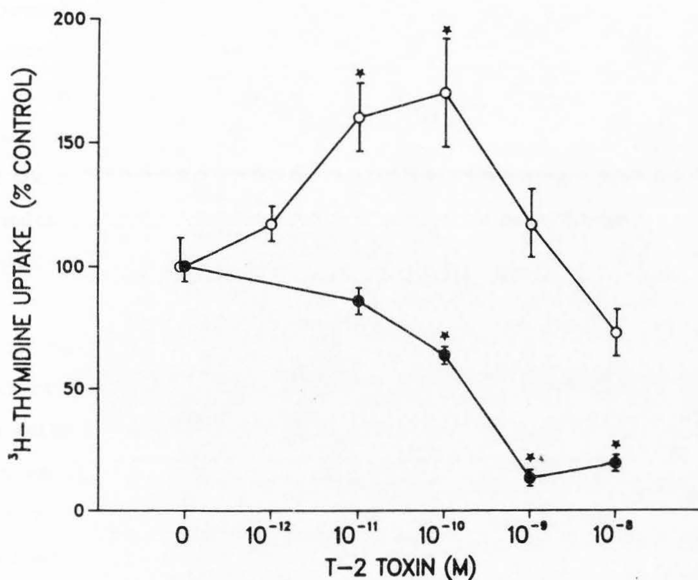


FIG. V-1. Uptake of [^{3}H]-TdR by murine (NFS/N) splenic cells treated with various concentrations of T-2 toxin for 24 (○) or 48 h (●). The 24-h treatment was begun 24 h after establishing cultures. The plotted points represent mean ± S.E.M. Points marked with an asterisk (*) differ significantly from control ($p<0.05$).

2. Twenty-four hour exposure to 10^{-8}M T-2 decreased cell viability. The viability of splenic cells decreased following exposure to 10^{-9}M or greater T-2 for 48 h. Exposure to 10^{-8}M T-2 for 24 or 48 h reduced cell viability by approximately 50 percent. [^{3}H]-TdR uptake was not affected similarly; [^{3}H]-TdR uptake following 48 h was much lower than that observed following 24 h. [^{3}H]-Thymidine uptake does not reflect differences in the uptake of thymidine by individual cells.

The time-associated effects of T-2 on lymphocyte mitogenesis have been summarized in Figs. V-3 and V-4. Only one concentration for each of the four mitogens has been illustrated in Fig. V-3. Except for Con A, results did not vary with mitogen concentration. All experiments were repeated three times with similar results.

Uptake of [^{3}H]-TdR increased in cells treated with T-2 toxin 24 h after PWM stimulation (Fig. V-3A). The increase was observed over the concentration range of 10^{-12}-10^{-9}M T-2. [^{3}H]-TdR uptake also increased in cells treated with LPS 24 h before T-2 was added. The increase was not as dramatic, however, as that observed for PWM- stimulated cells. The responses to PHA were decreased when T-2 was added after 24 h. The decrease occurred at all concentrations of toxin. Splenic cells exposed to 10^{-8}M T-2, after 24 h prior exposure to mitogen, did not respond.

Uptake of [^{3}H]-TdR decreased following 48 h exposure to T-2 in the presence of either LPS or PWM (Fig. V-3B). Lipopolysaccharide-responsive cells were affected by 10^{-12}M T-2. Pokeweed mitogen-sensitive cells appeared more resistant to the effects of the toxin; [^{3}H]-TdR uptake did not decrease until 10^{-11}M T-2. The response of splenocytes to PHA was not affected by less than 10^{-9}M T-2, at which point [^{3}H]-TdR uptake was

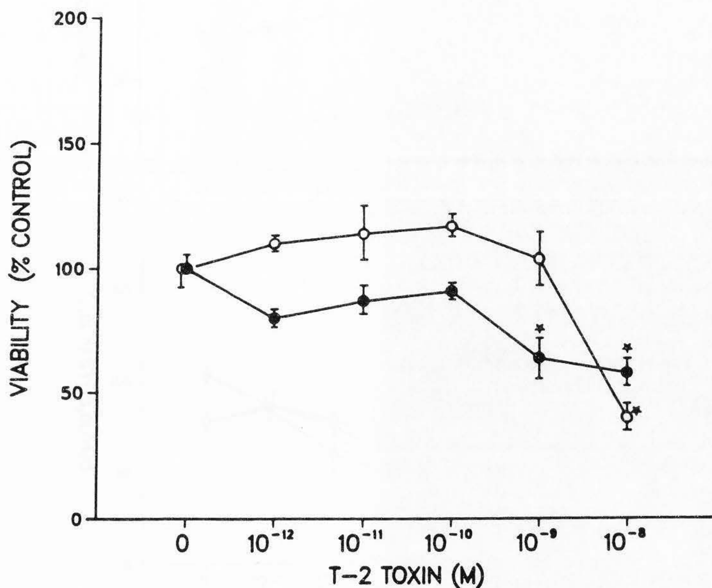


FIG. V-2. Viability of murine (NFS/N) splenic cells following 24 (○) or 48 h (●) exposure to various concentrations of T-2 toxin. The 24-h treatment began 24 h after establishing cultures. The plotted points represent mean ± S.E.M. Points marked with an asterisk (*) differ significantly ($p<0.05$) from control values.

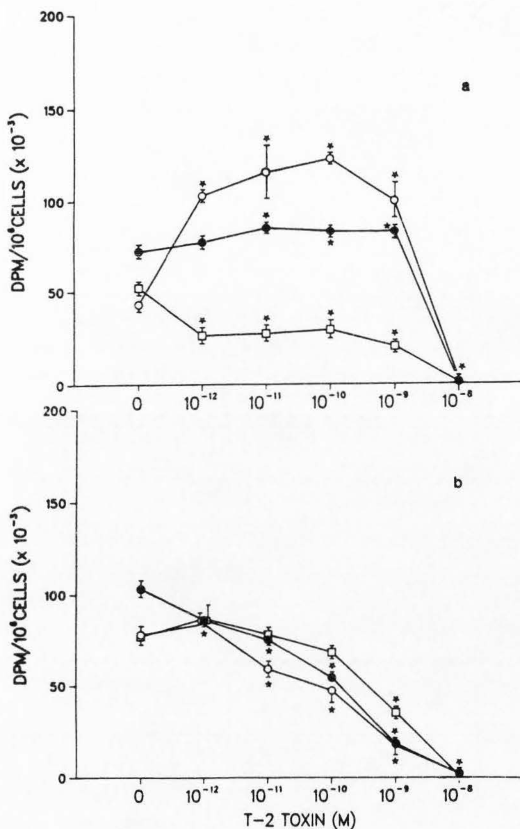


FIG. V-3. Mitogen responses of murine (NFS/N) splenic cells treated with various concentrations of T-2 toxin for 24 (a) or 48 h (b). The responses to LPS 0.16 ug (●), PWM 1.56 ul (○), and PHA 80 ng (□) have been indicated. Mitogens have been expressed as amounts per well . The plotted points are mean ± S.E.M. Points marked with an asterisk (*) differ significantly ($p<0.05$) from control values.

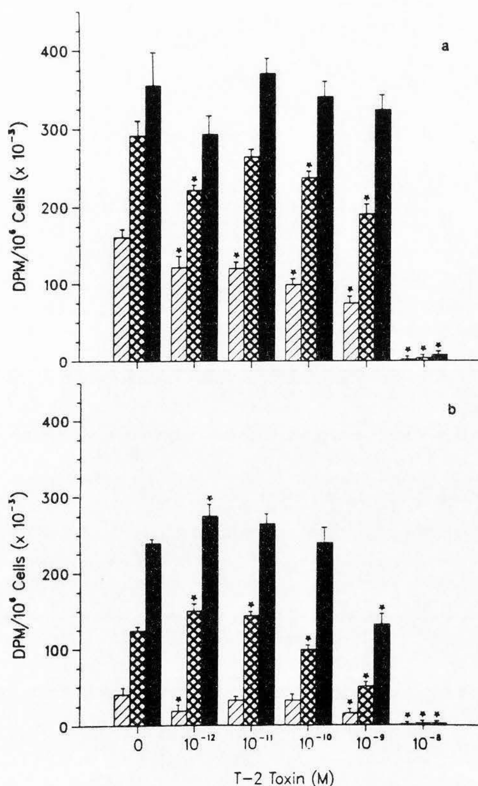


FIG. V-4. Con A responses of murine (NFS/N) splenic cells treated with various concentrations of T-2 toxin for 24 (a) or 48 h (b). The amounts of Con A per well were 20 (▨), 49 (▩), or 80 ng (■). The plotted points represent mean ± S.E.M. Points marked with an asterisk (*) differ significantly ($p<0.05$) from controls.

decreased. Again, as observed for 24 h exposures, 10^{-8}M T-2 abolished responsiveness to mitogens.

The relationship between Con A concentration, T-2 concentration, and time of T-2 exposure has been expanded (Fig. V-4). As was stated earlier, the use of different concentrations of Con A resulted in apparent differences in sensitivities to T-2. At the lowest concentration of Con A used in 24 h exposures, T-2 decreased the responsiveness of splenic lymphocytes. However, cells treated with the greatest concentration of Con A appeared relatively insensitive to the effects of T-2. Responses of cells in 48 h exposures were increased (10^{-12}-10^{-11}M T-2) when simultaneously exposed to the middle and greatest concentrations of Con A. ^{3}H-Thymidine uptake decreased slightly in cells exposed to the lowest concentration of Con A.

Figure V-5 presents the results of pre-treatment experiments for cells exposed to 10^{-9}M T-2. No differences were observed between control cultures and those treated with 10^{-10}M T-2. Ninety-minute exposure to 10^{-9}M T-2 at either 0 or 37°C resulted in increased [^{3}H]-TdR uptake by splenic cells (Fig. V-5a). Incubation of splenic cells at 0°C for 60 or 90 min increased the responses to both PWM and LPS (Fig. V-5b). The response to PHA increased following 15 min pre-treatment with T-2 and decreased when incubated for longer periods. When splenic cells were pre-treated with T-2 at 37°C the response to LPS increased following 90 min (Fig. V-5c). The response to Con A was reduced when incubated with T-2 for up to 30 m; longer incubations resulted in responses comparable to control values.

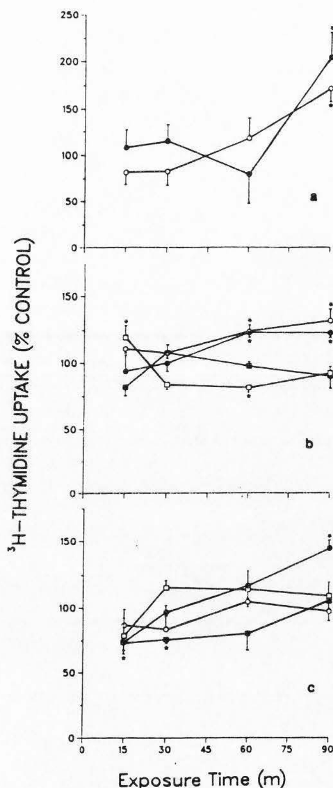


FIG. V-5. (a) The effect of T-2 toxin (10^{-9}M) pre-treatment exposure temperature, 0 (○) or 37°C (●), on [^{3}H]-TdR uptake by splenic cells. (b) The responses of splenic cells pre-treated with T-2 toxin at 0 or (C) 37°C to LPS (●), PWM (○), PHA (□), or Con A (■). (See text for mitogen concentrations.) The plotted points are mean ± S.E.M. Points marked with an asterisk (*) differ significantly ($p<0.05$) from control values.

Discussion

In the present study, we report an inverse, time-associated response pattern between cells responding to B and T-cell mitogens. Lafarge-Frayssinet et al. (1979) reported a similar inverse relationship. Three days after the last injection of a crude Fusarium extract the responses of murine splenocytes were either increased or decreased when cultured with either LPS or PHA, respectively. The responses to both mitogens were depressed on day 10. On day 20, the response to LPS was greatly increased while the PHA response was comparable to control values.

Nanomolar and subnanomolar concentrations of T-2 both inhibited and stimulated murine splenic lymphocytes in vitro. Modulation of lymphocyte responsiveness was dependent upon T-2 exposure time. The increased mitogen responses reported herein occurred at concentrations that were not observably cytotoxic. Gyongyossy-Issa and Khachatourians (1985) reported that 24-or 48-h exposure to 125 pg/ml T-2 reduced cell viability by 49 and 31 percent, respectively. DNA synthesis was also depressed by 125 pg/ml. (A concentration of 10^{-12}M corresponds to approximately 4.5 pg/ml.) T-2 alone had a mitogenic effect on murine splenocytes and dramatically increased the mitogenic response to PWM if addition of T-2 was delayed for 24 h. Similar phenomena were observed using splenic cells from mice treated for 2 or 4 weeks with T-2 (Taylor et al., 1985) where both a dose-related increase in thymidine uptake (no mitogen) and an elevated PWM response were reported. Delaying the addition of T-2 for 24 h allows sufficient time for mitogen activation of transformational

events. After 24 h, mitogen-stimulated cells progress to the S-phase of mitosis and actively synthesize DNA. (For a detailed review of the events in mitogenic transformation see Hume and Weidemann, 1980.) T-2 may interfere with a feedback mechanism controlling the suppression of DNA synthesis. It is doubtful that a time-dependent change in culture cellularity was accountable for the observations above. In a study of human peripheral lymphocytes, the percentages of B- and T- cells did not vary over a 3-day culture period (Hofman et al., 1982).

Pokeweed mitogen stimulated both T- and B-cells _in vitro_ (Greaves and Janossy, 1972). It has been reported by Kina et al., (1982) that the _in vitro_ response of murine splenocytes to PWM was regulated by T-cells and was macrophage dependent. Irradiation of T-cells prior to being mixed with B-cells dramatically increased the number of plaque-forming cells, presumably due to a decrease in viable suppressor T-cells. Suppressor cells have been reported to exert their negative effects 24 h after immunization (Jones et al., 1976). Presently, we report that treatment of cells with T-2 24 h after the addition of PWM significantly increased the response to PWM. At the same time, the responses to both of the T-cell mitogens, Con A and PHA, were depressed. The induction of T-suppressor cells by Con A has been described (Rich and Pierce, 1973). Our results indicate that the elevated response to PWM may have resulted from T-2 disruption of T-cell regulation. Increased Con A responses (48 h exposures) were associated with decreased LPS and PWM responses. Decreased Con A and PHA responses (24 h exposures) were associated with increased LPS and PWM responses.

Brief exposure to T-2 (10^{-9}M) resulted in increased uptake of [^{3}H]-TdR by splenic cells. T-2 was rapidly taken up by murine splenic cells (Gyongyossy-Issa and Khachatourians, 1984). The authors reported that T-2 was associated with cells at 0°C, although approximately 2.5 times more toxin was associated with cells incubated at 37°C. In the present study, stimulation of [^{3}H]-TdR uptake by LPS or PWM was enhanced by pre-treatment of splenic cells at 0°C with T-2.

The results following in vitro exposure of splenocytes to T-2 were similar to results following in vivo toxin exposure. These similarities facilitate the use of in vitro exposure in this system for evaluating the mechanism of the immunomodulatory effects of T-2.

CHAPTER VI

MURINE THYMOCYTE TOXICITY OF T-2 TOXIN *IN VITRO*. ITS UPTAKE AND EFFECT ON MITOGEN RESPONSE

Introduction

T-2 toxin (4,15-diacetoxy-8-(3-methylbutyryl-oxy)-12, 13-epoxy-trichothec-9-en-ol) is a cytotoxic and cytostatic (Ueno et al., 1973; Morel-Chany et al., 1981) and an immunomodulatory (Otokawa, 1983; Taylor et al., 1987b) trichothecene mycotoxin. Lymphocytes are sensitive to the radiomimetic effects of T-2. The toxin is also effective *in vitro*. The thymus, a primary lymphatic organ, is affected following *in vivo* treatment with T-2 (Hayes et al., 1980; Rosenstein et al., 1979, 1981; Taylor et al., 1985; Ueno, 1984). The decrease in thymus mass following T-2 exposure is often associated with cortical depletion. Lafarge-Frayssinet et al. (1980) observed that T-2 treatment of pregnant rats had a negative effect on the thymus of newborns. Radioactivity detected in the fetuses suggested that T-2 passed through the placenta. The *in vitro* effects of T-2 on mitogen responses of both human and murine splenic lymphocytes has been documented (Cooray, 1984; DiNinno et al., 1985; Forsell et al., 1985; and Taylor et al., 1985). Both increased and decreased (dose-related) responses to phytohaemagglutinin (PHA) following *in vitro* exposure of thymic cells to T-2 have been reported (Lafarge-Frayssinet et al., 1979). The association of T-2 with murine lymphocytes

has also been reported (Gyongyossy-Issa and Khachatourians, 1984). The authors stated that approximately 10^5 molecules of T-2 are bound per cell.

The thymus of young mice contains a large number of immature lymphocytes many of which will contribute to the T-cell repertoire of the animal. T-2 may interact with thymic cells pharmacologically, i.e., via a receptor-mediated process. The present investigation was undertaken to assess the uptake characteristics of T-2 by murine thymocytes as well as to demonstrate the toxin's _in vitro_ effects on the mitogenic responses of murine thymocytes to concanavalin A.

Methods

Media

RPMI 1640 (Gibco, Grand Island, NY) was the standard growth medium used in all experiments. Medium used in 24- and 48-h cultures was supplemented with 5% heat-inactivated and filtered fetal bovine serum (Hyclone Labs, Logan, UT), 100 units penicillin, and 100 ug streptomycin/ml (Gibco). Medium was buffered with sodium bicarbonate and the final pH adjusted to 7.4.

Thymic Cell Preparation and Culture

Thymic tissues were aseptically excised from either male CD-1 or NFS/N mice and used for uptake or mitogen response studies, respectively. Tissues were immediately immersed into ice-cold RPMI 1640. Thymuses from several animals were pooled for each experiment, teased apart, and aspirated through hypodermic needles of decreasing diameters (18, 21 and

25 gauge) to prepare a single-cell suspension. Connective fascia and cell clumps were separated during settling. Erythrocytes and dead cells were removed from thymic cell preparations used in mitogen-response assays by centrifugal separation through Ficoll-Hypaque (Parish et al., 1974). After separation, cells were washed twice in RPMI 1640. Cell viability was assessed using the trypan blue exclusion technique of Phillips (1973). All of the following experiments were repeated with consistent results; however, only representative data have been presented.

Uptake of [^{3}H]T-2 Toxin by Thymic Cells

[^{3}H]T-2 (Spec. Act. 11 Ci/mmol, Amersham Arlington Heights, IL) and T-2 (Romer Labs, Washington, MO) were diluted into RPMI containing 0.01% DMSO. Radiochemical and unlabeled T-2 purities were in excess of 95%. Dilutions of [^{3}H]-T2 were made producing a 1-20 nM concentration range. The respective activities of the [^{3}H]-T2 solutions and their molar concentrations, based upon a specific activity of 11 Ci/mmol, were verified. Incubations of thymic cells were done at either 0 or 37°C. The cells were contained in glass tubes and shaken for the duration of incubation. In preliminary studies the effect of preincubation with high specific activity of [^{3}H]-T2 followed by unlabeled T-2 was investigated. Preincubation had no influence on the uptake of labeled T-2 by thymic cells. The amounts of T-2 and various parameters measured have been indicated and described in figure legends. Cell-associated [^{3}H]T-2 was assay by rapid vacuum filtration onto glass fiber filters. Tubes and filters were washed repeatedly with ice-cold saline. The interactions of

dexamethasone (DEX, Sigma) and clonidine (CLD, Sigma) with T-2 were also investigated. The various concentrations of DEX or CLD have been indicated in figure legends.

24 and 48 Hour T-2 Toxin Exposure Experiments

Cells (NFS/N, 10^6) were seeded into wells of microtest tissue culture plates (Falcon Plastics, Oxnard, CA) and incubated for 48 h at 37°C in 5% CO_2. T-2 was added at the initiation of cultures (48 h exposure) or 24 h after the initiation of cultures (24 h exposure). Adding T-2 at the initiation of culture or after 24 h of mitogen-stimulation differentiated the effects of T-2 on early and late transformational events. Well volumes for 48 h exposures were 150 ul; well volumes for 24 h exposures were initially 150 ul and subsequently increased to 200 ul by the addition of T-2. Cell viability was assessed by thymidine uptake and trypan blue exclusion. Six hours prior to collection of cells, 0.5 uCi of $[^3H]$-thymidine ($[^3H]$-TdR, spec. act 2 Ci/mmol, New England Nuclear, Boston, MA) in 50 ul medium was added to each well. Cells were harvested onto glass microfiber filters and $[^3H]$ activity determined via liquid scintillation counting (Model 2660, Packard Instrument Co., Downers Grove, IL). Mean $[^3H]$-TdR uptake, expressed as DPM/10^6 cells, was based upon quadruplicate well counts. Similar experiments were conducted in the presence of concanavalin A type IV (Con A, 0.26 ug/ml, Sigma).

Statistical Analysis

Data were analyzed utilizing a one-way ANOVA design (Dixon and Massey, 1969). Normal distribution and equal variances were assumed. An

F statistic was chosen to test the hypothesis of equality of group means. Individual means were compared using the least significant difference (LSD) test.

Results

In order to assess [^{3}H]-T2 association with thymocytes, the following parameters were initially assessed; [^{3}H]-T2 concentration, cell number, and time. The amount of [^{3}H]-T2 associated with thymocytes was dependent upon cell number (Fig. VI-1). Cell number varied from 1-4 x 10^6 cells per tube. Cell associated activity increased proportionally to cell number. This experiment indicated that [^{3}H]-T2 associates with thymocytes. The time dependency of [^{3}H]-T2 association with thymocytes is presented in Fig. VI-2. The amount of cell-associated activity was time-dependent, reaching a plateau after 30 min. Based upon the information of Figs. VI-1 and VI-2, 2.0-2.5x10^6 cells per tube and a 12 min incubation period were used in additional studies.

The specificity of [^{3}H]-T2 association with thymocytes was determined in the presence of unlabeled T-2 at either 37 or 0°C (Fig. VI-3). Cells were incubated with either labeled T-2 (1-20 nM) alone or labeled T-2 in the presence of a 100-fold excess of unlabeled T-2 for 12 min. It was determined by trypan blue exclusion that cell viability was not affected by 12-min incubation in a 1.0 mM solution of T-2. The amount of cell-associated activity was reduced in the presence of unlabeled T-2. By convention, specific association is defined by the displacement of the labeled ligand by the unlabeled ligand, whereas

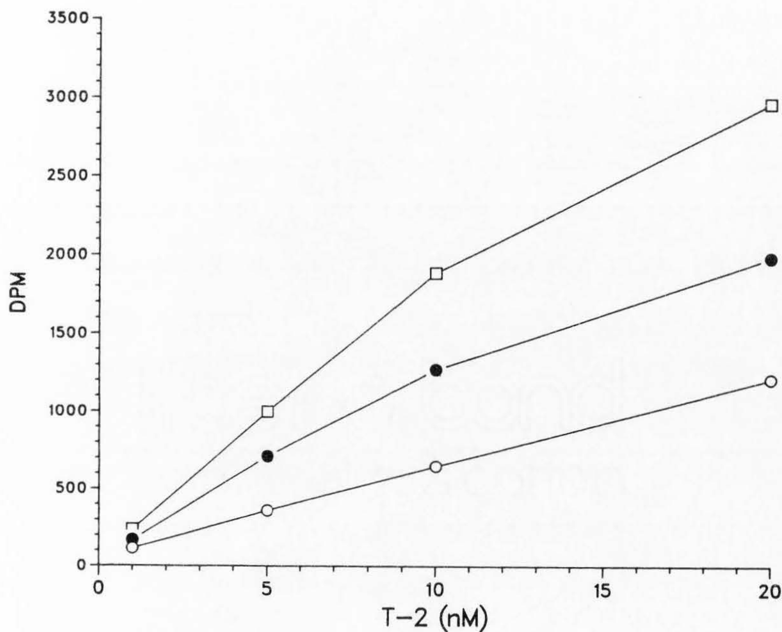


FIG. VI-1. The effect of thymic cell number on the uptake of [^{3}H]-T2. Thymic cells 1 (○), 2 (●), or 4 (□) x 10^6 were incubated (37°C) for 30 min in the presence of 1-20 nM [^{3}H]-T2. Values represent mean, n=2.

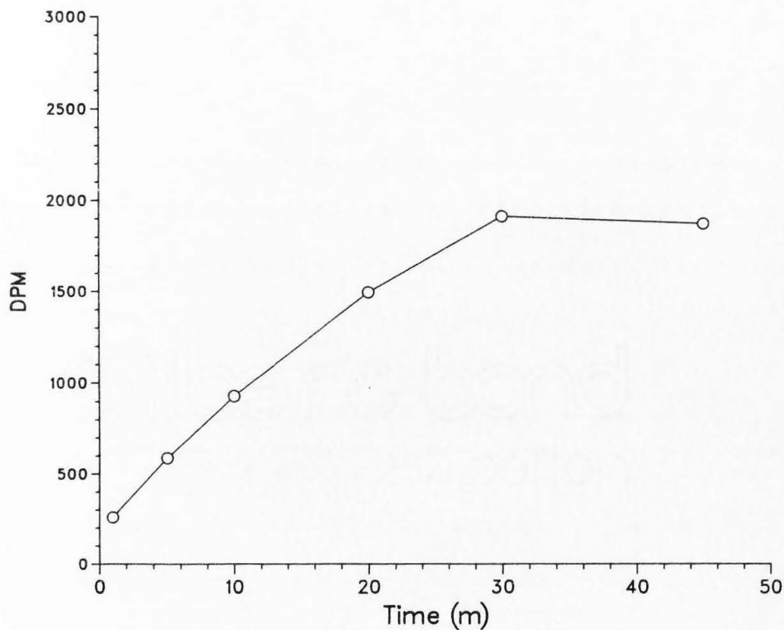


FIG. VI-2. The effect of incubation time on the uptake of [^{3}H]-T2 by thymic cells. 2.5 x 10^{6} thymic cells were incubated (37°C) with 5.0 nM [^{3}H]-T2 for 1-45 min. Values represent mean, n=2.

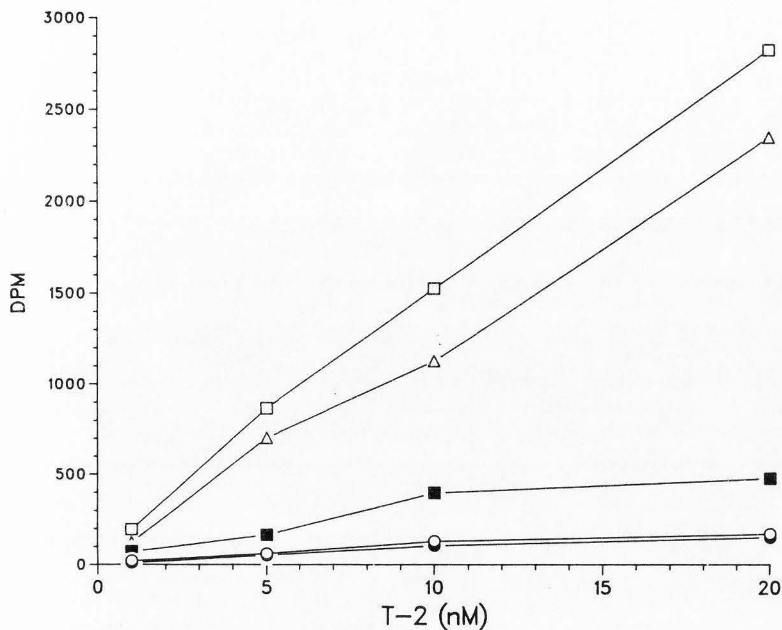


FIG. VI-3. The effect of excess unlabeled T-2 toxin and temperature on the uptake of [^{3}H]-T2 by thymic cells. 2.5x10^{6} thymic cells were incubated with [^{3}H]-T2 alone (□ ○) or followed by a 100-fold excess of unlabeled T-2 toxin (■ ●) at either 37°C (□■) or 0°C (○●) for 12 min. Specific association of T-2 toxin with thymocytes was determined (△). Values represent mean, n=2.

non-specific association in not displaced by the cold ligand. The specific association of T-2 with thymocytes was determined via the difference between the levels of [^{3}H] associated with cells incubated with labeled T-2 alone or both labeled and unlabeled T-2. When the difference was plotted the amount of T-2 associated with cells increased with increasing concentrations of T-2, a plateau was not observed. The amount of cell-associated [^{3}H]-T2 was drastically reduced if cells were incubated at 0°C. Very little difference was observed between cells incubated with labeled T-2 alone or labeled and unlabeled T-2 together at 0°C.

The association of [^{3}H]-T2 was reduced by the presence of unlabeled T-2 (Fig. VI-4). The amount of unlabeled T-2 was varied (20-1000nM) with respect to the concentration of [^{3}H]-T2 (5nM). Maximal displacement of [^{3}H]-T2 occured between 0.5 and 1.0 uM T-2. Approximately 70 percent of the [^{3}H]-T2 was displaced by 1.0 uM unlabeled T-2.

Simultaneous incubation of thymocytes with [^{3}H]-T2 and dexamethasone (10^{-5} M) reduced the amount of cell-associated activity (Fig. VI-5). The reduction was approximately 50 percent for all levels of labeled T-2 used (1-20nM). Dexamethasone did not affect the nonspecific association of T-2 with thymocytes. When thymic cells were incubated in the presence of dexamethasone (10^{-5} M) and labeled and a 100-fold excess of unlabeled T-2, no differences were observed in the amount of cell- associated activity (fig. VI-5). Clonidine did not affect the association of T-2 with thymic cells. As indicated in Fig. VI-6, 10^{-5} M clonidine had no effect on the association of labeled T-2 alone or its nonspecific

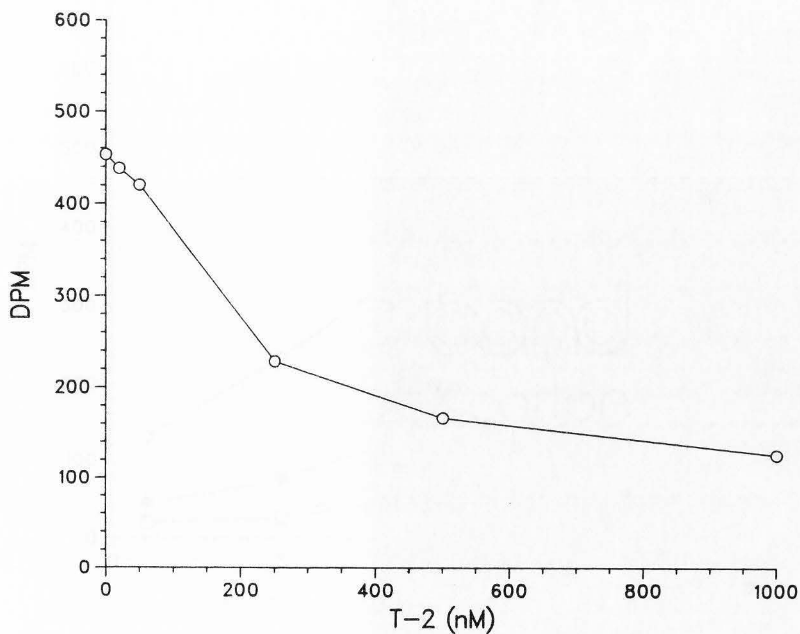


FIG. VI-4. The effect of unlabeled T-2 toxin on the association of $[^3H]$-T2 with thymic cells. $2x10^6$ thymic cells were incubated (37°C, 12 min) with 5nM $[^3H]$-T2 and followed by varying amounts of unlabeled T-2 toxin 20-1000 nM. Values represent mean, n=2.

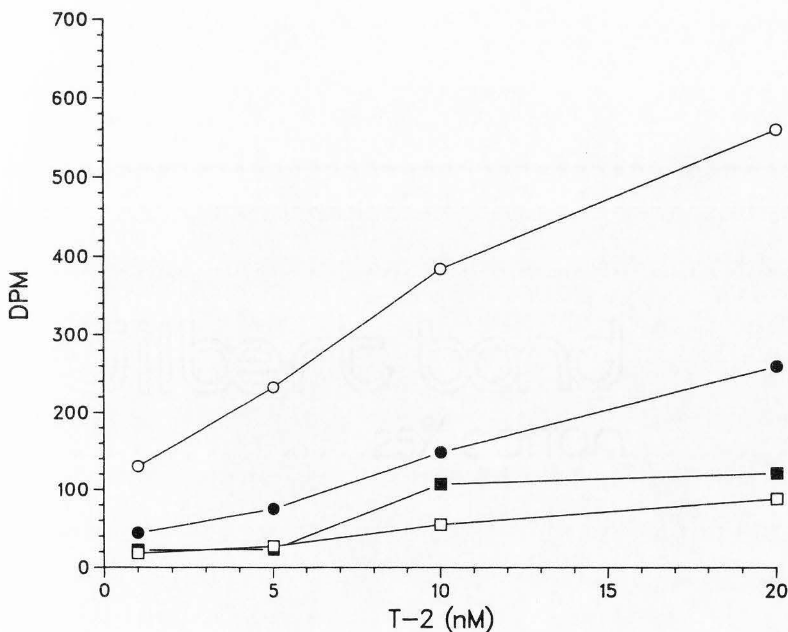


FIG. VI-5. The effect of Dexamethasone on the association of [^{3}H]-T2 with thymic cells. $2x10^{6}$ thymic cells were incubated (37°C, 12 min) with [^{3}H]-T2 alone (○) or with 10^{-5} M dexamethasone (●). Similarly [^{3}H]-T2 with a 100-fold excess of unlabeled T-2 toxin with (■) or without (□) 10^{-5} M dexamethasone. Values represent mean, n=2.

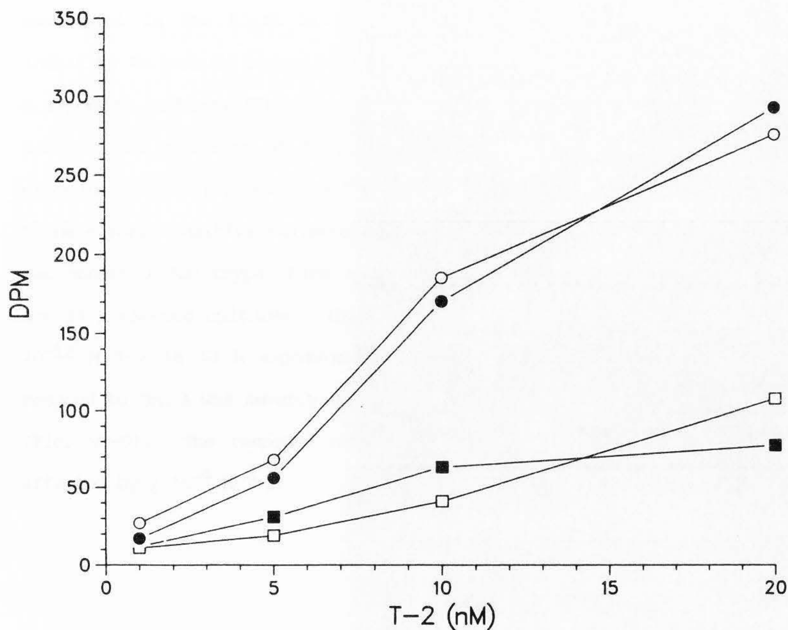


FIG. VI-6. The effect of clonidine of the association of [^{3}H]-T2 with thymic cells. 2×10^6 thymic cells were incubated (37°C, 12 min) with [^{3}H]-T2 alone (○) or with 10^{-5} M clonidine (●). Similarly, [^{3}H]-T2 with a 100-fold excess of unlabeled T-2 with (□) or without (■) 10^{-5} M clonidine. Values represent mean, n=2.

association with thymic cells, assessed in the presence of both labeled and nonlabeled T-2.

The viability of thymic cells, incubated in the presence of T-2 for 24 or 48 h was assessed via trypan blue exclusion and [^{3}H]-TdR uptake. Both 24 and 48 h exposed cultures were incubated for a total of 48 h, T-2 was added in the first 24 h after the initiation of cultures. The inability to exclude trypan blue indicated cell death occurred in the 48-h exposure cultures (Fig. VI-7). Cell death was attributed to exposure time in the presence of T-2, as no cell death was observed in 24 h exposures. Data for cell viability, based upon [^{3}H]-TdR uptake, appeared to be a more sensitive indicator of cell injury (Fig. VI-8). However, as was observed for trypan blue exclusion, no cell injury was observed in the 24 h exposed cultures. The amount of [^{3}H]-TdR uptake was reduced by 10^{-12} M T-2 in 48 h exposure cultures. The ability of thymocytes to respond to Con A was severly compromised by 48 h exposure to $>10^{-12}$ M T-2 (Fig. VI-9). The response to Con A in 24 h exposed cultures was not affected by $\leq 10^{-9}$ M T-2.

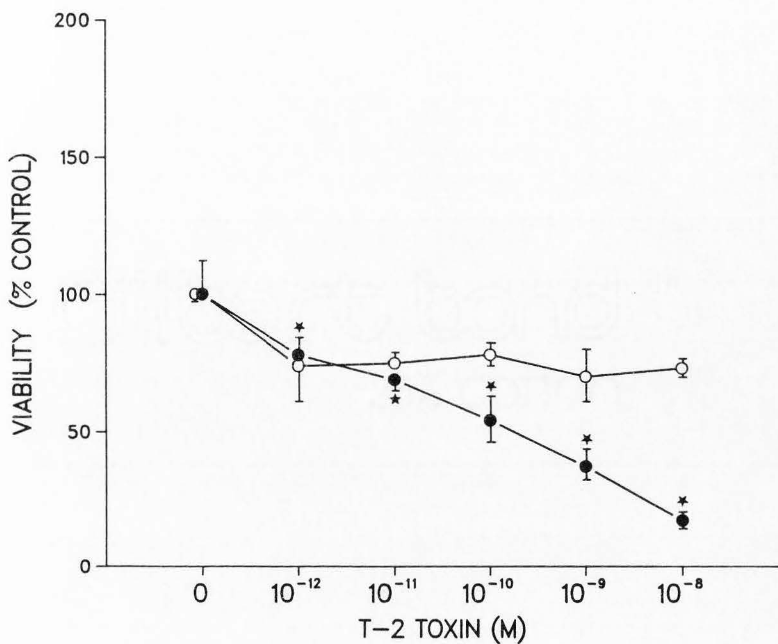


FIG. VI-7. The effect of 24 (○) and 48 h (●) exposure to T-2 toxin on thymocyte viability. Values represent mean ±S.D., n=4.

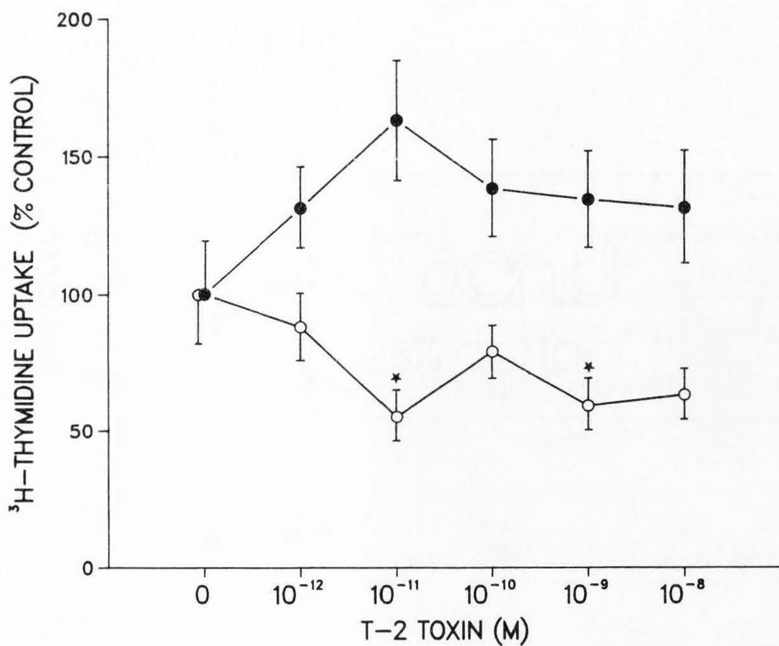


FIG. VI-8. The effect of 24 (●) and 48 h (○) exposure to T-2 toxin on the uptake of [^{3}H]-TdR by thymocytes. Values represent mean ±S.D., n=4.

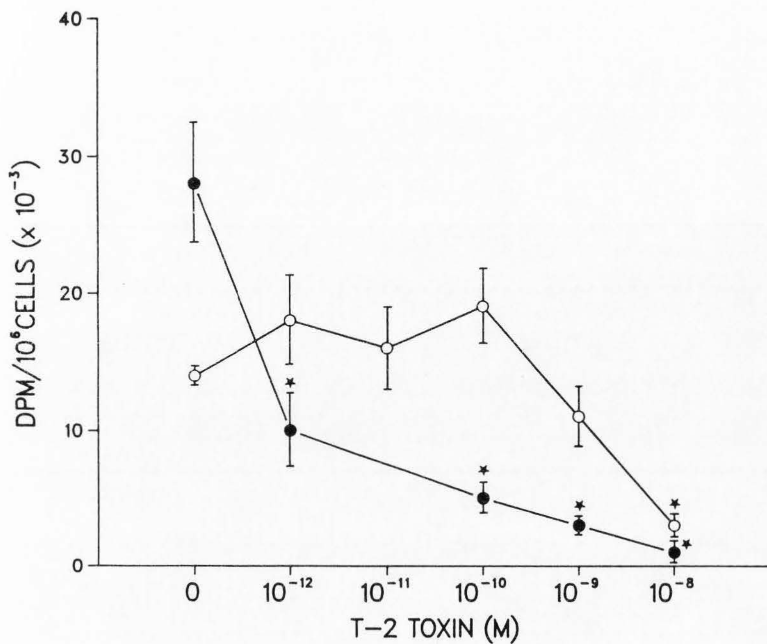


FIG. VI-9. The effect of 24 (○) and 48 h (●) exposure to T-2 toxin on the response of thymocytes to Con A. Values represent mean ±S.D., n=4.

Discussion

T-2 is an amphipathic molecule (Gyongyossy-Issa et al., 1986a). As such, it has been speculated that T-2 becomes incorporated into the outer half of membranes. Pronounced membrane alterations have been observed for erythrocytes incubated in the presence of T-2 (Gyongyossy-Issa et al., 1986b). The authors described changes in erythrocyte flexibility and decreased osmotic fragility.

A sensitive indicator of T-2 exposure is reduction in the cortical mass of the thymus, a phenomenon which may not be directly attributable to T-2. Increased corticosteroid production as a consequence of in vivo T-2 exposure has been reported (Lorenzana et al., 1985; Rafai and Tuboly, 1982) and described in previous chapters of this manuscript. Claman (1972) reviewed the thymolytic effects of corticosteroids; rodents are particularly sensitive to such effects. Lafarge-Frayssinet et al. (1980) observed that fetal thymic tissue was indeed a potential target of T-2 following maternal administration of the toxin. The thymic mass of neonates from treated mothers was reduced, and T-2 was detected within the thymic tissue. The present investigation suggests that T-2 associates with thymocytes and that such association may be of a specific pharmacologic nature. Cell association is dependent upon both the concentration of T-2 and cell number. An apparent specific association of T-2 with murine thymocytes was observed, as increasing the level of unlabeled T-2 reduced the association of $[^3H]$-T2 with thymocytes. Gyongyossy-Issa and Khachatourians (1984) reported similar observations using murine splenic cells.

T-2 may associate with thymocytes in an energy-independent, temperature-sensitive manner. The temperature sensitivity of T-2 association with thymocytes was described herein. The association of T-2 with splenocytes is relatively insensitive to the presence of various energy inhibitors and is reduced by decreasing incubation temperature (Gyongyossy-Issa et al., 1984). Much of the association of T-2 with thymocytes can be attributed to diffusion; the presently described phenomenon was dependent upon ligand concentration and temperature, and an uptake plateau was reached slowly. The large amount of unlabeled T-2 necessary to approach a background level of T-2 (Fig. VI-4) is also an indication of relative specific affinity of T-2 for thymocytes. Jarvelainen et al. (1985) reported that the release of ^{51}Cr from preloaded endothelial cells was reduced in the presence of 10^{-7} - 10^{-5} M DEX. The authors concluded that the reduction in loss of ^{51}Cr was due to membrane stabilization by DEX. The observation of reduced T-2 association with thymocytes in the presence of DEX may be a function of increased membrane stability by DEX and not an implication of pharmacologic similarity. However, nonspecific assocation of T-2 with thymocytes was not affected by DEX. Diffusion of T-2 across the cell membrane would be reduced if membrane permeability were reduced. Clonidine, an antagonist of α_2-adrenergic receptors, did not interfere with the association of T-2 with murine thymocytes.

Mitogenic activation of thymocytes, precursors of T-cell immunocompetence, are affected by very low levels of T-2 <u>in vitro</u>. The present investigation indicated a reduced response to Con A by thymocytes incubated in the presence of 10^{-12} M T-2 for the entire period of

mitogen treatment. A reduction in Con A response was not apparent with $<10^{-9}$ M T-2 in cultures exposed to T-2 24 h after cultures were established. These results suggest that the early events following mitogen exposure are quite sensitive to the effects of T-2.

It is not possible at present to conclude whether or not T-2 is the primary factor responsible for the reported observations of thymic cortical depletion. The association appears to be a diffusion-regulated process. The association of T-2 with thymocytes is sensitive to the presence of DEX, a synthetic corticosteroid. Circulating levels of corticosterone increase during exposure to T-2 and may alter the amount of T-2 entering thymocytes. Mitogen response of thymocytes is severely reduced if T-2 is present when the cells are initially activated. These phenomena support the possibility that T-2 exerts its toxic effect directly on thymic cells.

CHAPTER VII

SUMMARY

The immunomodulatory activity of T-2 was indicated to be associated with activation of the hypothalamic-pituitary-adrenal axis. Circulating levels of corticosterone increased dramatically following acute exposure to T-2. Elevated corticosterone levels were also observed following 2 and 4 weeks exposure to T-2. The level of hypothalamic norepinephrine increased in toxin-treated animals after 2 weeks exposure. The hormonal changes described above are indicative of the stress response, i.e., activation of the hypothalamic-pituitary-adrenal axis. The observed development of endotoxemia during acute exposure to T-2 may play a central role in the initial activation of the hypothalamic-pituitary-adrenal axis. Prolonged exposure to T-2 (2-4 weeks) was not associated with endotoxemia; therefore, the role of endotoxin in the maintenance of the stress response is dubious. The persistent elevation in corticosterone and the increased levels of hypothalamic norepinephrine may have been perpetuated by the development of the herein reported gastric pathology.

The importance of the adrenal glands in the defense against T-2 toxicity was demonstrated by the incidence of mortality observed for T-2 treated, adrenalectomized animals. The role of corticosterone in T-2 induced immunomodulation is complex. Toxin treatment alone elicits an

increase in circulating corticosterone levels. However, the level of corticosterone in antigen-challenged, T-2 treated mice was reduced. It is possible that the immunomodulatory effects of T-2 are related to the changes in thymic tissue as a consequence of increased levels of corticosterone. The immunomodulatory activity of T-2 on antibody production was reduced by removal of the adrenal gland. An attempt to discern the specific contribution of corticosterone in T-2 induced immunomodulation was made by treating animals with Metyrapone, an inhibitor of corticosterone biosynthesis. Metyrapone treatment did not reduce the levels of corticosterone in antigen–challenged animals. Metyrapone treatment alone led to increased responses to both T-dependent and T-independent antigens. The effects of T-2 on antibody response were not affected by Metyrapone treatment.

T-2 toxin was shown to interact directly with both splenic and thymic cells _in vitro_. An inverse, time-dependent response of cells responding to B- and T-cell mitogens was reported. Nanomolar and subnanomolar concentrations of T-2 both inhibited and stimulated murine splenic lymphocytes _in vitro_, respectively. Modulation of lymphocyte response was dependent upon the time of T-2 exposure during mitogen activation. T-2 alone had a mitogenic effect on murine splenocytes and dramatically increased the mitogenic response to PWM (a B-cell mitogen), if addition of T-2 was delayed for 24 h. At the same time, the responses to the T-cell mitogens Con A and PHA were depressed. The data indicate that the elevated response to PWM may have resulted from T-2 disruption of T-cell regulation.

The present investigation suggests that T-2 associates with thymocytes and that such association may be of a specific pharmacologic nature. Cell association was dependent upon both the concentration of T-2 and cell number. An apparent specific association of T-2 with murine thymocytes was postulated, since increasing the level of unlabeled T-2 reduced the association of $[^3H]$-T2 with thymocytes. Nonspecific association was also observed, as not all $[^3H]$-T2 could be displaced by the unlabeled ligand. The observed reduction in the amount of $[^3H]$-T2 associated with thymocytes in the presence of dexamethasone may be a function of increased membrane stability by dexamethasone. The alteration of T-2 association with thymocytes in the presence of a synthetic corticosteroid may have physiologic significance, as corticosteroid levels do increase in T-2 treated animals.

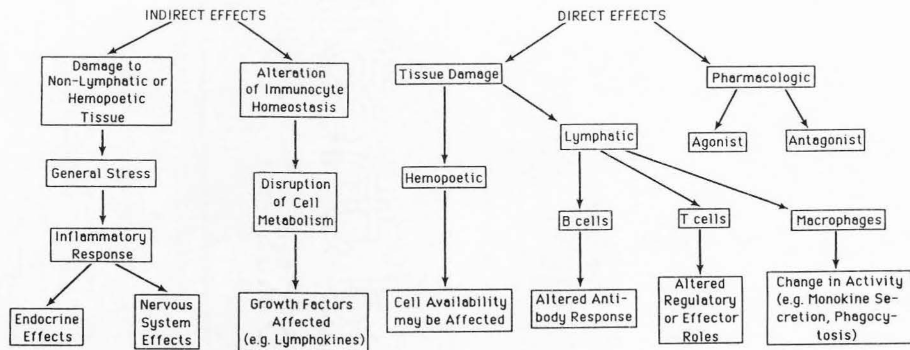
This investigation has provided new evidence supporting the role of hypothalamic-pituitary-adrenal axis activation following T-2 exposure. The adrenal gland was observed to be an important component in the _in vivo_ immunomodulation by T-2. _In vitro_, T-2 is also an immunomodulatory compound, as evidenced by the various responses of murine splenocytes. Thymic cells, compromised by _in vivo_ exposure to T-2, associate with T-2 and exhibit decreased activity following _in vitro_ exposure to the toxin.

EPILOGUE

The ultimate manifestation (i.e., immunomodulation) following T-2 exposure is undoubtedly a culmination of various insults. Trichothecene-associated immunomodulation may be discussed according to the scheme presented in the following diagram, Fig. VIII-1. Trichothecene mycotoxin-induced immunomodulation occurs through both indirect and direct routes. With both in vivo and in vitro systems T-2 has been demonstrated to be an immunomodulatory compound; both stimulatory and inhibitory effects have been observed. The disparity of effects may be related to the dose, frequency of exposure, and time of exposure. The response of the immune system to antigenic stimulation is a premeditated event in which various types of cells participate in a programmed sequence of events. Many investigations have pursued the T-cell regulatory mechanism as the target of trichothecene mycotoxins. One could speculate, based on studies in mice, that T cells are particularly sensitive to T-2 and that when T cells are damaged, at a dose sparing other lymphocytes, changes in particular T-cell and B-cell responses may occur.

While trichothecene mycotoxins are known to be immunotoxic by direct cytocidal effects or by inhibition of macromolecular synthesis, particularly protein and DNA syntheses, hormone-mediated reductions in immunoresponsiveness should also be considered as potentially

TRICHOTHECENE EXPOSURE



IMMUNOMODULATION

FIG. VIII-1. Various routes of immunomodulation following trichothecene mycotoxin exposure.

contributing. Elevation in serum concentrations of cortisol (Rafai and Tuboly, 1982), as well as epinephrine and norepinephrine (Lorenzana et al., 1985), have been reported in T-2 treated pigs. These hormones individually or together, even at physiological concentrations, can reduce mitogen-induced T-cell blastogenesis, inhibit interleukin 2 production by T cells, or inhibit the expression of Ia antigens and interleukin 1 production by macrophages (Cray et al., 1983; Gills et al., 1979; Snyder and Unanue, 1982 and Westley and Kelley, 1984).

Trichothecene mycotoxins are known to induce inflammatory responses in a variety of animal species. Some inflammatory mediators, such as protaglandins and histamine, may also play a role in the regulation of as immune responses. Prostaglandins and histamine, at physiologic concentrations, can induce a profound inhibition of lymphocyte response to mitogens (Chouaib et al., 1985; Suzuki and Huchet, 1981 and Al-Imara and Dale, 1985). Studies to date have shown elevations in plasma concentrations of certain protaglandins in pigs, rats, and guinea pigs in response to trichothecene treatment (Lorenzana et al., 1985 and Feuerstein et al., 1985).

Endotoxins, known for their stimulatory effects on splenic progenitor cells (Burgess and Nicola, 1983), are another factor that should be considered. Gastroenteritis is commonly observed in animals following sufficient exposure to trichothecene mycotoxins, and disruption of the gut mucosa may facilitate the absorption of endotoxins.

Trichothecene mycotoxins are potentially immunotoxic to animals. Chronic exposure of man to the toxins via contaminated food sources have been associated with a substantially increased incidence of bacterial

infections. Debilitating effects may not be directly correlated with mycotoxin exposure as immunosuppression, related to trichothecene exposure, may be inappropriately diagnosed as a primary problem of microbial infection. Long-term exposure to concentrations which fail to produce overt primary toxic effects may have far-reaching ramifications for both man and animals. Because of the importance of the immune system in surveillance for neoplastically transformed cells, chronic depression of immunocompetence may indeed exert effects not realized for many years.

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Science Teacher, secondary education, September 1979 to June 1980, Randolph, UT

PROFESSIONAL ORGANIZATIONS

American Society of Pharmacology and Experimental Therapeutics (student member)

Mountain-West Association of Toxicologists

Rocky Mountain Immunology Association

Society of Toxicology (student member)

OFFICES HELD

Mountain West Chapter, Society of Toxicology - executive council, student councilor, Fall 1985 - Spring 1986

Graduate Student Association, Utah State University - executive council, library representative, Fall 1984 - Spring 1985

CERTIFICATIONS

Secondary Education

AWARDS

N.I.H.-French C.N.R.S. Exchange Scientist Fellowship, 1987, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France

Gordon Conference, 1987, Mycotoxins and Phycotoxins, Plymouth State College, Plymouth, NH

S.O.T. Travel Award, 1986, New Orleans, LA

F.A.S.E.B., 1984, Summer Research Conference, Trichothecene Mycotoxins, Saxtons River, VT

N.I.H., 1983, N.I.H. Immunotoxicology Workshop, Research Triangle Park, NC

Gordon Conference, 1983, Trichothecene Mycotoxins, Plymouth State College, Plymouth, NH

PUBLICATIONS

Reddy, R.V., M.J. Taylor and R.P. Sharma. 1987. Studies of immune function of CD-1 mice exposed to Aflatoxin B_1. Toxicology 43:123-132.

Srisuchart, B., M.J. Taylor and R.P. Sharma. 1987. Alteration of humoral and cellular immunity in manganese chloride treated mice. Accepted, J Toxicol Environ Hlth.

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Sharma, R.P., M.J. Taylor and D.R. Bourcier. 1983. Subcellular distribution of alpha-chaconine in mouse hepatocytes. Drug Chem Toxicol 6(2):219-234.

Bourcier, D.R., W.M.Bracken, M.J. Taylor and R.P. Sharma. 1982. Cadmium-copper interaction: effect of copper pretreatment and cadmium-copper chronicexposure on the distribution and accumulation of cadmium, copper, zinc and iron in mice. Trace Sub Envi Health, vol XVI, edited by D.D. Hemphill, Univ Missouri, Columbia, pp 273-279.

CHAPTERS IN BOOKS

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Toxicology, edited by J.J. Haley and W.O. Berndt. Hemisphere, Wash. D.C. pp. 439-471.

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MANUSCRIPTS IN PREPARATION

Hughes, B.J., R.P. Sharma and M.J. Taylor. 1987. Potentiation of immune function by Verrucarin A, a macrocyclic trichothecene, in male CD-1 mice.

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Taylor, M.J., R.V. Reddy and R.P. Sharma. 1987. Immunotoxicologic evaluation of rubratoxin B in male CD-1 mice.

Taylor, M.J. Srisuchart, B., Coulombe, R.A. and Sharma, R.P. 1987. Immunological response following low level ethanol treatment in mice.

Taylor, M.J., Warren, R.P. and Sharma, R.P. 1987. Immunomodulatory activity of T-2 toxin in relation to adrenal corticosteriod activity.

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Taylor, M.J., S. Jayasekara and R.P. Sharma. 1987. T-2 toxin immunomodulation is mediated by a stress-like response. Toxicologist 7:34.

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Srisuchart, B., M.J. Taylor and R.P. Sharma. 1986. Alteration of humoral and cellular immunity in manganese chloride treated mice. Toxicologist 6:68.

Taylor, M.J., B. Srisuchart and R.P. Sharma. 1986. Immunological assessment of ethanol treated mice. Toxicologist 6:271.

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