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EFFECTS OF T-2 TOXIN ON VACCINAL IMMUNITY AGAINST MAREK'S DISEASE AND ON THE IMMUNE SYSTEM OF WHITE LEGHORN CHICKENS

By

Eric Kufuor-Mensah

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Center for Environmental Toxicology

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ABSTRACT

EFFECTS OF T-2 TOXIN ON VACCINAL IMMUNITY AGAINST MAREK'S DISEASE AND ON THE IMMUNE SYSTEM OF WHITE LEGHORN CHICKENS

By

Eric Kufuor-Mensah

Four trials were conducted to determine the effects of T-2 toxin on vaccinal immunity against Marek's disease (MD). Day-old, RPRL line $15I_5$ X 7_1 chicks were treated daily for seven days via crop gavage with T-2 toxin at a subclinical dose of 1.25 mg/kg body weight. Treated and untreated chicks were vaccinated with herpesvirus of turkeys (HVT) at hatch and challenged with JM strain of MD virus (MDV) at 8 days of age. Chickens were tested for HVT and MDV viremia and were observed for the development of MD lesions within 8 weeks of age. T-2 toxin significantly reduced body weight by 7 days after treatment. T-2 toxin shortened the incubation period for the development of MD lesions and mortality, but only in unvaccinated, challenged chickens. T-2 toxin also significantly reduced titres of HVT viremia within 7 days post-vaccination. However, the percent protection from MD in T-2 toxin-treated, HVT-vaccinated chickens ranged from 82%-95% and was comparable to that in untreated chickens (89%-100%). In a second experiment, the effect of T-2 toxin on

peripheral blood and splenic B- and T- lymphocyte subpopulations was evaluated using flow cytometry. Day-old, $15I_5$ x 7_1 chicks were treated daily for 7 days via crop gavage with T-2 toxin at a subclinical dose of 1.25 mg/kg body weight. Peripheral blood and splenic lymphocytes from T-2 toxin-treated and untreated control chicks were analysed for phenotypic expression of CD4, CD8, CD3 and IgM cell surface markers at 8-9, and 21-22 days of age. The percentages of both peripheral blood and splenic B-lymphocytes in T-2 toxin-treated chickens were significantly lower than that in untreated chickens but only at 8-9 days of age. However, at 21-22 days of age the percentages of B- and T-lymphocytes were comparable to untreated chickens. The data suggest that exposure of chickens to T-2 toxin may influence the development of a) HVT viremia; and b) MD lesions and mortality, but only in unvaccinated chickens. The data also suggest that T-2 toxin may severely deplete B-lymphocytes and relatively increase CD4 and CD3 T-lymphocytes.

Dedicated to the memory of my parents and brother,

Nana Twum Barima Apawu II

Madam Yaa Tenewaa

Dominic Apawu-Mensah.

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

KEY TO ABBREVIATIONS

T-2 toxin.....3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene
MD.....Marek's disease
MDV.....Marek's disease virus
PBS.....Phosphate buffered saline
HVT.....Herpesvirus of turkey
CEF.....Chick embryo fibroblasts
DEF.....Duck embryo fibroblasts
ETOH.....Ethanol
PC.....Post-challenge
PV.....Post-vaccination
PI.....Post-inoculation

INTRODUCTION

INTRODUCTION

Marek's disease (MD) is a herpesvirus-induced lymphoproliferative neoplastic disease of chickens characterized by infiltration of various nerve trunks and/or visceral organs with pleomorphic lymphoid cells (Calnek and Witter, 1991). Until the early 1970s, MD was an economically important disease causing losses of billions of dollars worldwide due to condemnations in broiler chickens and reduced egg production and mortality in layer chickens (Purchase, 1985). Several vaccines have been developed and used in the field but the one most widely used is HVT isolated by Witter et al., (1970). The introduction of vaccines, and the common practice of vaccinating 1-day-old chicks drastically reduced the economic losses associated with MD (Purchase, 1973). However, the Delmarva region of the United States, (parts of Delaware, Maryland and Virginia) has consistently experienced higher MD losses in broilers than in other regions (Purchase, 1985; Witter, 1989a). The introduction of serotype 2 MD virus (MDV) in bivalent vaccines has markedly reduced losses although data from the late 1980s have shown losses to be somewhat on the rise (Witter, 1989b).

Failure of vaccines to provide the expected protection levels ("vaccination breaks") has occasionally been reported in Asia, and the Middle East (Shieh, 1989; Davidson, 1989). Such vaccination breaks have been attributed to several factors including the emergence of very highly oncogenic strains of MDV (Eidson et al., 1978, 1981; Witter et al., 1980; Schat et al., 1981), inadequate methods of vaccine production or mismanagement of vaccines at hatcheries causing loss of vaccine titres during thawing, reconstitution and use (Thornton et al., 1975; Halvorson and Mitchell, 1979). Increased genetic susceptibility to MD or the presence of maternal antibodies against HVT have also been suggested as possible reasons of vaccination failures (Calnek and Smith, 1972; Spencer et al., 1974. In addition poultry operation practices such as multi-age rearing and insufficient cleaning between generations of chickens could also be responsible for early exposure to MDV before vaccinal immunity develops (Good, 1983; Price, 1983; Witter et al., 1984).

Infectious disease agents such as infectious bursal disease virus (Giambrone et al., 1976; Jen and Cho, 1980; Sharma, 1983), chicken anemia virus (Otaki et al., 1988) reticuloendotheliosis virus (Bulow, 1977); reovirus (Rosenberger, 1983); chemical immunosuppressants such as cyclophosphamide and corticosterone (Purchase and Sharma,

1974; Witter et al., 1976; Payne et al., 1978; Powell and Davidson, 1986); polychlorinated biphenyls (Halouzka and Jurajda, 1992) and mycotoxin such as aflatoxin (Edds et al., 1973; Batra et al., 1992) have been reported to influence the efficacy of vaccination, presumably through depression of cell-mediated and/or humoral immune response.

T-2 toxin has received much attention because of its adverse effects on humans and farm animals (Mayer, 1953a, b). T-2 toxin, a type A trichothecene, is a secondary metabolite produced primarily by *Fusarium* spp, which grows on cereal grains and contaminates agricultural products usually in temperate regions of North America, Europe and Asia (Scott, 1989; Russell et al., 1991; Wang et al., 1993; Chu and Li, 1994). Exposure to T-2 toxin has been associated with a variety of clinical syndromes in both humans and farm animals. T-2 toxin has been implicated as a major causative factor in fatal alimentary toxic aleukia in humans during World War II (Mayer, 1953a, b; Joffe, 1971; 1978), and red mold disease in humans and animals (Ueno et al., 1972; Saito and Ohtsubo, 1977). Fusariotoxicosis in lactating cows and poultry has also been reported (Bamburg et al., 1968; Wyatt et al., 1972; Hsu et al., 1977; Chi and Mirocha, 1978). In addition, T-2 toxin and its metabolites have been reported as possible constituents of "yellow

rain", a chemical warfare agent used in South East Asia (Haig, 1982; Watson et al., 1984).

T-2 toxin, has been reported as a potent immunotoxin which impairs the immune system through its ability to inhibit protein and DNA synthesis and thereby causing severe damage to actively dividing cells within the bone marrow, lymph nodes, spleen, thymus, bursa of Fabricius, and the gastrointestinal tract (Saito et al., 1969; Ueno et al., 1971; Wyatt et al., 1973; Lutsky et al., 1978; Hayes et al., 1980; Hoerr et al., 1981; LaFarge-Frayssinet et al., 1981; Thompson and Wannemacher, 1990). T-2 toxin has also been found to lower B and T cell numbers, inhibit lymphocyte transformation, decrease IgG and IgM antibody levels, and cause lymphocytolysis (Hayes et al., 1980; Jagadeesan et al., 1982; Rosenstein and LaFarge-Frayssinet, 1983; Cooray and Jonsson, 1990).

Previous studies have shown that repeated exposure to subclinical doses of T-2 toxin may cause immunosuppression and may decrease the resistance of exposed animals to various infectious diseases such as salmonellosis in chickens (Boonchuvit et al., 1975) and mice (Ziprin and McMurray, 1988; Tai and Pestka, 1988, 1990), tuberculosis (Kanai and Kondo, 1984; Ziprin and McMurray, 1988), listeriosis (Corrier and Ziprin, 1986), herpes simplex infection in mice (Friend et al., 1983), and aspergillosis

in rabbits (Niyo et al., 1988). In addition to decreased host resistance, T-2 toxin may impair host control of tumor cell growth by suppressing tumor defense mechanisms. (Schoental et al., 1979; Schiefer et al., 1987; Corrier and Norman, 1988).

The objectives of the present studies were to determine the effects of sublethal doses of T-2 toxin on vaccinal immunity against Marek's disease and to further characterize its effects on both peripheral blood and splenic B- and T-lymphocyte subpopulations of white leghorn chickens using flow cytometry.

LITERATURE REVIEW

LITERATURE REVIEW

T-2 Toxin

Historical Background

Interest in T-2 toxin and other trichothecene mycotoxins began after a widespread outbreak of alimentary toxic aleukia (ATA), or septic angina, in humans and animals in the Union of Soviet Socialist Republics (USSR) and in Eastern Europe during World War II (Joffe, 1986a). The characteristic symptoms of the disease were fever, necrotic angina, leukopenia, hemorrhage, exhaustion of bone marrow, sepsis, and up to 60% mortality (Mayer, 1953a,b; Joffe, 1978, 1986b). The cause of ATA was connected with a trichothecene compound, mainly T-2 toxin, which was isolated from authentic outbreak samples of cereal grains infected by *Fusarium poae* and *Fusarium sporotrichioides* (Joffe, 1971, 1986b; Joffe and Yagen, 1977, 1978; Lutsky and Mor 1981a).

The causative fungi capable of producing T-2 toxin and related toxic trichothecene-type compounds induced skin inflammation, diarrhea, vomiting, hemorrhages, feed refusal, depression of bone marrow, damage to hematopoietic tissues, leukocytosis, leukopenia and nervous disorders in experimental and farm animals (Ueno et al., 1972; Saito and

Ohtsubo 1974; Sato et al., 1975; Lustky and Mor, 1981b; Mirocha, C. J. 1983; Joffe, 1978; 1986b). Of the trichothecene mycotoxins, T-2 is one of the most highly toxic and has been the major compound studied in this group (Ueno, 1983). The seasonal occurrence of ATA, its endemic nature, and the composition of the affected population suggested the importance of climatic and ecological factors in producing toxins that were found in field grains naturally infected by *Fusarium* spp. (Joffe, 1986c).

The role of *Fusarium* strains and their toxins as causative agents in human disorders has acquired considerable significance after evidence that some potent mycotoxins of the trichothecene group (T-2 toxin, deoxynivalenol (DON or vomitoxin) and nivalenol (NIV) may have been used as "Yellow Rain" in chemical warfare in Southeast Asia (Haig, 1982; Watson et al., 1984)

Natural occurrence

T-2 toxin is a very potent biologically and chemically active secondary metabolite, produced worldwide in temperate climatic zones by various species of *Fusarium*, which infects cereal grains, feedstuffs, corn, wheat, oats, barley, and rice in the field or during storage (Joffe, 1986e; Scott, 1989; Keeler and Tu, 1991). The toxin has been associated with mycotoxicosis in farm animals and humans who ingest

moldy agricultural commodities (Bamburg et al., 1969; Hsu et al., 1977; Joffe, 1978, 1986d; Wang et al., 1993; Beardall and Miller, 1994).

The trichothecene mycotoxins have affected all parts of the world because they are naturally occurring contaminants of agricultural commodities (Vesonder, 1983; Ichinoe, et al., 1984; Scott, 1989). In any area of the world where cereal grains, forages, or corn are grown under required weather conditions, including but not limited to, ambient humidity, temperature, crop management, and geographical location, a genus or many genera of fungi may infect the plant (Joffe, 1986c). Overwintering conditions under low fluctuating temperatures appeared to promote *Fusarium* mycotoxin production in these grains.

Fusarium sp. generally colonize grains or cereals in the field, but most toxin production occurs in storage at cool temperatures, up to 15° C (Joffe and Yagen, 1977b; Joffe, 1978). Important environmental factors include high moisture or relative humidity and both warm and cool temperatures (Joffe, 1986c). Greater trichothecene production is usually found in years when the autumn is cool and wet, and harvest is delayed.

Although the appearance of fungi is not the ultimate factor in determining the presence of trichothecenes, it is

the most common indicator since they are responsible for trichothecene production. The toxicosis related to the presence of the naturally occurring trichothecene compounds was characterized as fusariotoxicosis in North America first by investigators at the University of Wisconsin where T-2 toxin produced by *F. tricinctum* in moldy corn was associated with illness and death in lactating cows (Hsu et al., 1972).

Species of *Fusarium* are common and widespread in nature and cause many important diseases of man and farm animals after consuming cereals that overwintered in the fields. A recent outbreak of human toxicosis caused by moldy rice contaminated with *Fusarium* and T-2 toxin was associated with heavy rainfall during rice harvest season in China (Wang et al., 1993). The chief symptoms were nausea, dizziness, vomiting, chills, abdominal distention and pain, thoracic stiffness, and diarrhea. *Fusarium heterosporium* and *F. graminearum* were the predominant fungi isolated from the moldy rice. The highest level of T-2 toxin detected was 420 ppb.

Simultaneous occurrences of fumonisin B, (FB1) and other mycotoxins in moldy corn collected in the People's Republic of China from regions with high incidences of esophageal cancer were recently reported by Chu and Li

(1994). Samples contained high levels of FB1 (18-155 ppm) and total type A trichothecenes (139-2030 ppb), including T-2 toxin, HT-2 toxin iso-neosolaniol, and monoacetoxyscirpenol. In addition, the concentration of total type B trichothecenes in some of the corn samples ranged from 470 to 5826 ppb.

Mold and mycotoxin contamination of mixed samples of corn is widespread in the midwestern corn belt of the U.S. In a survey of incidences of molds and mycotoxins in commercial animal feed mills in seven midwestern states in 1988-89, *Fusarium* sp. was found to be predominant. When assayed for mycotoxins, 19.5% of the samples were positive for at least one of the following: aflatoxin, zearalenone, T-2 toxin, or DON (Russell et al., 1991).

In another survey in Argentina, examination for *Fusarium* toxins in corn and milling byproducts revealed that 33% of the samples were contaminated by DON, its amount ranging from traces to 1200 µg/kg; 15% of the samples contained T-2 toxin at concentrations ranging from 900 to 2400 µg/kg (Saubois et al., 1992). Only a few samples (7%) contained diacetoxyscirpenol (DAS), NIV, and neosolaniol. DON contaminated only those byproducts destined for human consumption, whereas T-2 toxin was found in byproducts destined for both human and animal consumption.

A 3 year survey for the presence of mycotoxins in grains harvested in Atlantic Canada detected DON and zearalenone (Zea) in 53-62%, and 25-29% of samples, respectively, with little DAS, T-2 toxin, or HT-2 toxin found (Stratton et al., 1993).

Mycoflora and natural occurrences of mycotoxins are not confined to cereal grains. El-Maghraby and Abdel-Sater (1993) reported the contamination of tobacco and cigarettes in Egypt. DAS, T-2 and Zea were isolated from *F. moniliforme* infections of banana fruits in which 4 out of 40 samples had aflatoxin B_1 (15-20 µg/kg), Zea (5.5 µg/kg) and T-2 toxin at a level of 2.8 µg/kg (Chakrabarti and Ghosal, 1986). Trichothecene mycotoxins, including T-2 toxin, and DAS were detected in unusual locations such as the dust of ventilation systems in office buildings in urban areas of Montreal, Canada, reportedly affected by the "sick building syndrome" (Smoragiewicz et al, 1993).

There are toxigenic strains of *Fusarium* indigenous to the warmer regions of the U.S., and other tropical and semitropical areas of the world. Richardson et al., (1985) isolated *Fusarium spp.* from plant material grown in the hot, humid climate of North Carolina which tested positive for the production of mycotoxins, T-2 toxin, Zea, and DON. In South Africa, oat grain and barley were contaminated with *F.*

aluminatum which produced T-2 toxin in large amounts (0.8-2600 mg/kg at a relatively high (25° C) temperature (Rabie et al., 1986). Samples of moldy maize harvested from farms in Jos district, Nigeria, were contaminated by *Fusarium* mycotoxins, NIV, Fusarenon-X, T-2 toxin, and HT-2 toxin (Okoye, 1993).

Chemical Structure

T-2 toxin is a member of a large group of sesquiterpenoid fungal metabolites, called trichothecenes, whose common skeleton includes a tetracyclic ring system containing an epoxide group at C-12,13 and a double bond at C-9,10. The skeleton is thus characterized as 12, 13-epoxytrichothec-9-ene (Bamburg et al., 1968). The name for the structure of a tetracyclic skeleton composed of cyclohexane, cyclopentane and 6-membered oxyrane rings, and four methyl groups was proposed as "trichothecane" by Godtfredsen et al. (1967).

The structure of T-2 toxin is shown in Figure 1. All natural trichothecenes have the same stereochemistry: α at C-3 (R1), β at C-4 (R2) and C-15 (R3), α at C-7 (R4), and α at C-8 (R5) for groups A and B. The full systematic chemical name of T-2 toxin, therefore, is 3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene.

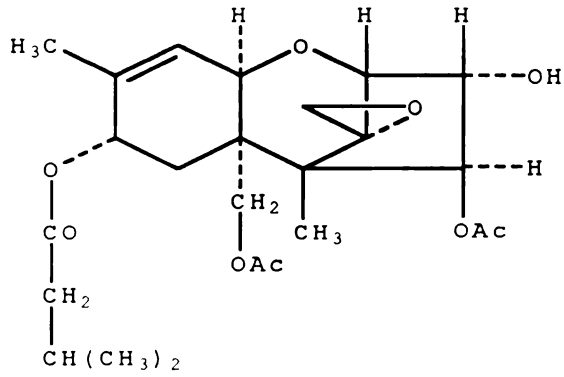

Figure 1: Chemical Structure of T-2 toxin

The trichothecenes are divided into simple and macrocyclic trichothecenes, according to their chemical characters depending on the presence of a macrocyclic ring linking at C-4 and C-5 with diesters or triesters, and on the fungi producing the trichothecene (Ueno, 1983). The simple trichothecenes are again divided into four types, namely, type A, B, C and D. Type A trichothecenes include T-2 toxin, DAS, HT-2 toxin, and others. T-2 toxin, the first to be recognized as a naturally occurring trichothecene, is the most highly toxic among these trichothecene mycotoxins (Ueno, 1983; Trenholm et al., 1989; Keeler and Tu, 1991; Sharma and Salunkhe, 1991). The type A trichothecenes are primarily produced by *F. sporotrichioides*, *F. tricinctum* and *F. poae*. Type B trichothecenes, characterized by the

presence of a ketone group at the C-8 position, include nivalenol, fusarenon-X and deoxynivalenol (vomitoxin or DON) and are produced primarily by *F. nivale* and *F. episphaeria*. Type C trichothecene is represented by crotocin produced by *Cephalosporium contocinigenum* and has an epoxide at the 7-8 position. Type D trichothecenes comprise the macrocyclic trichothecenes which bear a bridge of varying length and composition between carbons 4 and 5. Types A and B trichothecenes are the most important.

Physical properties

T-2 toxin readily crystallizes from purified ethyl acetate extracts as white crystals. Analysis and molecular weight determination verified the molecular formula as $C_{24}H_{34}O_9$ (Bamburg et al., 1968). The toxin is very stable in the solid state and freely soluble in moderately polar organic solvents, such as methanol, ethanol, chloroform, and dimethyl sulfoxide, but is only slightly soluble in water (Bamburg and Strong, 1971). T-2 toxin is relatively stable in methanol. However, it was transformed to several products after 22 days exposure to methanol (Wei and Chu, 1986). The stability of the toxin in aqueous media decreased with increasing temperature. The toxin was more stable at 4° C and least stable at 37° C, with formation of HT-2, T-2 triol, and T-2 tetraol (Trusal, 1985). Duffy and Reid

(1993) estimated the half-life of T-2 toxin in deuterated phosphate-buffered saline solutions under quasi-physiological conditions to be about 4 years and concluded that the contribution of nonezymatic degradation to the detoxification of T-2 toxin and its metabolites was negligible.

Mechanisms of Action

Inhibition of Protein and DNA Synthesis

Many *in vitro* and *in vivo* studies have been done in various systems in an effort to determine the mechanism of action of T-2 mycotoxin. The general conclusion from these studies was that T-2 toxin blocks cellular protein synthesis by binding to the 60S subunit on the ribosome and thereby inhibits initiation, elongation, and termination of protein synthesis in eukaryotic cells (Ueno et al., 1968; Bamburg, 1972, 1974; Cundliffe et al., 1974; Cannon et al., 1976b; Cundliffe and Davis, 1977). T-2 toxin was also found to interfere with peptidyl transferase activity which is required for elongation and termination at the transcription site in eukaryotic cells (Tate and Caskey, 1973; Wei and McLaughlin, 1974; Mclaughlin et al., 1977). Ueno et al. (1968, 1973) who originally described inhibition of protein synthesis as a dominant effect of the trichothecenes pointed out that T-2 toxin was a weak inhibitor of protein synthesis

in cell-free systems, but was very potent in intact cells. Bamburg (1972) reported there was no direct correlation between protein synthesis inhibition and cell growth and replication, and suggested other mechanisms of action may be involved.

When results of the various trichothecenes' ability to inhibit protein synthesis *in vitro* were compared with the results of whole animal lethality, it was shown that several of the trichothecenes were weak inhibitors of protein synthesis in Vero cells and rat spleen lymphocytes *in vitro* but were highly toxic *in vivo* (Thompson and Wannemacher, 1986). Thus, *in vitro* cell response of a given trichothecene is not always an accurate predictor of toxicity in whole animals and protein synthesis inhibition might not be the sole cellular mechanism of action for *in vivo* toxicity (Bamburg, 1972; Ueno et al., 1973b).

The degree of protein synthesis inhibition correlated with the amount but not the rate at which T-2 toxin is taken up by the mitochondria. The rate of uptake of labeled leucine into mitochondrial protein, using isolated rat liver mitochondria supplemented with an S-100 supernatant from rat liver and an external ATP-generating system, was unaffected by the addition of T-2 toxin and was not a rate-limiting step in incorporation. However, 0.02 μg/ml of T-2 toxin

decreased the rate of protein synthesis by 50% in isolated mitochondria (Pace et al., 1988).

T-2 toxin also interferes with mitochondrial functions, resulting in the inhibition of growth of yeast (Schappert and Khachatourians, 1983). A significant amount of [^{3}H]T-2 localized in the mitochondrial fraction within 15 minutes of its addition to an isolated perfused rat liver and inhibited the electron transport chain in mitochondria (Pace, 1983).

The effect of T-2 toxin on protein and DNA synthesis depends on the type of organ involved. Intraperitoneal injection of T-2 toxin in mice caused inhibition of DNA and protein synthesis in bone marrow, spleen, and thymus (Rosenstein and Lafarge-Frayssinet, 1983). *In vitro*, T-2 toxin inhibited DNA and protein synthesis of mice spleen cells and rat hepatoma cells stimulated with phytohemagglutinin. Thompson and Wannemacher, (1990) studied the *in vivo* effects of T-2 toxin on protein and DNA synthesis in serum, liver, heart, kidney, spleen, muscle, and intestine of rats and concluded that although the levels of [^{14}C]leucine and [^{3}H]thymidine incorporation in each of the tissues of normal rats were consistent, a significant degree of variability existed in the rates of incorporation among the various tissues. Muscle and heart had the lowest index of leucine incorporation, spleen, intestine, and

kidney had intermediate levels, whereas liver had the highest index of protein synthesis , over 10 times that of the muscle. In most tissues recovery was seen with time. DNA synthesis indices were highest in the spleen and intestine. The muscle, heart, kidney, and liver incorporated a 3- to 10-fold lower level of thymidine per mg protein (Thompson and Wannemacher, 1990).

Alteration of cell membrane function

Studies on cytotoxicity have shown that T-2 toxin exerts its toxic effect by intervening at the subcellular level and inhibiting biochemical functions such as DNA, RNA, and protein syntheses (Ueno et al., 1968; Bamburg 1972; Wei et al., 1974; Suneja et al., 1983, 1987; Pace et al., 1988). However, this does not account for all the cytotoxic effects of T-2 toxin because some studies suggest that T-2 toxin interacts with cell membranes and alters membrane function.

Since T-2 toxin is an amphipathic molecule, interactions with various kinds of membranes are expected (Gyongyossy-Issa et al., 1984). The plasma membrane plays a significant role in the uptake and interaction of T-2 toxin with yeast cells (Schappert and Khachatourians, 1984). Membrane-modulating agents such as ethanol, cetyltrimethylammonium bromide and heat were found to increase the sensitivity of the yeast, *Saccharomyces sp.*,

towards T-2 toxin. However, a yeast mutant with a reduced plasma membrane permeability was resistant to T-2 concentrations of up to 50 µg/ml. Bunner and Morris (1988) proposed alteration of multiple cell membrane functions as an important mechanism of action and reported that T-2 toxin had multiple effects on cell membrane functions at very low concentrations (0.4 pg/ml to 4 ng/ml) which were independent of protein synthesis. Uptake of calcium, rubidium (K^+) glucose, leucine and tyrosine was reduced within 10 minutes of exposure of L-6 myoblasts to T-2 toxin. Such reduced uptake included either direct or indirect effects of T-2 toxin on amino acid, nucleotide, and glucose transporters, as well as calcium and potassium channel activities (Bunner and Morris, 1988).

Lipid peroxidation of cell membranes

Free radical-mediated phospholipid peroxidation of membranes by T-2 toxin has been proposed by some workers. Tsuchida et al., (1984) measured the presence of lipid peroxides (malonaldehyde formation) using the 2-thiobarbituric acid (TBA) method in various tissues including the liver in rats treated with up to 4 mg/kg body weight of T-2 toxin. The TBA values increased in the liver 24 and 48 hours after administration of T-2 toxin, reaching values approximately 6 and 5 times higher than those of

time-matched controls. However, no changes were observed in the TBA values in the kidney, thymus, and jejunum, 24 hours after toxin administration, and increases of only 20-50% over the respective control values were recorded after 48 hours.

Vitamin E, a free radical scavenger possesses protective activity against the stimulation of hepatic peroxidation induced by T-2 toxin (Tsuchida et al., 1984). Rats were pretreated for 2 weeks with diets containing different amounts of DL-α-tocopheryl acetate (vitamin E). In the pretreated rats not given T-2 toxin, the hepatic TBA values in the vitamin E-deficient animals were about 188% above those in the vitamin E-supplemented rats (300 mg/kg of diet), whereas in the T-2 toxin-treated, vitamin E-deficient animals, the TBA values rose about 400% as compared with those in the supplemented animals (Tsuchida et al., 1984). T-2 toxin had consistently produced depressed concentrations of vitamin E in plasma of chicks. The addition of micelle-promoting compounds such as taurocholic and oleic acids alleviated depression in both plasma vitamin E and growth (Coffin and Combs, 1981). An increase in nuclear lipid peroxidation induced in rat liver by T-2 toxin administered orally for 5 days resulted in an increase in TBA and a decrease in activity of liver glutathione-S-transferase (Ahmed and Ram, 1986). Chang and Mar (1988) reported an

increase in conjugated diene formation in liver, spleen, kidney, thymus, and bone marrow when rats were treated with 3-6 mg/kg body weight of T-2 toxin, indicating an increased level of lipid peroxidation in these tissues. In contrast, Schuster et al., (1987) measured the formation of TBA reactive material in isolated hepatocytes and liver homogenates from rats, then also determined ethane exhalation *in vivo* after oral administration of T-2 toxin. No significant difference from the controls in these parameters was noted and the authors concluded that lipid peroxidation did not play a major role in the toxicity of T-2 toxin. This contradictory conclusion was explained by Schuster et al., (1987) as associated with the different methods used to assay TBA reactive material in the liver homogenates.

A free radical mechanism inducing lipid peroxidation was first reported by Segal et al., 1983 as a cause of hemolysis. Even though the amount of malonaldehyde production was not significant, specific free radical scavengers, including vitamin E, mannitol, and histidine, inhibited hemolysis caused by T-2 toxin. Rizzo et al., (1992) recently reported the involvement of the lipoperoxidation mechanism in the hemolytic activity of T-2 toxin on rat erythrocytes. The authors proposed that T-2 toxin exerts its toxicity on procaryotic cells in 3

different ways: by penetrating the phospholipid bilayers and acting at the subcellular level, by interacting with the cellular membranes, and by free radical-mediated phospholipid peroxidation. But most likely, more than one mechanism operates at the same time.

The T-2 toxin-induced hemolysis had several characteristics, all of which include an initial time-dependent, and long lag period before hemolysis starts.

The characteristic lag period and the hemolytic activity of T-2 toxin on human and guinea pig erythrocytes were observed by Gyongyossy-Issa et al., (1985, 1986a,b) In contrast, cattle erythrocytes were not susceptible (DeLoach et al., 1987). Subsequently, a species-specific hemolysis of mammalian erythrocytes caused by T-2 toxin has been proposed by Deloach et al., (1989). Pig, man, rabbit, guinea pig, horse, dog, rat, and mouse erythrocytes were all lysed to a varying degree, but cow, sheep, goat, buffalo, and deer erythrocytes were all resistant to hemolysis by T-2 toxin. Since erythrocytes from ruminant animals contain little or no phosphatidylcholine, perhaps the presence of phosphatidylcholine in the membranes is required for the hemolytic action of T-2 toxin (Deloach et al., 1989).

Altered Intercellular Communication

T-2 toxin, other trichothecenes, and other inhibitors of protein synthesis, regulate intercellular communication through superinduction of cellular proteins such as interleukin-1 (IL-1) production by macrophages and IL-2 synthesis by lymphocytes (Miller and Atkinson, 1987; Holt et al., 1988). Either the interference with rapid turnover of proteins that limit the translation of mRNA for interleukin synthesis or the impaired degradation of intracellular interleukins has been suggested as the mechanism of the superinduction (Miller and Atkinson, 1987). Cardiovascular lesions induced in rats after passive transfer of splenic cells from syngeneic rats treated with T-2 toxin may be mediated by IL production (Sherman et al., 1987).

Induction of apoptosis

T-2 toxin has recently been reported to induce apoptosis, morphological changes of nuclei characterized by fragmentation and the formation of an apoptotic nuclear body, in HL-60 human promyelocytic leukemia cells (Ueno et al., 1995). The toxin induced apoptosis at 10 ng/ml within 2-6 hr without significant cytotoxicity.

Pharmacokinetics

In vivo metabolic studies conducted in chicks administered tritium-labeled T-2 toxin by crop gavage have

shown that T-2 toxin and its metabolites were primarily excreted into the intestine through the bile, and that the liver was a major organ for the metabolism and excretion of the toxin and its metabolites into the feces via the bile (Chi et al., 1978a; Yoshizawa et al., 1980; Visconti and Mirocha, 1985; Giroir et al., 1991). The chicks excreted 60.5, and 81.6% of the recovered radioactivity at 24, and 48 hours, respectively, and the gastrointestinal (GI) tract contained 26.9, and 10.4% of the recovered radioactivity at 24, and 48 hours, respectively (Chi et al., 1978a). The abdominal fat and heart contained the least amount of radioactivity among the tissues analyzed. The radioactivity of ^{3}H-labeled T-2 toxin reached a maximum concentration 4 hours after dosing in most tissues except muscle, skin, and bile, in which the maximum radioactivity was attained at 12 hours. The specific radioactivity in the blood, muscle, skin, and heart was similar throughout the 48-hour period. The bile, including the gallbladder, contained the highest specific radioactivity among organs and tissues (except the GI tract) during the 48-hour period Chi et al., 1978a). The edible portions of the carcass contained only 0.06 and 0.04 ppm of T-2 or its metabolites at 24 and 48 hours, respectively, after dosing with 0.5 mg of T-2 toxin per kg body weight. This suggests that humans would be unlikely to be affected by consuming the muscle from chickens fed diets

containing concentrations of T-2 toxin likely to occur in natural outbreaks.

The transmission of radioactivity into eggs from laying white leghorn hens administered single or multiple doses of tritium-labeled T-2 toxin via gastric-intubation was reported in a separate study by Chi et al., (1978b). In single dosed birds, the maximum radioactivity in eggs occurred at 24 hours after dosing; the yolk and egg white contained 0.04 and 0.13% of the administered radioactivity, respectively. In multiple-dosed birds given 8 consecutive daily doses, the radioactivity in the yolk increased with each dose, whereas the radioactivity in the egg white increased rapidly until the third dose, and thereafter remained constant. Therefore, in both the single and multiple dosed birds the specific radioactivity of the egg white was greater than that of the yolk. The amount of residue transmitted into an egg in birds intubated daily with 1 mg/kg T-2 toxin for 8 consecutive days (equivalent to 1.6 ppm dietary T-2 toxin) was about 0.9 µg, suggesting little danger, if any, to public health from residues (Chi et al., 1978b).

Metabolism

The initial *in vitro* metabolism studies using hepatic homogenates indicated that T-2 toxin was rapidly transformed

by ester hydrolysis catalysed by esterases at C-4 to several products, including HT-2 toxin, 4-deacetylneosolaniol, neosolaniol and T-2 tetraol (Ellison and Kotsonis, 1974; Ohta et al., 1977, 1978; Yoshizawa et al., 1980a). Two oxidation products, 3′-hydroxy HT-2 toxin and 3′-hydroxy T-2 toxin, were further identified in liver homogenates supplemented with an NADPH-generating system from mice and monkeys (Yoshizawa et al., 1984) and from pigs and rats (Wei and Chu, 1985). This suggests that cytochrome P-450 catalyzes the hydroxylation reaction at the C-3′ position of T-2 and HT-2 toxin. In phenobarbital-induced rat liver microsomes, T-2 toxin is rapidly metabolized to 3′-hydroxy T-2 toxin, 3′-hydroxy HT-2 toxin, HT-2 toxin, and T-2 triol (Knupp et al., 1984). The metabolic profiles of T-2 toxin incubated with inducers and inhibitors of the cytochrome P-450-dependent monoxygenase system in rats, mice, rabbits, chickens, guinea pigs, cows, and pigs were studied by Knupp et al., (1987) and Kobayashi et al., (1987). The hepatic S-9 and microsomal fractions from various species hydroxylated T-2 toxin and HT-2 toxin, a deacetylated metabolite of T-2 toxin formed by reactions involving microsomal esterases, to form 3′-hydroxy T-2 toxin and 3′-hydroxy HT-2 toxin, respectively. This two hydroxylation reactions were catalyzed by the cytochrome P-450-dependent monoxygenase system. Species comparisons indicated that the rate of

hydroxylation reaction was highest in the hepatic microsomes of guinea pigs and mice. Microsomal fractions from chickens had a low activity in the hydrolysis and hydroxylation reactions. The major metabolite in microsomal preparations from control and phenobarbital-induced rats, rabbits, and mice was HT-2 toxin. In microsomes isolated from phenobarbital-treated chickens, 3'-hydroxy T-2 toxin was the major metabolite, and 30 and 79% of the added T-2 toxin remained unmetabolized at 60 minutes in incubations from phenobarbital-induced and control birds, respectively. The percentage of hydroxylated metabolites formed in the microsomal preparations of the species studied was significantly increased after phenobarbital treatment compared with the nontreated controls (Knupp et al., 1987). Treatment of rats with phenobarbital induced esterase and mixed function oxidase activity, the latter being increased to a greater extent. Two previously unidentified metabolites detected in chicken, rat, and mouse microsomal preparations were tentatively classified as isomers of 3'-hydroxy T-2 toxin.

Some cultured cell lines possess enzyme systems capable of limited metabolism of T-2 toxin to a variety of known and some yet unknown metabolites. Chinese hamster ovary cells (CHO), and African green monkey kidney cells (VERO) metabolized T-2 toxin to a greater degree and to a wider

variety of metabolites than the human fibroblasts and mouse connective tissue cells (L-929) (Trusal, 1986). In CHO, fibroblasts, and L-929 cells, the major metabolite was HT-2 toxin, whereas in VERO cells an unknown metabolite, more polar than T-2 toxin, was the major metabolite. Cell and media extracts of CHO and VERO cells contained small amounts of T-2 triol, T-2 tetraol, and several unknown metabolites.

The sensitivity of lymphoid cells to the uptake, metabolism and cytotoxic effects of T-2 toxin varies according to their degree of differentiation (Porcher et al., 1988). Of the human lymphoid cell lines studied, susceptible Dandi (B-cell lymphoma) cells, and resistant REH (non-differentiated non-B non-T cell leukemia) and KE37 (T-cell leukemia) cells took up 20 and 3% of the T-2 present in the medium, respectively, when the cells were incubated with [^{3}H]T-2 toxin. Metabolites recovered from the culture medium and cells included T-2 tetraol, T-2 triol, HT-2 toxin, neosolaniol, and T-2 toxin.

Studies on the survival rates of various lymphoid cells upon exposure to 400 μm T-2 toxin per 1 x 10^{7} cells per ml revealed that thymocytes and cells collected by peritoneal lavage were exquisitely sensitive to the cytotoxic effects of the toxin, with no survival after 5 to 6 hours exposure (DiNinno et al., 1985). Spleen cells had an intermediate

survival rate with a rapid decline in viability. Bone marrow cells were relatively resistant, with only a small but significant decline in viability. A large proportion of a heterogeneous population of bone marrow cells was resistant to T-2 toxin and had a 40-50% survival rate, suggesting that these cells would allow the lymphoid organs to be repopulated, even upon prolonged exposure to T-2 toxin.

Excretion

About 80% of orally administered ^{3}H-labeled T-2 toxin in broiler chickens was rapidly metabolized to more polar derivatives such as HT-2 toxin, neosolaniol, 4-deacetylneosolaniol, T-2 tetraol, T-2 toxin, and several unknown derivatives were eliminated in the excreta within 48 hours after administration (Yoshizawa et al., 1980). In a subsequent study, Visconti and Mirocha (1985), used gas chromatography-mass spectrometry to identify several of the unknown T-2 metabolites detected in the chicken excreta. Namely, 3′-hydroxy HT-2 toxin, the major metabolite present in excreta and organs, 3′-hydroxy T-2 toxin, 8-acetoxy and 15-acetoxy T-2 tetraol (also called 4-deacetyl neosolaniol) in addition to another monoacetylated isomer of T-2 tetraol were identified. Although most of the T-2 metabolites were found in the excreta, considerable amounts were also found in the liver. However, no trichothecenes were detected in

the heart and kidneys, and only trace amounts were detected in the lungs.

Pathotoxicologic effects of T-2 toxin in chickens

Clinical signs

Wyatt et al., (1972a) described a disease syndrome in 6-week-old broiler birds in several commercial flocks and fancy pigeons. The syndrome was characterized by raised proliferative caseous, yellowish-white, plaque-like lesions in the oral cavity. Morbidity varied from 10% to 25%, with up to 10% mortality and depressed growth rates in the remaining chicks. Later, Wyatt et al., (1972b) reproduced identical oral lesions in the chickens fed control feed which had been spiked with 4 and 16 ppm concentrations of T-2 toxin for three weeks. Chi et al. (1977a, 1978) also reported that similar oral lesions developed in birds fed contaminated diet. Speers et al. (1977) also reported the results of a 21-day feeding trial in which laying hens were fed purified T-2 toxin at 4, 8, and 16 ppm or corn invaded by *F. tricinctum*. Mouth lesions developed and their severity was proportional to the amount of toxin in the feed. These observations by Wyatt et al. (1972a,b); Chi et al. (1977a, 1978) and Speers et al. (1977) showed that appearance of oral lesions are excellent diagnostic indicators of T-2 intoxication in chickens. Large numbers of

bacteria, namely *Staphylococcus aureus*, *S. epidermitis*, and *Escherichia coli*, were isolated from the oral lesions (Hamilton et al., 1971; Wyatt et al., 1972a).

Neurological disturbances observed in chickens after ingestion of dietary T-2 toxin include abnormal positioning of the wings, hysteriod seizures, and an impaired righting reflex (Wyatt et al., 1973). Changes in brain catecholamine concentrations could be responsible for altered motor activity. Chi et al., (1981) administered T-2 toxin to 4-week-old chickens in a single dose of 2.5 mg/kg body weight and then determined the brain accumulations of dopamine, norepinephrine and serotonin over the next 48 hours. Although serotonin concentrations were not altered from those of controls, brain dopamine levels were almost double the control, while norepinephrine levels decreased by 25% in 25 hours after T-2 toxin administration. Since dopamine is a precursor to norepinephrine, it is possible that the toxin has some direct inhibitory effect on this conversion. The observed effects of T-2 toxin on brain monoamines and the resulting neurochemical imbalance may account for the physiological manifestation of trichothecene intoxication (MacDonald et al., 1988; Wang et al., 1993).

T-2 toxin acting as an emetic factor in moldy corn has occasionally been reported in ducklings (Ueno et al., 1974) and pigeons (Fairhurst et al., 1987; Ellison and Katsonis,

1973) but not in chickens. T-2 toxin at oral and intravenous sublethal doses of 0.72 and 0.15 mg/kg body weight, respectively, induced vomition in pigeons (Ellison and Katsonis, 1973). In an acute toxicity study in pigeons, Fairhurst et al., (1987) administered T-2 toxin sublingually in doses ranging from 0.2 to 10 mg/kg to pigeons. Vomiting began at 20-120 minutes and persisted for 3-4 hours, during which each pigeon vomited several times. The birds then became subdued and tremorous with marked ataxia; in some birds the wings were held in an abnormally low position, and most deaths occurred within 24 hours.

Acute Toxicosis

Saito et al., (1969) described the acute histological and hematological findings, both characterizing the "radiomimetic" effects of the toxic metabolites of *F. nivale* on proliferating cells when mice were injected subcutaneously or intraperitoneally, or given orally. Histological examination revealed marked cytotoxic changes in tissues with actively dividing cells, including the crypt cells and Paneth cells of small intestine, especially the duodenum, germ center of the lymphoid follicles in the spleen and lymph node, cortex of the thymus, and the hematopoietic cells of the bone marrow. The affected cells had degenerative changes, atypical mitosis, pyknosis,

hyperchromatosis of the nuclear membrane and fragmentation or karyorrhexis of the nuclei. It is now acknowledged that the trichothecenes affect multiple tissue target sites, with predominant lesions occurring in the gastrointestinal tract, lymphoid tissues, and the hematopoietic tissue of the bone marrow (Ueno, 1977; Hayes et al., 1980; Hoerr et al., 1981b; Bamburg, 1983; Otokawa, 1983). Experimental administration of either culture preparations of *Fusarium* or purified T-2 toxin, by various routes, produced the clinical picture of intoxication in rabbits (Gentry and Cooper, 1981), cats (Sato et al., 1975; Lutsky et al., 1978), rats and mice (Ueno, 1977; Schoental et al., 1979; Hayes et al., 1980), monkeys (Rukimini et al., 1980; Jagadeesan et al., 1982) chickens, ducks and pigeons (Wyatt et al., 1973, 1975; Chi et al., 1977; Joffe and Yagen, 1978; Hoerr et al., 1981, 1982a,b; Fairhurst et al., 1987), pheasants (Huff et al., 1992), bobwhite and Japanese quail (Ruff et al., 1992), and guinea pigs (DeNicola et al., 1978).

Hoerr et al., (1981b) reported the "radiomimetic" effects of T-2 toxin when 7-day-old broiler chicks were administered T-2 toxin via crop gavage, at a single dose of 2.0-2.5 mg/kg body weight. Severe, acute necrosis and depletion of lymphoid tissue and bone marrow were observed within 24 hours after treatment. Cell repletion with partial or complete restoration occurred by 72 to 168 hours

in the more severely necrotic tissues. Necrosis of tips of villi and crypts in duodenum, multiple foci of hepatocellular coagulative necrosis, and necrosis of feather and follicular epithelium were described.

Coagulation disorders

T-2 toxin is known to cause hematological disorders and produce serious coagulopathies as measured by various clotting assays in chickens and guinea pigs (Doerr et al., 1974, 1981; Cosgriff et al., 1984). Growth inhibition doses of T-2 toxin caused deficiency of coagulation factors VII, X, prothrombin and fibrinogen which led to prolonged prothrombin and activated partial thromboplastin clotting times. However pretreatment of animals with vitamin K had no effect , indicating that T-2 toxin does not act as a vitamin K antagonist. Platelet aggregation in whole blood was depressed in response to adenosine diphosphate and collagen. A sublethal dose of T-2 toxin affected the kallikrein-kinin system by depletion of prekallikrein, which indicated increased bradykinin levels in plasma (Johnsen et al., 1988).

Estimation of acute toxicity

The 10-day median lethal dose for 1-day-old broiler chicks ranged from 5.03 ± 0.25 to 5.25 ± 0.21 mg/kg body weight (Chi et al., 1977). In addition, the 10-day median

lethal dose for 8-week-old broiler chicks and laying hens was 4.97 ± 0.25 and 6.27 ± 0.42 mg/kg body weight, respectively. Within 4 hours after dosing, birds developed asthenia, inappetence, diarrhea, and panting. Death occurred within 48 hours after T-2 toxin administration. Sublethal doses of T-2 toxin resulted in a decrease in weight gain and feed consumption. The abdominal cavities of birds given lethal doses contained a white, chalk-like material, which covered the viscera. No salient pathologic or clinical signs were observed at 10 and 20 days after administration of T-2 toxin.

Hoerr and Carlton, (1981a) estimated the 72-hour single oral dose LD_{50} and 14 daily oral doses LD_{50} of T-2 toxin administered to 7-day-old broiler chicks as 4.0 and 2.90 mg/kg body weight, respectively.

Subacute and chronic effects

T-2 toxin caused losses in weight gain when fed either as commercial feed contaminated with *Fusarium poae* or *Fusarium sporotrichioides* or as pure T-2 toxin added to a balanced diet. Doses as low as 4 ppm of pure T-2 toxin in feed caused reduced feed consumption and decreased weight gains in broiler chicks (Wyatt et al., 1973; Chi et al., 1977a). Sublethal doses of T-2 toxin decreased feed consumption and weight gain proportionately with the amounts

of toxins administered (Wyatt et al., 1973; Chi et al., 1977a, 1978). The most marked effects occurred within the first 10 days of the treatment during a 30-day experiment. Other toxins that have been shown to have adverse effects on weight gains include HT-2 toxin, T-2 tetraol and deacetyl HT-2 toxin, all of which are metabolic byproducts of T-2 toxin (Chi et al., 1978).

T-2 toxin not only affects weight gain but often reduces egg production and quality. In subacute studies, single comb white leghorn laying hens fed T-2 toxin at a dose of up to 8 ppm significantly decreased feed consumption, egg production, and egg shell thickness (Wyatt et al., 1975; Chi et al., 1977). The fertility and progeny performance were not depressed by feeding T-2 toxin, but the hatchability of fertile eggs of hens fed 2 and 8 ppm was significantly lowered. Speers et al., (1977) also observed loss of body weight and decreased egg production when laying hens were fed purified T-2 toxin consumed at the rate of 16 ppm. Egg production of 8-month-old laying hens decreased precipitously during a 5-day period after the birds began to consume feed containing T-2 toxin and HT-2 toxin at 3.5 and 0.7 mg/kg, respectively (Shlosberg et al., 1984). However, when these hens were given uncontaminated feed, production returned almost to the expected value within 12 days.

In broiler chicks, T-2 toxin administered at the rate of 14 daily doses ranging from 1.5 to 3.0 mg/kg body weight/day caused emaciation, decreased body weight and hematocrit, malformed feathers, pale yellow beaks and legs and death (Hoerr et al., (1982). Lymphoid organs were atrophic, bone marrow was pale red or yellow, the liver was discolored yellow, and the crop mucosa was ulcerated.

During the first three weeks of a six-week trial, mice fed a balanced semipurified diet containing 20 ppm T-2 toxin had hypoplasia of lymhoid tissues, bone marrow, and splenic red pulp, resulting in anemia, lymphopenia, and eosinophilia (Hayes et al., 1980). After continued exposure to T-2 toxin, hematopoietic cells regenerated in bone marrow and splenic red pulp and became hyperplastic by six weeks. However, all lymphoid tissues remained atrophic. Granulopoiesis and thrombopoiesis occurred before erythropoiesis. The suppression of hematopoiesis was transient and did not lead to hematopoietic failure (Hayes et al., 1980).

Immunologic effects

Immunosuppression

The broad immunosuppressive effects of T-2 toxin and other trichothecenes on humoral and cell mediated immunity have been well documented (Otokawa, 1983; Taylor et al.,

1989; Corrier, 1991; Sharma, 1993; Pestka and Bondy, 1994a,b).

Exposure to trichothecene mycotoxins has been reported to cause severe damage to actively dividing cells within the bone marrow, lymph nodes, spleen, thymus, bursa of Fabricius, and the gastrointestinal tract (Saito et al., 1969; Ueno et al., 1971; Wyatt et al., 1973; Lutsky et al., 1978; Hayes et al., 1980; Hoerr et al., 1981; LaFarge-Frayssinet et al., 1981). Repeated exposure of animals to T-2 toxin has been shown to cause immunosuppression and may decrease their resistance to various infectious diseases such as salmonellosis in chickens (Boonchuvit, et al., 1975) and mice (Ziprin and McMurray, 1988; Tai and Pestka, 1988, 1990), tuberculosis (Kanai and Kondo, 1984; Ziprin and McMurray, 1988), listeriosis (Corrier and Ziprin, 1986), and herpes simplex virus infection in mice (Friend et al., 1983), and aspergillosis in rabbits (Niyo et al., 1988).

The increased susceptibility of T-2 toxin-exposed animals to gram-negative bacterial infections may be due to increased endotoxin sensitivity associated with increased absorption of the endotoxin (Taylor et al., 1991). Acute simultaneous exposure to T-2 toxin (per os) and endotoxin (intraperitoneal) in mice resulted in increased mortality, hypothermia, TNF-alpha production and thymic atrophy

compared to treatment with either T-2 toxin or endotoxin alone. It has also been shown that T-2 toxin toxemia is associated with elevated plasma levels of eicosanoids through its effect on the cyclooxygenase pathway of arachidonic acid with increased release of prostaglandin, PGE_2 and thromboxane (Shohami and Feuerstein, 1986) which play an important role in the pathophysiology of bacterial endotoxemia (Feuerstein et al., 1981; Slotman et al., 1985).

In addition to decreased host resistance, T-2 toxin may also suppress tumor defense mechanisms and impair host control of tumor cell growth (Schoental et al., 1979; Schiefer et al., 1987; Corrier and Norman, 1988).

Immunomodulation

The mechanisms by which T-2 toxin and other trichothecenes exert their specific effect on immunological function have been best characterized in the mouse model (Taylor et al., 1989). T-2 toxin and the other trichothecenes may induce other immunomodulating effects in addition to immunosuppression (Taylor et al., 1989; Pestka and Bondy, 1994).

Humoral immunity

Humoral immunity can be both stimulated or inhibited by T-2 toxin and the other trichothecenes. The effect of T-2

toxin on antibody production appeared dependent on the type and dose of antigen, and the frequency of toxin administration. Chronic exposure of mice to T-2 toxin enhanced a dose-dependent antibody response to T-cell-independent antigens (polyvinylpyrrolidone and dinitrophenyl-ficoll), but depressed antibody response to T-cell-dependent antigen, sheep red blood cells, (SRBC) (Otokawa et al., 1979, 1983; Lafarge-Frayssinet et al., 1979; Rosenstein et al., 1979, 1981). However, Holt and DeLoach (1988) reported that T-2 toxin suppressed the antibody response to both T-cell-dependent antigen, (SRBC), and also the T-cell-independent antigen, TNP-Lipopolysaccharide. In further contrast, dietary exposure of CD-1 mice to T-2 toxin did not alter T-cell-independent antibody responses to DNP-Ficoll or *Escherichia coli* lipopolysaccharide (LPS), but at 10 ppm, T-2 toxin enhanced the T-cell-dependent responses (Tomar et al., 1988a). In total, these findings showed that T-2 toxin can modulate immune response and that this modulation is attributable to the direct toxic effects of the toxin on the cells of the immune system.

Mice sensitized to SRBC and given intraperitoneal injection of a single dose of T-2 toxin (3 mg/kg body weight), 2-3 days after antigen (SRBC) sensitization developed higher SRBC titres than controls (Masuko et al.,

1977). The titres of antibodies to SRBC measured 8 or 15 days after SRBC sensitization were also elevated in mice intraperitoneally injected with T-2 toxin at a dose of 3 mg/kg body weight, either 2 days before or on the same day as the SRBC sensitization.

T-2 toxin may enhance or depress *in vitro* lymphoblastogenic response of B- and T-lymphocytes to various mitogens (Lafarge-Frayssinet et al., 1979; Buening et al., 1982; Friend et al., 1983; Holt et al., 1988b; Tomar et al., 1988a). T-2 toxin given at very low dose (0.05-1 ng/ml), increased mitogenic responses of splenic B- and T-lymphocytes to phytohemagglutinin (PTH) and LPS. However, at higher doses (2-10 ng/ml) of T-2 toxin, mitogenic responses of splenic B- and T-lymphocytes from animals stimulated by PTH, LPS and concanavalin A (Con A) mitogens were depressed. These results suggested that both B- and T-lymphocytes were sensitive to T-2 toxin. The effect on lymphoblastogenic response was transient when CD-1 mice were repeatedly exposed to low levels of T-2 toxin (Taylor et al, 1985). Tomar et al., (1988b) reported that various lymphocyte subpopulations of human peripheral blood have different susceptibilities to T-2 toxin. Mitogenic response was inhibited at a lower concentration (1.6 ng/ml) of Con A mitogen as compared to PTH (2.4 ng/ml) and pokeweed mitogens (PWM). The effects of T-2 toxin on

mitogenic responses of murine splenic cells *in vitro* appeared to be dependent on the type of mitogen used in the lymphoblastogenesis assay (Taylor et al., 1987). Murine splenic cells cultured with various mitogens and exposed to T-2 toxin (10^{-11} to 10^{-10}M) after 24 hr increased ^{3}H-thymidine uptake by splenic cells. Stimulation by PWM increased dramatically while the response to LPS was increased to a lesser extent. Conversely, exposure to T-2 toxin decreased T-cell responses to both PHA and Con A (Taylor et al., 1987).

T-2 toxin affected the levels of specific classes of immunoglobulins when various experimental and domestic animals were repeatedly exposed to low levels of T-2 toxin. A chronic dietary exposure of mice to T-2 toxin caused a significant dose-dependent increase in the number of spontaneous antibody-secreting cells in the spleen as detected by the protein A plaque assay (Cooray and Lindahl-Kiessling, 1987; Pestka and Bondy, 1990; Schiefer et al., 1987). The reduction in the suppression of B-cell growth and stimulation of B-cells caused by erythropoiesis or activated macrophages could be responsible for the increase in antibody production (Cooray and Lindahl-Kiessling, 1987). In contrast, repeated exposure of mice to T-2 toxin administered orally or injected intraperitoneally at a dose of 2.5 mg/kg body weight caused a decrease in titres of

antibodies to SRBC and plaque forming cells (Rosenstein et al., 1979; Taylor et al., 1985). Dose-dependent antibody production assessed by the number of plaque-forming cells, was not affected by T-2 toxin up to an *in vivo* dose of 0.5 mg/kg body weight (Taylor et al., 1985). However, the number of plaques per 10^6 splenic cells was depressed at a dose of 2.5 mg/kg body weight.

T-2 toxin has been shown to decrease immunoglobulin synthesis and antibody response in domestic and laboratory animals. Long term exposure to dietary T-2 toxin decreased antibodies to SRBC (IgG and IgM levels) in CD-I mice (Taylor et al., 1985) and in monkeys (Jagadeesan et al., 1982). The levels of immunoglobulin G, M, and A were significantly lower in T-2 toxin-treated calves than in untreated controls (Mann et al., 1982, 1983, 1984; Osweiler et al., 1981).

Using peanut agglutinin (PNA) receptors and surface immunoglobulin (SIG) as surface markers for bovine T- and B-cells respectively, Mann et al., (1984) reported that subclinical levels of T-2 toxin at 0.3 and 0.5 mg/kg/day for 56 and 18 days, respectively, caused a slight increase in (SIG^+) B-cells. However, T-cell (PNA^+) numbers were not affected at the low dose or transiently reduced in calves treated at 0.5 mg/kg T-2 toxin fed orally for 28 days. Antibody synthesis is also regulated by T cells.

Cell-mediated immunity

Delayed-type hypersensitivity

Masuko et al., (1977) and Otokawa et al., (1979), reported an enhancing effect of T-2 toxin on the development of delayed-type hypersensitivity (DTH) to SRBC cells in mice. The dose, timing, and frequency of T-2 toxin administration were crucial factors in the enhancing effect of T-2 toxin. When mice received 3 mg/kg T-2 toxin 2 or 3 days before sensitization with subcutaneous injection of SRBC, no appreciable effect was observed on the DTH response (footpad swelling) (Masuko et al., 1977). Sensitization of mice with the toxin administered 3 hours before antigen injection did not cause significant depression in DTH responses by 7 and 14 days later. However, when the toxin was injected 2 days after sensitization, marked enhancement of DTH response was observed. In mice treated with the toxin 3 days after sensitization, DTH responses were also enhanced but to a lower degree than those treated on day 2. The optimal time for toxin administration was 2 days after antigen stimulation.

A single dose of T-2 toxin larger than 3 mg/kg body weight was required to induce significant enhancement of DTH response (Masuko et al., 1977). The administration of the

toxin only slightly affected or caused no appreciable suppressive effect on the hemagglutinating antibody response at the time of the DTH response (Masuko et al., 1977). These findings led to the hypothesis that T-2 toxin seems to preferentially inhibit proliferation of a subset of T lymphocytes which have a suppressor function. When spleen cells from mice, which had received a tolerogenic high dose of SRBC 14 days earlier, were transferred to unsensitized mice, DTH response of the recipients was significantly suppressed on day 7, while spleen cells from mice further treated with T-2 toxin 2 days later showed significantly less suppressive activity in the recipients (Otokawa et al., 1979).

Results of these spleen cell transfer experiments showed that suppressor cells in the DTH response were produced by an intravenous injection of a large dose of sRBC, but decreased by T-2 toxin treatment. The decreased suppressor activity in the spleen seems to explain the enhancement of DTH response observed in mice treated with T-2 toxin after the intravenous injection (Otokawa et al., 1979).

T-2 toxin could not completely inhibit the development of suppressor cells for DTH since there was partial recovery of tolerance by T-2 toxin. The inhibition of tolerance induction by T-2 toxin may likely be due to the

susceptibility of the suppressor cells or their precursors to T-2 toxin (Otokawa et al., 1979). T-2 toxin probably has an inhibitory effect on the generation of suppressor cells by inhibiting development of their precursors, since T-2 toxin was effective only when administered within a few days after antigen stimulation (Otokawa et al., 1979). In contrast, Taylor et al., (1985) reported a dose-dependent depression in DTH when low levels of T-2 toxin (0.02-5.0 mg/kg body weight) was fed intermittently to CD-1 mice sensitized intraperitoneally on day 14 and challenged 5 days later.

Topical application of T-2 toxin inhibits the contact hypersensitivity response in mice (Blaylock et al., 1993). T-2 toxin reduced the ear-swelling response to oxazolone challenge when the toxin was applied topically at or within 1 hr. after challenge. The toxin significantly reduced MHC class 11(1A) expression and antigen presentation. One mechanism of action of T-2 toxin in reducing the contact hypersensitivity response is via inhibition of protein synthesis and effective antigen presentation by epidermal Langerhans cells (Blaylock et al., 1993).

Graft rejection

T-2 toxin caused delayed graft rejection when mice were given daily intraperitoneal injections of the toxin at 0.75

mg/kg body weight for 7 days prior to receiving allografts and for 2 to 4 times a week for 20 days after the graft (Rosenstein et al., 1979).

Role of Macrophages

T-2 toxin administered as a single dose that caused marked lymphoid depletion, and suppressed or enhanced *in vivo* macrophage phagocytic activity in antigenically sensitized mice (Corrier et al., 1987a). Enhancement or suppression of phagocytosis was a function of the time T-2 toxin was administered in relation to antigenic stimulation or challenge. T-2 toxin had no effect on the viability or phagocytic activity of resident peritoneal macrophages to sRBC in nonsensitized mice (Corrier et al., 1987a). Thus, resident peritoneal macrophages are resistant to toxic effects of T-2 toxin *in vivo*. However, a significant increase in phagocytic activity occurred in cells from mice treated with toxin and subsequently sensitized with SRBC. In contrast, phagocytosis of SRBC was significantly suppressed in cells from mice treated with T-2 toxin after sensitization (Corrier et al, 1987a).

In vitro viability studies have shown that exposure of alveolar macrophages to submicromolar concentrations of T-2 toxin for 20 hr influenced alveolar macrophage viability, cell number, and viability index (Gerberick and Sorenson, 1983). In addition, T-2 toxin is toxic to alveolar

macrophages in monolayer cultures and inhibits macrophage phagocytic activity *in vitro* (Gerberick et al., 1984).

Both single-dose and successive treatments of mice with T-2 toxin by oral gavage enhanced the respiratory burst activity of macrophages and pre-inoculation treatment with T-2 toxin also caused a significant increase in the number of peritoneal cells (Cooray and Jonsson, 1990).

Cell-mediated resistance

Treatment of mice with T-2 toxin after *Listeria* challenge suppressed resistance to listeriosis, leading to rapid growth of *Listeria* and significant increases in mortality (Corrier and Ziprin, 1986b; Corrier et al., 1987b). Conversely, pretreatment of mice with T-2 toxin prior to challenge with *Listeria* markedly enhanced resistance to listeriosis, as shown by significant reduction in *Listeria*-induced mortality in T-2 toxin-treated mice (Corrier and Ziprin, 1986a). The incidence of *Listeria*-induced mortality was dependent on T-2 toxin dose, and progressively decreased with increasing dose.

Despite the fact that T-2 toxin caused significantly reduced thymus and spleen weights, bone marrow cellularity, the total number of circulating leukocytes, lymphocytes, and neutrophils, the enhanced resistance to listeriosis was accompanied by a significant increase in the influx of

macrophages into *Listeria*-elicited peritoneal exudates (Corrier et al., 1987b). In addition, *in vivo* phagocytosis of SRBC by peritoneal macrophages was significantly increased in T-2 toxin-treated mice that were sensitized with SRBC. The enhancement of resistance to listeriosis in mice pretreated with T-2 toxin was associated with increased migration or activation of macrophage effector cells (Corrier et al., 1987b). Increased concentration of serum amyloid P-component, an acute-phase reactant protein that increases macrophage listericidal activity have also been associated with enhanced resistance (Singh et al., 1986; Ziprin et al., 1987). In contrast, T-2 toxin had no effect on either the course of infection or serum amyloid P-component concentration in mice challenged with *Salmonella typhimurium* (Ziprin et al., 1987). In another study, Cooray and Jonsson, 1990, reported that T-2 toxin had a modulatory effect on the cell-mediated immune system. Pretreatment of mice with a single dose of T-2 toxin by oral gavage caused an enhanced resistance to common mastitis pathogens, *E. coli* or *S. aureus*. This enhanced resistance was associated with migration and activation of macrophages into sites of bacterial infection. In addition, T-2 toxin reduced the virulence of both pathogens.

The exact mechanism of action involved in T-2 toxin-enhanced resistance to listeriosis is not known. One theory

that had been proposed to explain resistance to listeriosis in thymus-deprived mice (Cheers and Waller, 1975; Newborg and North, 1980) and in mice treated with cytotoxic drug Cyclophosphamide (Tripathy and Mackaness, 1969; Luster et al., 1981) was attributed to the removal of a radiosensitive population of regulatory T cells. Suppressor T cells are short-lived lymphocytes that are more susceptible to cytotoxic agents than are other subpopulations of lymphocytes (Mitsuoka et al., 1976; Chan et al., 1977). Therefore, enhanced host resistance in mice treated with T-2 toxin prior to *Listeria*-challenge may have been caused by depletion or impaired function of a regulatory T suppressor cell population and a subsequent enhancement of macrophage activity.

Exposure to T-2 toxin may also inhibit cell-mediated immune function by causing defects at multiple hematopoietic sites or compartments. Mice exposed to T-2 toxin had an apparent dose-dependent delay in thymocyte maturation or differentiation, characterized by significant increases in the percentage of double-negative ($CD4^-8^-$) thymocytes and significant decreases in the percentage of double-positive ($CD4^+8^+$) thymocytes (Smith et al., 1994). The effects of T-2 toxin on multiple hematopoietic compartments involved in the production of T-lymphocytes may contribute to the peripheral T-cell lymphocytopenia and T-cell mediated

immunosuppression produced by the T-2 toxin. Thymic atrophy was characterized by significant reduction in the total number of cells within all phenotypes defined by CD4 and CD8 cell surface antigen expression. The bone marrow from T-2 toxin-treated mice had a highly significant hypocellularity, indicating that this hematopoietic compartment may also be a target. A non-significant reduction in overall splenic celularity was observed. However, there was a significant decrease in the total number of both B- and T-lymphocytes present within the spleen (Smith et al., 1994).

Prenatal exposure of mice to T-2 toxin resulted in significant fetal thymic atrophy and a significant reduction in CD44 and CD45 fetal liver prolymphoid cell subpopulations (Lafarge-Frayssinet et al., 1990; Holladay et al., 1993). These results showed that T-2 toxin easily passes the placental barrier and that lymphocyte progenitors, compared with thymocytes, represent highly sensitive targets for T-2 toxin, and are therefore responsible for thymic atrophy (Holladay et al 1993). The authors concluded that direct cytotoxic effects of T-2 toxin had limited effect on thymic atrophy, as evident by expression of CD4, CD8, and TCR cell-surface antigens.

T-2 toxin has also been shown to impair migration-chemotaxis and phagocytosis of neutrophils in humans, cattle

and rats (Buening et al., 1982; Gerberick and Sorenson, 1983; Yarom et al., 1984) and rabbits (Niyo et al., 1988).

Antiviral Activity

T-2 toxin and the other trichothecene mycotoxins (diacetoxyscirpenol and neosolaniol) inhibit herpes simplex virus type 2 (HSV-2) replication in human epidermoid carcinoma No. 2 (HEp-2) cells by blocking viral protein syntheses and not by inhibiting adsorption and penetration of virions into the host cells (Okazaki et al., 1988a). The addition of these toxins within 4 hours after HSV-2 infection was necessary for the inhibition of virus replication. Viral polypeptides synthesized in HSV-2-infected cells treated with the toxins was analyzed by immunoblotting using rabbit antiserum to HSV-2. The results showed that syntheses of early viral proteins were greatly inhibited when the toxins were added 1 hr after infection. Late viral proteins were also inhibited by the addition of T-2 toxin and the other trichothecenes, 4 to 6 hr after infection. The toxins added after the completion of the late viral protein syntheses did not significantly affect the HSV-2 replication. However, viral RNA synthesis was not inhibited when the toxins were added 1 hour after infection (Okazaki et al., 1988a). The inhibition of HSV-2 replication by the trichothecenes is closely correlated with blocking of viral protein synthesis (Okazaki et al., 1988b). In time

course studies of virus growth and viral protein synthesis in HSV-2-infected HEp-2 cells, the presence of T-2 toxin and other trichothecenes completely inhibited viral plaque formation. In HSV-2-infected cells treated with the toxins, neither virus replication nor virus-specific polypeptides was noted although two major nonspecific polypeptides were detected in treated cells as wells as in mock-treated cells.

Okazaki et al. (1992) reported that both hydrolysis at the C-4 position, hydroxylation at the C-3′ position, and hydrolysis at the C-8 position of T-2 toxin are responsible for the reduction in antiviral activity. The metabolic conversion of T-2 toxin to 3′-hydroxy HT-2 toxin or to T-2 tetraol decreases the antiviral activity. In plaque-reduction tests, the HSV-2 plaque formation was inhibited by the trichothecenes in a dose-dependent manner.

Effect of T-2 toxin on vaccinal immunity

Despite the fact of the broad immunosuppressive effects of T-2 toxin in various domestic animals, poultry and laboratory animals, there are very few studies on the effect of the toxin on vaccinal immunity. Repeated exposure of mice to T-2 toxin prior to vaccination with BCG (*Mycobacterium bovis*) against *Mycobacterium tuberculosis* resulted in depressed vaccinal immunity, as evidenced by increased multiplication with larger viable counts of organisms

recovered from the lungs and spleen as compared to the non-treated BCG group (Kanai and Kondo, 1984). In contrast, Ziprin and McMurray, (1988) reported a consistent and significant influence of BCG in reducing the number of virulent tubercle bacilli present in the lungs, but T-2 toxin treatment did not influence the efficacy of the BCG vaccine. The number of organisms found in the lungs of mice that received T-2 toxin without subsequent BCG vaccination was not significantly different from the number present in untreated mice. Similarly, the number of organisms found in the lungs of T-2 toxin-treated vaccinated mice did not differ significantly from the number found in vaccinated mice that had not been treated with toxin. In another study, a single oral treatment with T-2 toxin 7 days before intraperitoneal challenge with *Mycobacterium bovis* decreased resistance to infection (Ziprin and McMurray, 1989). In addition, T-2 toxin treatment had no effect on BCG infection when this was initiated by inhalation of microbial aerosol.

No apparent effect of T-2 toxin on either antibody production or the size of the thymus, spleen or the bursa of Fabricius in chickens immunized with a bacterin against *Pasteurella multocida* and treated with 10 ppm of T-2 toxin for 4 weeks after hatch (Richard et al., 1978).

Marek's Disease

Marek's disease (MD) is a herpesvirus-induced lymphoproliferative neoplastic disease of chickens characterized by infiltration of various nerve trunks and visceral organs with pleomorphic lymphoid cells. The disease has recently been reviewed by Witter and Calnek, 1991. Marek's disease is economically important only in chickens. Turkeys, quails and other species have limited susceptibility. The disease most commonly occurs in young, sexually immature chickens 2 to 7 months old, but can occur at virtually any age beyond 3 weeks. The disease occurs throughout the world and virtually all flocks are exposed to the causative virus (Witter and Calnek, 1991).

Etiology

Marek's disease virus is divided into 3 serotypes based on indirect immunofluorescence assays and agar gel precipitin tests (Bulow and Biggs, 1975a,b; Schat and Calnek, 1978). Lee et al. (1983) confirmed this serotype classification through the use of serotype-specific monoclonal antibodies. The oncogenic viruses form serotype 1, whereas the nononcogenic viruses belong to serotype 2. Herpesvirus of turkeys (HVT) isolates are classified as serotype 3. The oncogenic strains of Marek's disease virus (MDV) are further classified into 3 pathotypic groups,

namely very virulent (vvMDV), virulent (vMDV) and mild (mMDV). The vvMDV strains are very highly oncogenic and may induce visceral and neural lesions in vaccinated, genetically susceptible and in non-vaccinated, genetically resistant strains of chickens (Witter et al, 1980; Schat et al, 1982; Powell and Lombardini, 1986). vMDV strains are oncogenic and cause high incidences of MD in susceptible strains, but none or low incidences of lesions in resistant strains of chickens (Purchase and Biggs, 1967; Eidson and Schmittle, 1968). The mMDV strain causes minimal lesions, often only in very susceptible strains of chickens (Rispens et al., 1972; Smith and Calnek, 1973).

Serotype 2 MDV is non-oncogenic; however, strains such as SB-1 may cause cytolytic infections in lymphoid organs (Schat et al., 1978; Calnek et al., 1979).

Witter et al., (1970) isolated a non-oncogenic herpesvirus from turkeys and designated it herpesvirus of turkeys (HVT). HVT is highly effective as a cell-associated or cell-free vaccine in preventing MD (Okazaki et al., 1970; Witter et al., 1970; Calnek et al., 1970b; Purchase et al., 1972a,b). The relative ability of HVT to protect against MD is the basis for the differentiation between very virulent and virulent serotype 1 strains. Bivalent vaccines that include both serotype 2 and serotype 3 in their formulation are also protective against MD (Witter, 1987, 1989).

Pathogenesis

Several extensive reviews of the pathogenesis of MD have been published (Payne and Rennie, 1973; Payne et al, 1976; Murthy and Calnek, 1978; Calnek, 1985; Calnek and Witter, 1991).

During the pathogenesis of MDV, four major virus-host cell interactions occur. These include: (a) entrance of virus into the cell after infection with cell-free virus leading to the establishment of infection; (b) virus replication resulting in cytolytic infection that coincide with permanent immunosuppression; (c) establishment of latent infection; and (d) transformation of infected lymphoid cells that may or may not progress to lymphoma formation.

Exposure to MDV, generally by inhalation, leads to the first significant event in major lymphoid organs such as the spleen, thymus, and bursa of Fabricius. The virus is carried to the lymphoid organs, possibly by alveolar macrophages, even though macrophages are not susceptible to infection (Bulow and Klasen, 1983). In the lymphoid organs, a semi-productive or productive-restrictive infection develops in which viral internal antigens (VIA) can be detected by immunofluorescence or precipitin tests, and naked intranuclear virions can be found by electron microscopy. Complete replication in which enveloped virions

are produced occurs rarely; however, virus can be isolated. Cytolysis is a consequence of this infection.

B-lymphocytes are the primary lymphocytic target cells for the early cytolytic infection in lymphoid organs, although a few T lymphocytes may also be infected (Shek et al., 1983; Calnek et al., 1984). Shek et al. (1983) showed by dual fluorescence studies that the cells containing VIA were mostly positive for surface IgM(μ) and Ia (T-lymphocyte). Calnek et al. (1984) also described *in vitro* infection of lymphocytes in which the B lymphocytes became infected and underwent a productive-restrictive infection. Thymocytes were significantly less susceptible to infection (Calnek et al., 1985). This type of infection can be detected *in vivo* in lymphocytes, epithelial cells, and occasionally tumor cells. *In vitro* infection of permissible cell cultures like chicken kidney cells (CKC), duck embryo fibroblasts (DEF), chick embryo fibroblasts (CEF), or B lymphocytes can result in a productive-restrictive response. The maximal cytolytic response of cells in the spleen generally occurs between 3-5 days post-infection (PI). The necrotizing effects of this early infection provoke an acute inflammation characterized by infiltration by macrophages and granulocytes, and by reticulum cell hyperplasia

resulting in an enlarged spleen (Payne et al., 1976). Ultimately, there is atrophy of the bursa and thymus.

During the 5-7 day PI period, there is a switch from cytolytic to latent infection. The latent infection primarily involves Ia-bearing (activated) T lymphocytes, but some B lymphocytes may also be latently infected (Shek et al., 1983; Calnek et al., 1984).

The latent infection is characterized by an absence of VIA or other features of productive-restrictive infection. However, virus may be rescued after co-cultivation of the cells on permissive monolayer cell cultures with VIA expression demonstrated after 24-48 hours of cultivation. This period marks the first detection of cell-associated viremia (Adldinger and Calnek, 1973; Payne and Rennie, 1973), the first appearance of lymphocytes bearing the putative tumor-associated antigen, membrane associated tumor surface antigen (MATSA), (Powell et al., 1974; Witter et al., 1975) in the spleen (5 days PI) then in the bursa of Fabricius and thymus (7 days PI)(Murthy et al., 1978). Coinciding with the switch to latency, the first antibody and cell-mediated immune responses become evident (Higgins and Calnek, 1975; Sharma et al., 1978). Latently-infected peripheral blood lymphocytes are means by which MDV is disseminated to other tissues after the early phase of cytolytic infection in the lymphoid organs. During the

second week of infection, localized foci of infection appear in many tissues of epithelial origin such as kidney, pancreas, adrenal gland, and proventriculus. Focal necrosis, with VIA and intranuclear inclusion bodies, results from these productive-restrictive infections which appear to spread from cell to cell causing inflammatory reactions around the affected cells (Calnek, 1986).

In the skin, cells of the feather follicle epithelium (FFE) undergo extensive infection and all cells surrounding the feather shaft are nearly involved. Unlike virtually all other infected tissues, the FFE infection is characterized as a fully-productive infection resulting in the production of enveloped virions which are infectious in the cell-free state (Calnek et al., 1970).

At about 7 days PI, there is a transient loss of mitogen responsiveness which is followed by a permanent immunosuppression involving both humoral and cell-mediated immunity (CMI). This phase is associated with the reappearance of cytolytic infection in lymphoid organs, and infiltrations of mononuclear cells in nerves (Lee et al., 1978a,b; Schat et al., 1978; Powell, 1980).

Two types of neuropathy associated with MDV infections have been described by Payne (1981). The earliest changes consisted of infiltrative macrophages and a few small

lymphocytes which progress by 2-3 weeks PI to predominantly lymphoid cells. Ultimately, neoplastic infiltrations with large neoplastic lymphocytes mixed with pleomorphic lymphoid cells termed "A-type" lesions can be seen. Demyelination may also develop at that time, and some A-type lesions may become largely replaced by "B-type" lesions, characterized by edema and sparse cellular infiltrations associated with inflammation.

The ultimate response in the disease is neoplastic transformation of infected T-lymphocytes. These cells are characterized as Ia-bearing, lymphoblastic T-lymphocytes which carry multiple copies of the MDV genome but appear to have only very limited expression of that genome. Transformed cells, which express MATSA, are the basic offending cells in MD lymphomas, but there are many other cellular constituents of tumors such as B-cells, T-cells, macrophages, and plasma cells. Grossly observable lymphomas involving any of a wide variety of visceral organs, skin, muscle, and nerves, can be seen as early as the 3rd week PI or may not appear until several weeks later, depending on various factors such as virulence of the MDV strain and host susceptibility (Sevoian et al., 1962; Payne and Biggs, 1967).

The other known cellular alteration associated with MDV infection occurs in cells of the arterial wall (Fabricant et

al., 1978). Fatty-proliferative lesions involving smooth muscle cells remarkably like those in human atherosclerosis appear to result from a non-productive MDV infection which may alter lipid metabolism (Fabricant et al., 1981).

Clinical signs

Signs associated with Marek's disease have been described by Biggs (1968) and recently reviewed by Calnek and Witter, (1991). In general, they range from asymmetric progressive paresis to complete paralysis of one or more of the extremities. Signs may vary from bird to bird, depending on the nerves affected. Lameness or paralysis of the legs, wings, neck, eyelids, or other parts of the body have been reported. Vagal involvement can result in paralysis and dilation of the crop and/or gasping. Other signs include muscular atrophy, diarrhea, weight loss, possible anemia, and blindness resulting from involvement of the iris. Young chickens affected by the acute form may show signs of anorexia and depression for 1-2 days with early and high mortality (Biggs and Payne, 1967; Darcel, 1973). About 8-12 days PI with MDV, a temporary paralytic syndrome (transient paralysis) may occur for 1-2 days and followed by recovery has been described by Cho et al., (1970); Kenzy et al., (1973). Swayne et al., (1989a,b) characterized transient paralysis as vasculitis with

intramural pseudocyst formation primarily in the cerebellar white matter and also in the nuclei of medulla resulting in leakage of IgG and albumin and vacuolation of the neuropile (vasogenic edema) in brains from chickens with clinical signs of MD-induced transient paralysis.

Gross lesions

The pathologic changes observed in MD have been well summarized (Payne et al., 1976; Payne, 1985). Nerve lesions are the most prominent feature of MD. The affected nerves are characterized by localized or diffuse enlargement, loss of cross-striations and grey or yellow discoloration with an occasional edematous appearance. Visceral organs, primarily the gonads, liver, lungs, heart muscle, and skin, may have diffuse or focal grayish-white lymphoid tumors. The thymus and bursa of Fabricius are usually atrophic. A diagnostic feature in MD is the diffuse involvement of the bursa of Fabricius as opposed to the multinodular tumors characteristic of lymphoid leukosis, another important lymphoproliferative disease commonly seen in chickens.

Control of Marek's Disease by Vaccination

The principles of vaccination have been reviewed by Witter, (1985). Three classes of viruses have been used to protect chickens against MD, namely (a) herpesvirus of turkeys (HVT) originally isolated from turkeys (Kawamura et

al., 1969; Witter et al., 1970). HVT has been established as the standard worldwide vaccine for MD because of its high protective efficacy, lack of oncogenicity, its self-limiting infection, good replication ability *in vivo* or *in vitro*, and its availability as cell-free and cell-associated preparations. Serotype 2 MDV, a non-oncogenic strain of MDV (Zander et al., 1972; Schat and Calnek, 1978) and attenuated serotype 1 MDV (Churchill et al., 1969; Rispens et al., 1972) have also been used to vaccinate chickens against MD.

The SB-1 strain of serotype 2 MDV is usually used in combination with HVT as a bivalent vaccine (Witter, 1987), since SB-1 and HVT vaccines administered together afforded greater protection than did either virus alone (Schat and Calnek, 1982; Witter, 1982). Outbreaks associated with very virulent MDV (vvMDV) strains in flocks vaccinated with HVT can often be controlled by vaccination with polyvalent vaccines composed of all three viral serotypes or with bivalent vaccines (serotypes 2 and 3) (Witter, 1982; Calnek et al., 1983; Witter and Lee, 1984). The improved efficacy of a bivalent vaccine against challenge with vvMDV has been documented (Schat et al., 1982; Vielitz and Landgraf, 1985). The phenomenon through which two vaccines protect better than either alone has been termed "protective synergism" and is best demonstrated with viruses of serotype 2 and 3 (Witter, 1987, 1989). The FC126 strain of HVT coupled with

either strain SB-1 or strain 301B/1 of serotype 2 MDV is the most common bivalent MD vaccine used in the field.

Vaccinal Immunity Against Marek's Disease

Specific immune responses involved in protection against MD following vaccination have been reviewed by Payne et al., 1976; Payne, 1982. It was originally proposed that immunity to MD is directed against both viral and tumor-associated antigens and both humoral and cell-mediated immune mechanisms appear to be involved. The key points discussed included the abrogation of vaccinal immunity by chemical immunosuppression (Purchase and Sharma, 1974; Payne et al., 1978), induction of immune responses against viral and tumor-associated antigen and induction of protective immunity with inactivated vaccines. Immunity induced by vaccination seems to have antiviral and antitumor components, and Payne et al. (1976, 1978) proposed a "two-step" mechanism of resistance. The first step involved humoral and cell-mediated immune responses directed against viral antigens which envisaged an initial resistance to virus replication and spread (Purchase et al. 1972; Payne et al., 1976). The reduced level of virus activity would subsequently result in a lowered incidence of malignant transformation, and at the same time the lymphoid system would be spared the immunosuppressive effects of MDV. The

second step consisted of cell-mediated immunity against MD tumor antigens (MATSA), which have been shown to be generated by HVT on lymphocytes and thus might induce anti-tumor cell-mediated immunity in the vaccinated chicks leading to immunological rejection of transformed cells (Witter et al., 1976; Powell and Rennie, 1978; Schat and Calnek, 1978; Sharma et al., 1978).

Both virus- and tumor-specific antigens stimulate immune responses. Successful immunization against MD using inactivated viral or tumor antigens have been reported in experiments where it was possible to dissociate the immune responses to the two types of antigens. Inactivated soluble or insoluble proteins extracted from infected cultured cells, or inactivated whole infected cells, were found to protect against MD by inducing immunity against viral antigens (Kaaden et al., 1974; Lesnik et al., 1975). Similarly, inactivated lymphoblastoid line cells were partially protective by virtue of an immune response against transformed cells (Powell, 1975; Powell and Rowell, 1977; Murthy and Calnek, 1979). Both types of inactivated vaccine inhibited the appearance of MATSA-bearing cells and prevented lymphomas from developing.

The immunity induced by viral antigens was characterized by the development of neutralizing antibodies, lowering of MD viremia, inhibition of virus replication and

the early degenerative changes in lymphoid organs (Calnek, 1972; Payne and Rennie, 1973; Burgoyne and Witter, 1973; Higgins and Calnek, 1975).

The importance of the immune system, humoral or cell-mediated, in vaccinal immunity against Marek's disease is indicated by the development of various immune responses against viral and tumor antigens in vaccinated chicks and by the abrogation of vaccinal immunity by surgical or chemical bursectomy and thymectomy. T-cell-mediated immune response appear relatively more important than humoral responses since immunity was abrogated by surgical thymectomy (Gupta et al., 1982) or temporarily abrogated by cyclophosphamide treatment (Purchase and Sharma, 1974; Payne et al., 1978), but was not affected (Gupta et al., 1982; Else, 1974) or was only slightly affected (Rennie et al., 1980) by bursectomy. Immunity to viral antigens may be more readily induced and thus more important than immunity to tumor antigens since chickens inoculated with inactivated preparations of fibroblasts productively-infected with virulent serotype 1 were better protected against MD lymphomas than chickens inoculated with inactivated lymphoma or lymphoma line cells bearing tumor-associated antigens with few or no viral antigens (Powell and Rowell, 1977; Murthy and Calnek, 1979). Thus, it is probable that normally all branches of the immune system are involved in resistance to MD and there may

be no absolute requirement for any form of immune response in order to confer resistance to host. Under appropriate experimental conditions, a degree of resistance might be provided by cell-mediated immunity, antibodies, macrophages, or natural killer cells alone.

Humoral immunity against viral antigens may also play an important role in vaccinal immunity. Virus neutralizing antibody titres were correlated with resistance among different genetic lines of chickens (Calnek, 1972). Bursectomy was partially effective in reducing the protective efficacy of serotype 3 vaccine in one trial (Rennie et al., 1980). Antibodies induced by vaccine viruses are active in antibody-dependent cellular cytotoxicity reactions (Kodama et al., 1979; Ross, 1980).

Cell-mediated immune responses directed against tumor-associated antigens are also an important component in protection induced by vaccine viruses. Infection with vaccine viruses induces lymphocytes cytotoxic to target cells from MD lymphoblastoid cell lines and induces resistance to virus-negative MD tumor transplants (Mason and Jensen, 1971; Sharma, 1977; Sharma et al., 1978)). Vaccines consisting of inactivated preparations of lymphoma cells and lymphoblastoid MD tumor cell lines have provided resistance to tumor induction (Powell, 1975; Powell and Rowell, 1977; Murthy and Calnek, 1979).

Humoral immune responses to tumor-associated antigens have not been detected in chickens following vaccination. However, antibodies can be induced following hyperimmunization with lymphoma cells (Witter et al., 1975; Stephens et al., 1980).

Vaccinal immunity appears to be based primarily on cellular responses to viral antigens, supplemented by humoral antiviral and cellular anti-tumor immune responses. Also contributing are natural immune mechanisms such as natural killer cells and macrophages. Natural killer cell activity was reported to be inhibited in susceptible chickens during the development of MD, but activity was enhanced in genetically resistant or vaccinated chickens following infection with MDV (Sharma and Coulson, 1979; Sharma, 1981, 1983). Silica treatment of chicks induced proliferation of macrophages in the peripheral blood and consequently restricted virus replication or spread. In contrast, inoculation of antimacrophage serum suppressed macrophage function *in vivo* and was associated with elevated virus titres and increased tumor incidence, supporting an immunosurveillant role for macrophages (Haffer et al., 1979).

Marek's disease vaccine failure

Stress factors associated with Marek's vaccine failure

Until the early 1970s, MD was an economically important disease causing losses of billions of dollars worldwide due to condemnations in broiler chickens and reduced egg production and mortality in layer chickens (Purchase, 1985). The introduction of vaccines, and the common practice of vaccinating 1-day-old chicks drastically reduced the economic losses associated with MD (Purchase, 1973). Several vaccines have been developed but the one most widely used is HVT isolated by Witter et al., (1970).

Failure of vaccines to provide the expected protection levels ("vaccination breaks") have occasionally been reported. Such vaccination breaks have been attributed to several factors including inadequate methods of vaccine production or mismanagement of vaccines at hatcheries causing loss of vaccine titres during thawing, reconstitution and use (Thornton et al., 1975; Halvorson and Mitchell, 1979). The influence of genetic resistance to MD or the presence of maternal antibodies against HVT have also been suggested as possible reasons of vaccination failures (Calnek and Smith, 1972; Spencer et al., 1974). The common poultry operation practices such as multi-age rearing and insufficient cleaning between generations of chickens could

also be responsible for early exposure to MDV before vaccinal immunity develops (Good, 1983; Price, 1983; Witter et al., 1984).

Okazaki et al. (1973) investigated the possibility that vaccination breaks were caused by strains of MDV against which HVT was unable to protect. The authors failed to isolate any MDV strains able to depress HVT vaccinal immunity. However, many researchers since then have isolated highly oncogenic strains of MDV (Eidson et al., 1978, 1981; Witter et al., 1980; Schat et al., 1981). Chickens vaccinated with HVT and subsequently challenged with such highly oncogenic viruses were indeed poorly protected (Eidson et al., 1981; Schat et al., 1982; Witter, 1982).

Susceptibility of chickens to pathogens may be the result of a vaccine-mediated impaired immune system. Commercially used vaccines (HVT, bivalent HVT + SB-1 or Rispens strain of serotype 1) caused a transient reduction in resistance to pathogenic *E. coli* infection in vaccinated chicks (Friedman et al., 1992). The increased susceptibility was associated with a transient dysfunction of B-lymphocytes, demonstrated by diminished response to B-lymphocyte-specific mitogen *in vitro* and by reduced antibody production to bovine serum albumin *in vivo*. The bivalent vaccines appeared most damaging. Using flow cytometric

analysis of peripheral blood mononuclear cells, Yamamoto et al. (1995) reported a drastic decrease in $CD4^+$ T-lymphocytes following a transient increase in vaccinated chickens challenged with virulent MDV. In addition, titres of anti-sRBC antibodies in MD-vaccinated chickens challenged with MDV were lower although statistically not significant than in untreated control chickens.

Stress from infectious disease, chemical immunosuppressants or other causes may also influence the efficacy of vaccination, presumably through depression of cell-mediated and/or humoral immune responses.

Infectious bursal disease virus

Infectious bursal disease virus (IBDV), a birnavirus, known to induce long-lasting B-lymphocyte immunosuppression, has been suggested as a likely cause of MD vaccine failures in the field. Giambrone et al. (1976) reported that chickens naturally exposed at hatch to IBDV were less protected by HVT vaccine against challenge with vMDV at 2 weeks of age than chickens not exposed to IBDV. It was reported that 20.7% of the HVT-vaccinatd chickens exposed to IBDV had gross MD lesions, compared with MD lesions in 2.99% in HVT-vaccinated chickens reared in an environment free of IBDV. Further, 56% of the unvaccinated-challenged chickens

infected with IBDV had MD lesions, compared with 18.1% MD lesions in unvaccinated controls not exposed to IBDV.

In another study Jen and Cho, (1980) reported the effects of IBDV on the development and level of HVT viremia and virus-neutralizing antibodies in chickens vaccinated with HVT. IBDV inoculated simultaneously with HVT at one day of age or inoculated 3 weeks after vaccination with HVT had no effect on HVT viremia. However, virus neutralizing antibody response to HVT was significantly suppressed when vaccinated chickens were exposed to IBDV either at the time of vaccination or at 3 weeks postvaccination (PV). This suppression of virus-neutralizing antibody response to HVT may result in a reduction of antiviral immunity against MDV and possibly reducing the host immune response directed against viral antigens.

The effect of IBDV on MD vaccinal immunity depended on the time IBDV and HVT were administered. Chickens inoculated at hatch with IBDV and HVT vaccine were poorly protected against challenge at 1 week with virulent MDV (Sharma, 1983). However, if vaccination with HVT preceded IBDV inoculation such as by embryonal vaccination followed by exposure to IBDV at hatch, or by vaccination at hatch and IBDV exposure at 5 days or longer, then the vaccinal efficacy of HVT was not influenced. Further, the inhibitory effect of IBDV was transient: chickens that received IBDV

and HVT simultaneously at hatch were poorly protected against challenge with vMDV at 1 week but were protected against challenge at 2 weeks of age. IBDV interfered with vaccine efficacy of HVT in chickens lacking maternal antibody to IBDV but did not do so in chickens that had such antibodies at the time of exposure to IBDV (Sharma, 1983).

Chicken anemia virus

Chicken anemia virus (CAV) is a circovirus which causes infection characterized by necrosis, lymphoid depletion and atrophy of the thymus, bursa of Fabricius and spleen with reticulum cell hyperplasia (Yuasa et al., 1979; Goryo et al., 1985). In addition, the virus causes severe depletion of the erythrocytic and granulocytic series of the bone marrow. CAV depressed protective vaccinal immunity against Marek's disease when vaccinated chicks were inoculated with CAV at 1 to 14 days of age (Otaki et al., 1988). The response of splenic lymphocytes to mitogen stimulation was more severely depressed in chicks dually inoculated with HVT and CAV than in chicks inoculated with CAV alone. These findings suggest that the depressive effect of CAV infection on vaccinal immunity may be due to severe impairment of T cell-mediated immunity resulting from enhanced pathogenicity of CAV in vaccinated chicks.

Reticuloendotheliosis virus (REV) and Reovirus

REV is not a naturally occurring disease in chickens, however, accidental inoculation of chickens with REV as by using vaccines contaminated with REV has been reported (Bulow, 1977). MD vaccines contaminated with REV have been shown to interfere with induction of antibodies to HVT and induction of protection by HVT against challenge with MDV (Bulow, 1977). Simultaneous vaccination of chickens with serotype 3 and attenuated reovirus vaccines resulted in poor protection against natural MD exposure in the field, compared to vaccination with serotype 3 alone (Rosenberger, 1983).

Cyclophosphamide and Corticosterone

Cyclophosphamide (Cy), induces severe and long-lasting destructive effect on B-lymphocyte function. The initial effect of Cy on T-lymphocytes is marked but transient (Lerman and Weidanz, 1970; Sharma and Lee, 1977). There is also evidence that certain T-suppressor cell populations are extremely sensitive to Cy. Thus, Cy-induced immunosuppression is probably due to a direct cytotoxic effect of the drug on immunocompetent lymphocytes, particularly those that have undergone antigenic differentiation and division (Dean et al., 1979; Shand, 1979). Treatment of chicks with Cy abolished protection

against MD afforded by vaccination with HVT (Purchase and Sharma, 1974). The authors suggested that the absence of protection was most likely a result of obliteration of B-lymphocyte function, which is markedly suppressed by the drug. This result is contrary to those of Else (1974) who reported that surgical bursectomy plus X-irradiation did not interfere with vaccinal immunity to Marek's disease and that humoral immunity was not essential for vaccinal immunity. Cy treatment of chicks abrogated the induction of immunity against Marek's disease by the HVT, but immunity eventually developed if a sufficient interval was allowed to elapse between vaccination and challenge (Payne et al., 1978). This delay may have allowed the recovery of the T-dependent lymphoid system from the transient suppression caused by Cy. HVT-vaccinated, Cy-treated chicks had a severe suppression of antibody and immunoglobulin production even when immunity to MD was present. This suggests that resistance was not mediated by humoral immunity, but the drug interfered with vaccinal immunity by an effect on the thymus and cell-mediated immunity. Powell and Davidson, (1986) induced clinical MD in HVT-vaccinated, MDV-challenged chickens that had been treated with either betamethasone or corticosterone. Both types of treatment significantly increased the incidence of MD. The titres of leukocyte-associated MD viremia and the incidence of cells in the

peripheral blood expressing MATSA were also increased. Cy did not have such effect. In contrast, Witter et al. (1976) reported the effects of maternal antibodies and Cy on HVT vaccination. The viremic responses were maximal between 8 and 15 days after inoculation in all groups but titres of viremia at 22 and 29 days were substantially lower. Virus titres were generally higher in chickens lacking maternal antibodies and in chickens given higher virus doses. In chickens treated with Cy, the virus titres from the 8th through the 15th days were lower than in chickens not treated with Cy. This effect was confirmed by the direct kidney culture method of virus isolation. By the 22nd day, however, virus titres in Cy-treated chickens were equal to those of the comparable untreated group (Witter et al., 1976).

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) have been reported to suppress immune responses and alter host defense mechanisms (Kerkvliet, 1984). The most common findings in laboratory animals exposed orally or cutaneously to sublethal doses have been described as severe atrophy of primary and secondary lymphoid organs, lower circulating immunoglobulin levels, and decreased specific antibody responses to antigens (Thomas and Hinsdill, 1978; Vos et al., 1980).

Chickens fed PCB and inoculated subcutaneously with a nononcogenic strain of MDV had a marked reduction in body weight and cytolysis and atrophy of lymphoid organs (Halouzka and Jurajda, 1992). A synergistic suppressive effect on the lymphoid organs was characterized by a marked reduction in the production of active MD-precipitating antibodies and a reduced incidence of microscopic MD lesions in peripheral nerves, skin and gonads.

Aflatoxins

Aflatoxins constitute a highly toxic group of mycotoxins, produced by *Aspergillus flavus and A. parasiticus*. Aflatoxins are recognized worldwide as potent hepatotoxins and hepatocarcinogens (Lancaster et al., 1961; Newberne and Wogan, 1968). The growth of the molds on cereal grains and production of toxins require high humidity and temperature conditions that are prevalent in tropical and subtropical areas, but may be occasionally found in colder regions. One of the pronounced effects of aflatoxins is its ability to impair the immune system.

Aflatoxins have been reported to increase the severity of avian infectious diseases such as salmonellosis (Boonchuvit and Hamilton, 1975), crop mycosis (Hamilton and Harris, 1971), aspergillosis (Richard et al., 1973) and cecal coccidiosis (Edds and Simpson, 1976). Edds et al.,

1973, reported that previous exposure to aflatoxin B1 increased susceptibility and mortality from cecal coccidiosis in chickens but did not interfere with the protection afforded by coccidiostat. However, chickens vaccinated against MD and fed aflatoxin B1 (AFB1) seemed more resistant to challenge with MDV than did nonvaccinated control chickens. In addition, vaccinated and nonvaccinated groups of chickens given AFB1 and subsequently exposed to cecal coccidiosis were more susceptible to challenge inoculation with MD virus than were similar groups of chickens not given AFB1.

Batra et al. (1991) reported a significantly higher frequency and increased severity of gross and microscopic lesions of MD in birds that had been vaccinated with HVT and challenged with MDV after been treated with AFB1 in the feed. The protective efficacy of HVT vaccine, as judged on the basis of gross and histopathological lesions was 86.1% and 77.3% in normally fed birds, compared with 37.6% and 8% percent in chickens fed AFB1.

CHAPTER 1

EFFECTS OF T-2 TOXIN ON VACCINAL IMMUNITY AGAINST MAREK'S DISEASE

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EFFECTS OF T-2 TOXIN ON VACCINAL IMMUNITY AGAINST MAREK'S DISEASE.

Abstract

Four trials were conducted to determine the effects of T-2 toxin on vaccinal immunity against Marek's disease (MD). Day-old, maternal antibody-negative white leghorn chicks of Regional Poultry Research Laboratory (RPRL) line $15I_5$ X 7_1 were treated daily for seven days via crop gavage with T-2 toxin at a sublethal dose of 1.25 mg/kg body weight. Treated and untreated chicks were also vaccinated with herpesvirus of turkeys (HVT) at hatch and were challenged with JM strain of MD virus (MDV) at 8 days of age. Chickens were tested for HVT viremia at 1 week post-vaccination (PV) immediately before challenge, and for HVT and MDV viremia at 3 weeks post-challenge. Chickens were observed for the development of MD lesions or mortality within 8 weeks of age. T-2 toxin significantly reduced body weight within 7 days after treatment. T-2 toxin shortened the incubation period for the development of MD lesions and mortality, but only in unvaccinated, challenged chickens. T-2 toxin also

days PV. However, the percent MD protection in T-2 toxin-treated, HVT-vaccinated chickens ranged from 82%-96% and was comparable to that in, HVT-vaccinated control chickens (89%-100%). The data suggest that exposure of chickens to T-2 toxin may influence the development of: a) HVT viremia; and b) MD lesions and mortality, but only in unvaccinated chickens.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by serotype 1 MD virus (MDV) (Calnek and Witter, 1991). MD occurs worldwide and was first controlled by vaccinating chickens with live attenuated serotype 1 MDV (Churchill et al., 1969; Rispens et al., 1972) or serotype 3 MDV, a nonpathogenic herpesvirus of turkeys (HVT)(Witter et al., 1970). Later, serotype 2 vaccines were developed from nonpathogenic MDV (Schat and Calnek, 1978). Since the introduction of vaccines, excessive MD losses have been noted sporadically among vaccinated broiler and layer flocks in the Delmarva region of the United States (Purchase, 1985) and in various parts of the world (Davidson et al., 1989; Shieh, 1989; Zanella et al., 1989). Losses in vaccinated flocks have been attributed to the emergence and increasing prevalence of MDV strains of increased virulence, the very virulent MDV (vvMDV) strains (Witter et al., 1980; Powell

and Lombardini, 1986; Eidson et al., 1978, 1981; Schat et al., 1981, 1982). Polyvalent vaccines containing viruses of several serotypes, but especially serotypes 2 and 3, have provided better protection than most monovalent vaccines against vvMDV strains in commercial flocks (Witter et al., 1984).

Failure of vaccines to provide expected protection has also been attributed to other factors, such as inadequate vaccine production methods (Thornton et al., 1975), poor management of vaccines at the hatcheries (Halvorson and Mitchel, 1979), influence of genetic resistance to MD (Spencer et al., 1974), the presence of maternal antibodies against HVT and other polyvalent vaccines (Calnek and Smith, 1972; Witter and Lee, 1984), insufficient cleaning and sanitation (Good 1983; Price 1983; Witter et al., 1984), and early exposure to MDV before vaccinal immunity develops (Witter and Lee, 1984; Sharma and Burmester, 1982; Okazaki et al., 1970). Stress from infectious agents such as infectious bursal disease virus (Sharma 1983; Giambrone et al., 1976; Jen and Cho, 1980), reticuloendotheliosis virus (REV) (Bulow, 1977), chicken anemia virus (Otaki et al., 1988), or from chemical immunomodulators such as cyclophosphamide (Purchase and Sharma, 1974; Witter et al., 1976; Payne et al., 1978), corticosterone or betamethasone (Powell and Davidson, 1986), aflatoxins (Edds et al., 1973;

Batra et al., 1991) and polychlorinated biphenyls (Halsuzka and Iurajda, 1992) have been shown to influence MD vaccinal immunity.

Mycotoxins such as T-2 toxin have received much attention because of possible adverse effects on the immune system of humans and farm animals (Mayer, 1953a, b). T-2 toxin, a very potent immunotoxic type A trichothecene, is a secondary metabolite produced primarily by *Fusarium spp*, which grows on cereal grains and contaminates agricultural products usually in temperate regions of North America, Europe and Asia (Scott, 1989; Russell et al., 1991; Wang et al., 1993; Chu and Li, 1994). Exposure of both humans and farm animals to T-2 toxin has been associated with a variety of clinical syndromes. T-2 toxin has also been implicated as a major causative factor in fatal alimentary toxic aleukia in humans during World War II (Mayer, 1953; Joffe, 1978), and red mold disease in humans and animals (Ueno et al., 1972; Saito and Ohtsubo, 1977). Fusariotoxicosis in lactating cows and poultry has also been reported (Wyatt et al., 1972; Hsu et al., 1972).

T-2 toxin has also been shown to impair the immune system through its ability to inhibit protein and DNA synthesis and thereby cause severe damage to actively dividing cells within the bone marrow, lymph nodes, spleen, thymus, bursa of Fabricius, and the gastrointestinal tract

(Saito et al., 1969; Ueno et al., 1971; Wyatt et al., 1973; Lutsky et al., 1978; Hayes et al., 1980; Hoerr et al., 1981; LaFarge-Frayssinet et al., 1981). T-2 toxin has been found to lower B and T cell numbers, inhibit lymphocyte transformation, decrease IgG and IgM antibody levels, and cause lymphocytolysis (Hayes et al., 1980; Jagadeesan et al., 1982; Rosenstein and LaFarge-Frayssinet, 1983; Cooray and Jonsson, 1990). Repeated exposure to T-2 toxin also causes immunosuppression and decreases the resistance of exposed animals to various infectious diseases such as salmonellosis in chickens (Boonchuvit et al., 1975) and mice (Ziprin and McMurray, 1988; Tai and Pestka, 1988, 1990), tuberculosis (Kanai and Kondo, 1984; Ziprin and McMurray, 1988), listeriosis (Corrier and Ziprin, 1986), herpes simplex infection in mice (Friend et al., 1983), and aspergillosis in rabbits (Niyo et al., 1988). In addition to decreased host resistance, T-2 toxin may also suppress tumor defense mechanisms and impair host control of tumor cell growth (Schoental et al., 1979; Schiefer et al., 1987; Corrier and Norman, 1988).

The objective of this study was to determine the effects of T-2 toxin on vaccinal immunity against MD in white leghorn chickens.

Materials and Methods

Chickens. Chickens used in this study were F_1 progeny of RPRL line $15I_5$ males and line 7_1 females. The breeder chickens are known to be free of antibodies to MDV, HVT, avian leukosis virus, REV, and other common poultry pathogens. Chickens were maintained in isolators provided with negative pressure for the duration of the trials.

Viruses. The vaccine virus, apathogenic serotype 3, FC 126 strain of HVT (Witter et al., 1970) propagated in chick embryo fibroblasts (CEF) was used. The JM strain of MDV (JM-MDV) propagated in duck embryo fibroblasts (DEF) was used as the challenge virus (Stephens et al., 1976).

T-2 toxin. T-2 toxin, a trichothecene secondary metabolite of several *Fusarium* species, was obtained in crystalline form from Sigma Chemical Company (St. Louis, Missouri).

The T-2 toxin stock was dissolved in 100% ethanol at a concentration of 2.5 mg/ml on the day of use and stored in the freezer at -20^{0}C until used within a 1 week treatment period.

Experimental design. Four trials were conducted to determine the effects of T-2 toxin on MD vaccinal immunity in maternal antibody-negative, RPRL $15I_5$ X 7_1 chickens. Day-

old chicks were divided into six groups, each group consisted of 20-27 chicks (Table 1.1). Chicks in each treatment group were held in a separate isolator. At 1 day of age, chicks in groups 1, 2 and 3 were treated daily, for seven consecutive days via crop gavage with T-2 toxin at a sublethal dose of 1.25 mg/kg body weight. T-2 toxin dose was based on the average weight of the chicks used in each treatment group. Chicks in groups 4, 5 and 6 were given a mixture of ethanol and phosphate buffered saline (PBS) and were maintained as untreated controls. In addition, at 1 day of age, chicks in groups 3 and 4 were vaccinated against MD with FC 126 strain of HVT at a dose of 2000 pfu/chick. At the end of T-2 toxin treatment (at 8 days of age), lymphoid organs from five chicks from each of the treated and untreated groups were evaluated microscopically. At 8 days PV with HVT, chicks from groups 2 and 3 (T-2 toxin-treated) and chicks in groups 4 and 5 (untreated) were challenged with JM strain of MDV at a dose of 500 pfu/chick. Chicks in groups 1 and 6 (T-2 toxin-treated and untreated) were maintained as unvaccinated unchallenged controls. At 1 week PV, the chicks were tested for HVT viremia immediately before challenge, and for HVT and MD viremia at 3 weeks PC. The chicks were observed for MD lesions and mortality within 8 weeks PC.

Table 1.1: Experimental design

	Age -- Treatment[A]		
	1 day		8 days
Group No.	T-2 toxin	HVT	MDV
1	+	-	-
2	+	-	+
3	+	+	+
4	-	+	+
5	-	-	+
6	-	-	-

[A] Chicks treated via crop gavage with T-2 toxin (1.25mg/kg body weight) for 7 consecutive days after hatch. Chicks in groups 4, 5 and 6 were treated with ETOH/PBS. Chicks were vaccinated with strain FC126 of HVT at a dose of 2000 pfu/chick. Chicks were challenged with JM strain of MDV at a dose of 500 pfu/chick. At day 8, chicks were tested for HVT viremia and at 3 weeks of age, chicks were tested for both HVT and MDV viremia. Chickens were observed for MDV-induced lesions and mortality within 8 weeks of age. No. of chicks per group = 20-27

Virus assays. At 1 week post-vaccination (PV), and at 3 weeks post-challenge (PC), peripheral blood monocytes (PBM) were obtained from chickens in various groups. PBM were used in co-cultivation assays for HVT and MDV by standard procedures using CEF and DEF cultures, respectively (Witter et al., 1969, 1970). Briefly, at 8 days PV, blood was obtained from chickens representing various treated groups. One milliliter of blood from each chicken collected using syringes containing 1 ml Alsever's solution, was placed into centrifuge tubes containing 8 ml Alsever's solution and centrifuged at 750 rpm for 15 minutes. PBMs were collected and centrifuged at 800-900 rpm for 10 minutes. The pellet was resuspended in Leib-McCoy media supplemented with 1% calf serum.

Duplicate CEF (for HVT assay) and DEF (for MDV assay) monolayer cultures were inoculated with 2×10^6 PBMs per 35 mm culture plate. Inoculated plates were maintained in Leib-McCoy medium supplemented with 4% calf serum for the first 24 hours, and with 1% calf serum thereafter. The plates were incubated at 37°C in 3% CO_2 atmosphere. Following inoculation, the medium was changed after 24 hours, and at about 48-hour intervals thereafter. At 5 to 7 days PI, cultures were examined for characteristic viral plaques under the light microscope. Viremia titres recorded as plaque forming units (pfu) of sample was determined as the

average number of plaques detected in 2, 35mm culture plates. In cases where discrimination between HVT and MDV was necessary (at 3 weeks PC), MDV viral plaques were visualized by indirect immunofluorescent (IFA) staining using monoclonal antibodies specific for serotype 1 MDV (Lee et al., 1983).

Pathology. Chickens that died or were moribund during the trials and those killed at the end of the trial were necropsied and examined for gross neural and visceral lesions of MD. In cases where gross lesions were absent, gonads, vagus nerves, and sciatic plexuses were examined histologically. Tissues were fixed in 10% formalin and stained with hematoxylin and eosin.

Statistics. Data on body weights and MD viremia titres (square root transformation) were analysed for statistical significant difference by analysis of variance and student-Newman-Keuls (SNK) test. Data on HVT viremia titres (square root transformation) were analysed by Student "t" test. The percent incidence of MD mortality or lesions, and percent protection were analysed by computing the interaction chi-square for the MD lesion frequency in a) T-2 toxin-treated, challenged chickens compared with that of untreated, challenged chickens; b) T-2 toxin-treated, vaccinated and

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challenged chickens compared with that of the untreated, vaccinated, and challenged chickens.

Protection tests. The protective index was calculated as the percent MD lesions in T-2 toxin-treated, unvaccinated, challenged chickens minus the percent MD lesions in T-2 toxin-treated, vaccinated, challenged chickens divided by the percent MD lesions in T-2 toxin-treated, unvaccinated, challenged chickens multiplied by 100. The protective index is equivalent to the percent protection.

Indirect immunofluorescence assay. The immunofluorescence technique used in this study has previously been described by Sharma, 1989. Briefly, at 7 days PI, the medium of DEF monolayer culture plates was discarded, and the plates were gently washed with PBS. One ml of 1:1 acetone-alcohol mixture was added to plates to fix the cells; the plates were air dried.

Five hundred microlitres of 1:200 dilution of MDV serotype 1-specific monoclonal antibody (provided by Dr. L.F. Lee, USDA-ARS, Avian Disease and Oncology Laboratory, East Lansing, MI) was added to each plate and incubated at 37°C for 30 minutes.

After incubation, excess antibody was discarded and the plates were washed and rinsed three times with distilled

water and PBS, respectively. Five hundred microlitres of 1:50 dilution of FITC-conjugated rabbit anti-mouse antibody was added to the plates and incubated at 37°C for 30 minutes. The plates were then washed and rinsed three times with distilled water and PBS respectively, and a few drops of glycerine-PBS solution were added. Excess mixture was discarded and the plates were blotted dry. The plaques were counted by using a dark-field microscope with ultraviolet illumination.

Results

Effect of T-2 toxin on body weight. After 7 consecutive daily doses of 1.25 mg/kg body weight of T-2 toxin administered via crop gavage, the mean body weight of T-2 toxin-treated chickens was significantly lower than that of untreated chickens, regardless of vaccination with HVT (Table 1.2). There was also mild to moderate or severe lymphoid depletion of lymphoid organs including thymus, spleen and the bursa of Fabricius. The bursa of Fabricius was the most severely affected. A few chicks had dry necrosis of the toes. No other significant gross lesions were noted in the remaining visceral organs.

Table 1.2. Effect of T-2 toxin on body weight of 7-day-old, $15I_5 \times 7_1$ chickens unvaccinated or vaccinated with HVT at hatch.

Treatment[C]		Trial No. -- Mean body weight[AB] (gm)			
T-2 toxin	HVT	1	2	3	4
+	-	43.04 ± 3.05[a]	50.04 ± 4.47[a]	51.50 ± 5.9[a]	42.50 ± 5.00[a]
+	-	40.94 ± 6.01[a]	46.18 ± 4.74[b]	49.40 ± 5.6[ab]	37.20 ± 4.80[b]
+	+	43.47 ± 4.22[a]	48.67 ± 3.92[ab]	46.10 ± 6.2[b]	35.30 ± 8.60[b]
-	+	56.65 ± 3.44[b]	57.30 ± 4.47[c]	62.20 ± 6.1[c]	47.20 ± 3.80[c]
-	-	55.70 ± 6.25[b]	60.32 ± 3.04[d]	60.80 ± 9.5[c]	53.90 ± 4.60[d]

T-2 toxin was given at a daily dose of 1.25mg/kg body weight for seven consecutive days after hatch

[A]Mean values within a column followed by different superscript letters are significantly different ($P<0.05$).

[B]Means and standard deviation.

[C]20-27 chicks were used per treatment group.

Effects of T-2 toxin on development of HVT and MDV Viremia. Typical cytopathic effects (plaques) of HVT and MDV were detected in monolayer cultures of CEF and DEF, respectively (Figures 1.1, 1.2). Indirect immunofluorescent staining of DEF monolayer cultures, was used to differentiate between HVT and MDV plaques using MDV serotype 1-specific monoclonal antibody (Lee et al., 1983). Figure 1.3. shows a typical immunofluorescent MDV plaque.

The effect of T-2 toxin on the development of HVT viremia in chickens vaccinated with HVT at hatch and challenged with MDV at 8 days of age is shown in Table 1.3. At 8 days of age, in trials 2, 3, and 4, but not in trial 1, the titre of HVT viremia in T-2 toxin-treated chickens was significantly lower than that in untreated chickens. By 4 weeks of age the titres of HVT viremia in T-2 toxin-treated chickens was comparable to that in untreated chickens, regardless of challenge with MDV.

The effect of T-2 toxin or HVT on the development of MDV-induced viremia in chickens challenged with MDV at 8 days of age is shown in Table 1.4. The titres of MDV viremia in HVT-vaccinated, MDV-challenged chickens that had been treated with T-2 toxin was comparable to that in the untreated group. The titres of MDV in T-2 toxin-treated, MDV-challenged chickens was consistently higher than that in

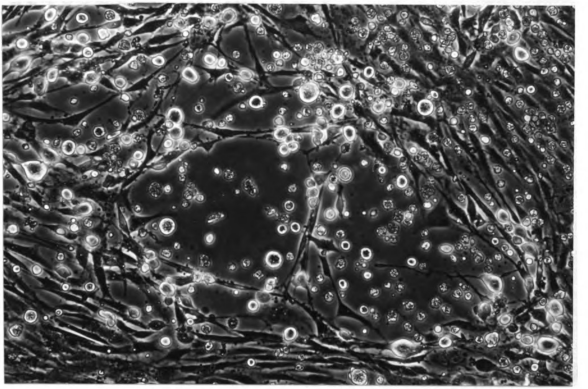

Figure 1.1. Typical plaque morphology of HVT in CEF at 5 days PI with PBMs from 8-day-old, T-2 toxin-treated, $15I_5$ X 7_1 chicks that had been vaccinated with HVT at hatch (X 354).

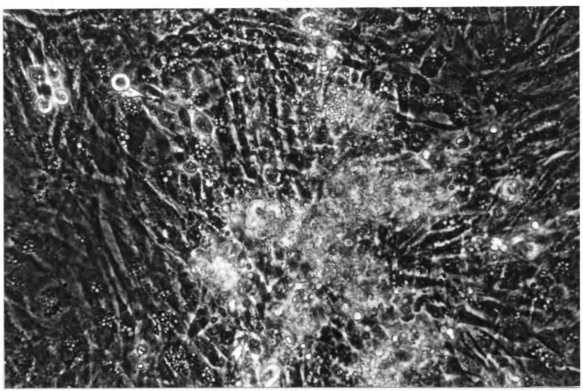

Figure 1.2. Typical plaque morphology of MDV in DEF at 7 days PI with PBMs from 4-week-old, T-2 toxin-treated chickens vaccinated with HVT at hatch and challenged with MDV at 8 days of age (X 354).

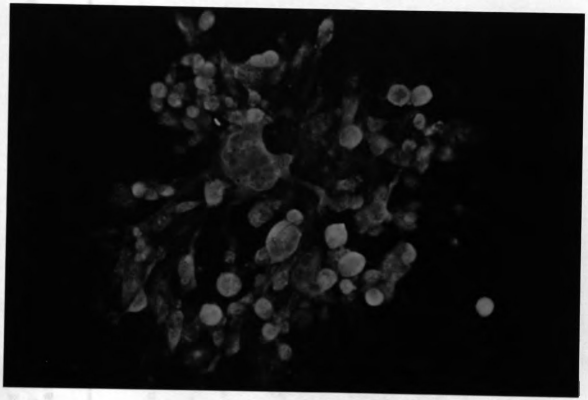

Figure 1.3. Typical IFA-stained (using serotype 1 MDV-specific mab) MDV plaque morphology in DEF at 7 days PI with PBMs from 4-week-old, T-2 toxin-treated chickens vaccinated with HVT at hatch and challenged with MDV at 8 days of age (X 360).

Table 1.3. Effect of T-2 toxin on development of HVT viremia in 1- and 4-week-old, $15I_5$ X 7_1 chickens vaccinated with HVT at hatch, and challenged with MDV at 8 days of age.

Treatment				Trial No. -- HVT Viremia[A] Titre (PFU)			
T-2	HVT	MDV	Age in weeks	1	2	3	4
+	+	-	1	2.18 ± 1.13	2.16 ± 1.04*	2.02 ± 1.68*	1.26 ± 0.51*
-	+	-	1	3.22 ± 1.84	6.74 ± 2.41	4.52 ± 2.13	5.33 ± 2.47
+	+	+	4	3.49 ± 1.28	ND	4.29 ± 2.57	3.99 ± 2.42
-	+	+	4	3.60 ± 0.69	ND	3.28 ± 0.99	2.83 ± 0.85

T-2 toxin was given at a daily dose of 1.25mg/kg body weight for seven consecutive days after hatch.
[A]Plaque forming units (means and standard deviations after square root transformation).
*Mean value significantly different ($P<0.05$).
n=12-13 chicks per treatment group tested.
ND=not determined

Table 1.4. Development of MDV-induced viremia in 4-week-old, T-2 toxin-treated chickens vaccinated with HVT at hatch and challenged with MDV at 8 days of age.

Treatment			Trial No. -- MDV Viremia[AB] Titre (PFU)			
T-2	HVT	MDV	1	2	3	4[C]
+	+	+	3.51 ± 1.31[b]	ND	3.15 ± 3.72[b]	1.68 ± 1.10[b]
-	+	+	3.61 ± 0.69[b]	ND	2.23 ± 1.65[b]	1.62 ± 1.5[b]
+	-	+	9.34 ± 3.92[a]	ND	16.51 ± 7.41[a]	20.22 ± 9.07[a]
-	-	+	8.11 ± 4.55[a]	ND	12.84 ± 3.33[a]	15.74 ± 4.08[a]

T-2 toxin was given at a daily dose of 1.25mg/kg body weight for seven consecutive days after hatch.
[A]Plaque forming units (means and standard deviations after square root transformation).
[B]Mean values within a column followed by different superscript letters are significantly different ($P<0.05$).
[C]MDV viremia tested at 4 wks post-challenge.
n=12-13 chicks per treatment group tested
ND=not determined

untreated chickens however, the difference was not statistically significant. At 3 weeks post-challenge, the MDV viremia titre in vaccinated, challenged chickens was significantly lower than that in unvaccinated challenged chickens, regardless of treatment with T-2 toxin (Table 1.4).

Necropsy Findings. Chickens that died or were moribund following challenge with MDV were necropsied. The gross changes commonly noted were the classical MD lesions including enlarged or edematous peripheral nerves involving the vagus, sciatic, and femoral nerves, and the brachial plexus with loss of cross-striations. The enlargements were segmental or uniformly diffuse along the length of the affected nerves. The bursa of Fabricius and the thymus were atrophied in affected chickens. Single or multiple greyish-white lymphoma nodules were present in one or several visceral organs including gonads, lungs, proventriculus, liver, heart, spleen adrenal glands and the bursa of Fabricius. The lymphoma nodules or infiltrates consisted of pleomorphic neoplastic lymphoid cells, plasma cells and mature lymphocytes (Figure 1.4).

Effect of T-2 toxin on Incidence of MD-Induced Mortality and Lesions in HVT-vaccinated and unvaccinated chickens . At

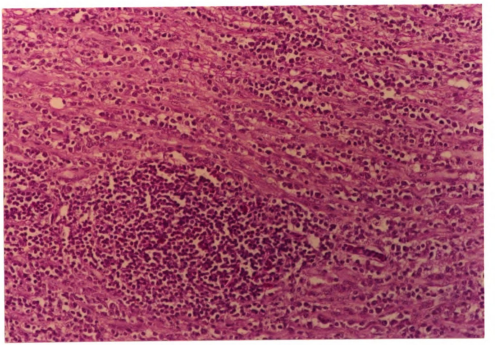

Figure 1.4. Photomicrograph of sciatic nerve from a 4-week-old, $15I_5$ X 7_1 chicken treated with T-2 toxin (1.25 mg/kg body weight) for 7 consecutive days after hatch and challenged with MDV at 8 days of age. Notice a metastatic lymphoma nodule and neoplastic cellular infiltrates (H&E X 242).

4 weeks PC in 3 of 4 trials, the incidence of MD-induced mortality and lesions in MDV-challenged chickens was significantly higher in T-2 toxin-treated chickens (56%, 32%, 67%) than in untreated chickens (10%, 16%, 35%) (Table 1.5). However, the incidence of MD-induced mortality and lesions was comparable in all groups vaccinated with HVT, regardless of treatment with T-2 toxin. At 8 weeks PC, the percent MD mortality and lesions in T-2 toxin-treated chickens (94%) was comparable to that in untreated chickens (100%) (Table 1.5). The incidence of MD mortality and lesions in HVT vaccinated group ranged from 0%-18%, regardless of treatment with T-2 toxin.

Percent Protection by HVT. At termination of the trials (8 weeks PC), the percent protection in all HVT vaccinated groups ranged from 82%-100% regardless of treatment with T-2 toxin (Table 1.6). By 8 weeks PC, the incidence of MD in vaccinated groups varied from 94%-100%, regardless of treatment with T-2 toxin.

Table 1.5. Percent MD mortality and lesions in T-2 toxin-treated and untreated chickens within 8 weeks post-challenge with MDV at 8 days of age.

Treatment			Trial No... Weeks PC...Percent Mortality/Lesions[A]							
T-2	HVT	MDV	1		2		3		4	
			4 wks	8 wks	4 wks	8 wks	4 wks	8 wks	4 wks	8 wks
+	-	-	0	0	0	0	0	0[c]	0	0
+	-	+	56*	100	9	100	32*	100	67*	94
-	-	+	10	100	13	95	16	100	35	100
+	+	+	0	18	0	4	4	4[c]	0	5
-	+	+	0	11	0	0	0	0[c]	0	0
-	-	-	0	0	0	0	0	0	0	0

[A]*significantly different (P<0.05)
[B]chicks were vaccinated with HVT at hatch, treated with T-2 toxin at a daily dose of 1.25mg/kg body weight for 7 consecutive days after hatch, and challenged with MDV at 8 days of age.
[c]57-86% of chicks accidentally suffocated in isolators at 5-1/2 weeks and necropsied
n=20-27 chicks per treatment group

Table 1.6. Percent MD protection in T-2 toxin-treated, HVT-vaccinated $15I_5$ X 7_1 chickens challenged with MDV at 8 days of age.

Treatment			Trial No. -- Percent Protection[A]			
T-2	HVT	MDV	1	2	3	4
+	+	+	82	96	96	95
-	+	+	89	100	100	100
-	-	-	0	0	0	0

T-2 toxin was given at a daily dose of 1.25mg/kg body weight for 7 consecutive days after hatch, and vaccinated with HVT at hatch
[A]Percent MD protection at 8 weeks PC.

Discussion

Data from this study suggest that exposure of newly hatched chicks to T-2 toxin may lower the titre of HVT viremia at 1 week PV at hatch. The data also suggest that T-2 toxin may increase the incidence of MD lesions and mortality within 4 weeks of age, but only in unvaccinated chickens. The data confirmed the negative effects of T-2 toxin on body weight gain.

Analysis of viremia results revealed that exposure of newly hatched chicks to T-2 toxin may lower titres of HVT viremia at 1 week, but not at 3 weeks PV. These results are similar to those reported by Witter et al. (1976) in which treatment of HVT-vaccinated chickens with cyclophosphamide (Cy) at a dose of 4 mg per day for the first 4 days after hatch reduced HVT viremia titres at 8 and 15, but not at 22 days of age. In contrast, Sharma et al., 1980, reported that immunosuppression of HVT-vaccinated chickens by neonatal surgical thymectomy and gamma-irradiation increased the titre of HVT.

Previous studies have shown a relationship between the level of HVT viremia and vaccinal immunity against MD. A correlation between HVT viremia and protection of chickens against MD was reported by Patrascu et al. (1972). Okazaki et al. (1973) indicated that an early establishment of HVT

viremia in vaccinated chickens was necessary for protection against MD in the field. Cho et al. (1976) and Riddell et al. (1978) reported in surveys that the incidence of detectable HVT viremia in vaccinated chickens was significantly lower in the MD-affected than in the healthy penmates. It is generally accepted that cell-mediated antiviral immunity generated by HVT vaccination plays a major role in the vaccinal immunity against MD (Gupta et al., 1982; Purchase and Sharma, 1974; Payne et al., 1978). In the present study, although T-2 toxin caused moderate to severe lymphoid depletion in the thymus, spleen and bursa of Fabricius, chickens treated with T-2 toxin were still able to mount antiviral immunity leading to protection against MD challenge.

The reduced titres of HVT viremia in T-2 toxin-treated chicks in the present study could be explained by the antiviral effect of T-2 toxin on HVT. Okazaki et al. (1988b) reported that the inhibition of HSV-2 replication *in vitro* was closely correlated with T-2 toxin-induced blocking of early and late viral protein synthesis. The finding that by 3 weeks PV, the level of HVT viremia in T-2 toxin-treated chickens was comparable to that of untreated chickens could be due to the lack of the antiviral effect of T-2 toxin as the toxin concentration wanes within 1 week after treatment and the immune system regenerates. The metabolic conversion

of T-2 toxin to its by-products decreases the antiviral activity of T-2 toxin in the HSV-2 plaque formation assay (Okazaki et al., 1992).

Analysis of MDV viremia results revealed a consistent increase, although statistically not significant, in titres of MDV at 3 weeks PC, but only in chickens treated with T-2 toxin. Interestingly, the higher titres of MDV viremia were accompanied by an increased incidence of MD mortality and lesions within 4 weeks PC, but only in unvaccinated groups. This finding is consistent with previous studies in which immunosuppressed chickens developed high levels of MDV viremia titres (Sharma et al., 1977; Powell and Davison, 1986). High levels of MDV viremia have been shown to be closely related to lesion formation and MD mortality (Witter et al., 1971). As expected, titres of MDV were significantly lower in HVT-vaccinated chickens than in nonvaccinated controls as has been previously shown by Witter et al. (1976).

MDV infection is known to initially cause severe depletion of lymphocytes in the thymus, bursa of Fabricius, and spleen resulting in the suppression of both humoral and CMI responses to antigenic stimulation (Payne and Rennie, 1973; Burg et al., 1971; Evans and Patterson, 1971; Jakowaki et al., 1973; Purchase et al., 1968). This MDV-induced immunosuppression combined with the immunosuppression caused

by T-2 toxin could explain the early development of MD lesions and mortality in unvaccinated T-2 treated chickens.

Furthermore, T-2 toxin has been shown to cause immunosuppression in chickens and other animals making them more susceptible to secondary bacterial and viral infections (Boonchuvit et al., 1975; Corrier and Ziprin, 1986; Friend et al., 1983). Data from the present study suggest that although a significant decrease in HVT viremia was noted in T-2 toxin-treated chickens, the percent MD protection was comparable to that in untreated chicks. This finding is consistent with those of Witter and Offenbecker (1978) who reported good immunity in vaccinated chickens which lacked readily detectable HVT viremia. In contrast, however, Okazaki et al. (1973) and Cho et al. (1976) reported low HVT titres in birds that subsequently developed MD in the field suggesting that immunity to MD depends on maintenance of high levels of HVT viremia.

Analysis of data on body weight confirmed the negative effects of T-2 toxin on body weight gain. T-2 toxin has been shown to reduce body weight gains of white leghorn and broiler chickens (Wyatt et al., 1972, 1973; Chi et al., 1977; Hoerr et al., 1982). Furthermore, the weight gain and feed consumption are usually more dramatically decreased during the first 3 week period during T-2 toxin treatment and were associated with feed refusal or reduced feed intake

(Chi et al., 1977). The decreased weight gain and feed consumption caused by T-2 toxin is usually proportional to the amount of the toxin given.

Commercial broiler chicken flocks in several countries around the world including Europe are not vaccinated against MD, as losses from MD during the growing period are usually 0.1% or less (Purchase, 1985). Data from the present study should have some practical implications as subchronic exposure to T-2 toxin enhanced the development of MD lesions and may be very important in inducing early incidence of mortality within 4 weeks of age, but only in unvaccinated chickens. However, in other countries where such commercial broiler chickens are routinely vaccinated against MD, subchronic exposure to T-2 toxin may not pose such a problem.

CHAPTER 2

FLOW CYTOMETRIC ANALYSIS OF PERIPHERAL BLOOD AND SPLENIC B- AND T-LYMPHOCYTE SUBPOPULATIONS IN CHICKENS TREATED WITH T-2 TOXIN

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Abstract

Four trials were conducted to determine the effects of T-2 toxin on peripheral blood and splenic B- and T- lymphocyte subpopulations in white leghorn chickens. Day-old chicks of Regional Poultry Research Laboratory (RPRL) line $15I_5$ X 7_1 were treated with T-2 toxin daily for seven days via crop gavage at a sublethal dose of 1.25 mg/kg body weight. At 8-9 and 21-22 days of age, single-cell suspensions of pooled samples of blood and splenic lymphocytes from T-2 toxin-treated and untreated control chickens were analysed for the presence of CD4, CD8, CD3 and IgM cell surface markers using monoclonal antibodies and flow cytometry. T-2 toxin significantly reduced the body weight of treated chickens. The percentage of both peripheral blood and splenic B-lymphocytes in T-2 toxin-treated chickens was significantly lower than that in untreated chickens, but only at 8-9 days

of age. An increase in the percentage of CD4 T-lymphocytes in both peripheral blood and spleen, and CD3 T-lymphocytes in spleen was noted in T-2 toxin-treated chickens, but only at 8-9 days of age. The data suggest that exposure of chickens to T-2 toxin may cause a severe depletion of B-lymphocytes and a relative increase in CD4 and CD3 T-lymphocytes.

Introduction

T-2 toxin, a trichothecene mycotoxin, has been shown to cause health problems in humans and animals (Mayer, 1953a, b; Joffe, 1986; Watson et al., 1984; Wang et al., 1993). The toxin is a secondary metabolite produced primarily by *Fusarium* spp. which grows on cereal grains and contaminates agricultural products in temperate regions of North America, Europe and Asia (Saito and Ohtsubo, 1974; Ueno, 1983; Vesonder, 1983; Scott, 1989; Wang et al., 1993; Beardall and Miller, 1994).

The cytotoxic effects of T-2 toxin have been shown to be associated with inhibition of protein and DNA synthesis due to the effect of the toxin on peptidyl transferase (Ueno et al., 1968; Bamburg, 1974; Cundliffe et al., 1974; Cundliffe and Davis, 1977; Wei and McLaughling, 1974; Pace et al., 1988). T-2 toxin has been shown to have a "radiomimetic" effect on rapidly proliferating cells such as

lymphoid organs, hematopoietic cells, and the gastrointestinal epithelium (Saito et al., 1969; Ueno, 1977; Hoerr et al., 1981b; Mirocha, 1983). Although T-2 toxin is known to inhibit synthesis of macromolecules such as proteins and DNA, its effect on the immune system has been shown to be either immunostimulatory or immunosuppressive.

The immunotoxic effects of T-2 toxin have been reviewed by many authors (Bamburg, 1983; Otokawa, 1983; Taylor et al., 1989; Sharma and Kim, 1991; Sharma, 1993). Acute and subacute exposure of chickens to T-2 toxin have been shown to cause necrosis and depletion of lymphocytes in thymus, spleen, bursa of Fabricius and cecal tonsils. (Wyatt et al., 1973; Richard et al., 1978; Hoerr et al., 1981, 1982a,b).

Low concentrations of T-2 toxin have been shown to increase mitogen-induced B-cell stimulation, but not T-cell responses (Taylor et al., 1985, 1987). T-2 toxin has also been shown to enhance delayed-type hypersensitivity and allograft rejection time responses (Masuko et al., 1977; Otokawa et al., 1979; Rosenstein et al., 1979).

The effect of T-2 toxin on antibody production has been shown to be inconsistent and difficult to interpret. Repeated lower doses of T-2 toxin decreased antibody production in response to T-dependent antigens (Rosenstein et al., 1979, 1981) and enhanced antibody responses to T-

independent antigens (Rosenstein et al., 1981; Cooray and Kiessling, 1987). Thus, T-2 toxin appears to selectively inhibit specific immune functions that involve T-cell regulation. It is worth noting that the regulation of immune responses is accomplished by the coordinated participation of various types of cells in the lymphoid tissues. Therfore in order to further investigate the mechanism of action of T-2 toxin on immune responses, it is essential to evaluate the cytotoxicity of T-2 toxin to various types of cells participating in immune responses. Selective depletion of B-lymphocytes from peripheral blood and spleen by T-2 toxin may impair antibody production without the involvement of cell-mediated immune responses. Since CD4 T-lymphocyte depletion may reduce antibody production whereas CD8 T-lymphocyte depletion may cause an increase in antibody production, it is important to determine the effect of T-2 toxin on specific subpopulation(s) of T-lymphocytes in lymphoid tissues.

The objective of this study was to determine effects of T-2 toxin on peripheral blood and splenic B- and T-lymphocyte subpopulations.

Materials and Methods

Chickens. Chickens were F_1 progeny of RPRL line $15I_5$ males and 7_1 females. The breeder chickens are known to be free

of antibodies to MDV, herpesvirus of turkeys, avian leukosis virus, REV and other common poultry pathogens. Treated and untreated chickens were maintained in separate isolators provided with negative pressure for the duration of the trials.

T-2 Toxin. T-2 toxin, a trichothecene secondary metabolite of several *Fusarium* sp. was obtained in crystalline form from Sigma Chemical Company (St. Louis, Missouri). The T-2 toxin stock was dissolved in 100% ethanol at a concentration of 2.5 mg per 1 ml, stored in the freezer at -20°C, and used within one week. Control untreated chickens were treated with ethanol:PBS mixture.

Experimental design. In each of the four trials conducted, 132-187 day-old chicks were allotted into 2 groups as shown in Table 2.1. Chicks in group 1 were divided into 2 lots, each containing 36-55 chicks. Chicks in lot 1A were treated with ethanol-PBS vehicle and maintained as untreated controls in separate isolators. Chicks in lot 1B were treated with T-2 toxin at hatch for 7 consecutive days via crop gavage at a dose of 1.25 mg/kg body weight. T-2 toxin dose was based on the average weight of the chicks used in each treatment group. Chicks in group 2 were divided into 2 lots, 2A, 2B. The protocol used to treat chicks in group 2

Table 2.1. Experimental design

Group #	Lot #	T-2 toxin	Age at Analysis (days)	
			Blood	Spleen
1	1A	-	8	9
	1B	+	8	9
2	2A	-	21	22
	2B	+	21	22

Day-old chicks were divided into 2 groups containing 2 lots each. Chicks in group 1, lot 1A were treated with ethanol/PBS vehicle via crop gavage and maintained as untreated controls in separate isolator. Chicks in lot 1B were treated with T-2 toxin at a dose of 1.25 mg/kg body weight for 7 consecutive days after hatch. At 8 and 9 days of age, single-cell suspensions of PBMs and spleen respectively were prepared from pooled samples of peripheral blood and spleen from T-2 toxin-treated and untreated chicks for single color flow cytometric analysis. Chicks in group 2 were treated as shown in group 1, maintained in 2 isolators until 21 and 22 days of age when pooled samples of PBMs and spleen, respectively were prepared for analysis.

with T-2 toxin was identical to that used in group 1. The chicks were maintained in different isolators until 3 weeks of age.

At 8 days of age (end of the T-2 toxin treatment), 2-3 chicks from each of treated and untreated lots in group 1 were selected at random and euthanatized for histopathologic evaluation of the lymphoid organs. Blood was also collected from 30 chicks from each of treated and untreated groups using Alsever's solution. Single cell suspensions of the peripheral blood were prepared from pools of 5 chicks per sample. At 9 days of age, chicks that were bled were euthanatized and spleens were aseptically collected. Five spleens per sample were pooled for analysis.

At 21 days and 22 days of age, PBMs and splenic lymphocytes, respectively were obtained from 18 chicks (3 chicks per pool) from each of treated and untreated lots in group 2. Except in trial 1, PBMs (collected at 8 and 21 days of age) and splenic lymphocytes (collected at 9 and 22 days of age) were tested. Chicks used in trial 1 were tested only at 21 and 22 days of age.

Preparation of single-cell suspensions.

Peripheral blood mononuclear cells (PBMs). Multiple pooled blood samples (3-5 chicks per pool) were obtained from the right jugular vein in Alsever's solution and placed on ice.

Five millilitres of pooled blood samples were layered over 6 ml of Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Missouri) and centrifuged at 2000 rpm for 20 minutes. The buffy coat interface was removed with a Pasteur pipette and washed three times with RPMI culture medium.

Spleen. Spleens were aseptically collected (3-5 spleens per pooled sample) in centrifuge tubes containing RPMI and placed on ice. The spleens were homogenized using 40μ mesh, and the single cell suspension obtained was sieved through cotton gauze and cleared on Ficoll-Hypaque at 2000 rpm for 20 minutes. The buffy coat interface was collected using a Pasteur pipette, and washed three times in RPMI culture medium.

The viability of cell suspensions was determined using erythrosin.

Immunofluorescent Staining. Monoclonal antibodies (mab) for CT4, CT8 and CT3, the avian homologues of mammalian CD4, CD8 and CD3, respectively (Southern Biotechnology, Birmingham, Alabama) were used. Fluorescein isothiocyanate-conjugated goat anti-mouse (FITC-GAM) for CT4, CT8, CT3 T-lymphocyte subpopulations, and FITC-goat anti-chick (FITC-GAC (IgM) for B-lymphocytes were used as secondary

antibodies. A single-color staining procedure was performed, as described by Chan et al., 1988.

Briefly, 2-3 x 10^6 PBMs or splenic lymphocytes per sample were incubated with the respective appropriate dilutions (CD3: 1:50, CD4 and CD8: 1:100) of mab for 45 minutes in microtitre plates on ice. The cells were washed three times with sodium azide-bovine serum albumin solution and then incubated with FITC-GAM (1:2000) or FITC-GAC (1:500) antibodies for 30 minutes. To facilitate the exclusion of dead cells after the final wash, the samples were transferred into test tubes and propidium iodide was added to the cell suspensions immediately before analysis. Percentages of B- lymphocytes and T-lymphocyte subpopulations were determined from 10^4 cells per sample by analysis in a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, California).

Statistical Analysis. Data for calculated means for individual samples were analysed with Student's t-test with significance determined at $p \leq 0.05$.

Results

Effect of T-2 toxin on body weight. In all trials, the mean body weight of chicks treated with T-2 toxin at hatch

for 7 consecutive days was significantly different from that of untreated chicks (Table 2.2).

Effect of T-2 toxin on lymphoid organs. Histologically, mild to moderate lymphoid depletion of the thymus and spleen, and severe lymphoid depletion of the bursa of Fabricius were noted at 8 days of age. In the thymus lymphoid depletion was primarily noted in the cortex. In addition, the spleen had reticuloendothelial cell hyperplasia. The bursa of Fabricius had lymphoid depletion of follicles with increased proliferation of interfollicular connective tissue. At 21 days of age, the histologic appearance of the lymphoid organs from treated chickens were comparable to that of untreated control chickens.

Effect of T-2 toxin on B- and T-lymphocytes in peripheral blood. At 8 days of age, the percentage of B lymphocytes in peripheral blood was significantly lower in T-2 toxin-treated chickens than in untreated control chickens (Table 2.3). In contrast, the percentage of CD4 T lymphocytes was significantly higher in T-2 toxin-treated chickens than in untreated chickens. As for CD8 and CD3 T lymphocytes, no consistent effect of T-2 toxin was noted.

At 2 weeks post-treatment (21 days of age), except in trial 3, the percentage of B lymphocytes in T-2 toxin-

Table 2.2. Effect of T-2 toxin on body weight of 7-day-old, $15I_5 \times 7_1$ chicks treated with T-2 toxin for 7 consecutive days after hatch.

			Trial No....Mean Body Weight (gm)			
Group #	Lot #	T-2 toxin	1	2	3	4
1	1A	-	62.70 ± 3.10	60.98 ± 3.46	61.73 ± 4.67	57.37 ± 7.25
	1B	+	46.68 ± 5.10*	49.90 ± 5.93*	47.14 ± 5.29*	44.78 ± 3.86*
2	2A	-	61.97 ± 4.72	59.33 ± 3.50	63.53 ± 3.98	58.33 ± 3.68
	2B	+	45.39 ± 4.77*	45.66 ± 6.33*	48.77 ± 6.73*	45.10 ± 5.63*

*mean value significantly different from control group ($p \leq 0.05$)
No. of chicks per treatment group=27-55

Table 2.3. Effect of T-2 toxin on B- and T-lymphocyte subpopulations in peripheral blood of 8-day-old, $15I_5 \times 7_1$ chicks treated with toxin for 7 consecutive days after hatch.

		Lymphocyte Subpopulations[A] (%)			
		T-lymphocytes			B-lymphocytes
Trial No.	T-2 toxin	CD4	CD8	CD3	
2	-	10.23 ± 1.89	7.12 ± 1.03	22.13 ± 2.96	3.99 ± 0.78
	+	10.87 ± 3.27	7.05 ± 0.96	20.14 ± 4.18	0.72 ± 0.31*
3	-	11.26 ± 1.39	5.97 ± 0.79	20.03 ± 2.04	3.84 ± 0.16
	+	16.50 ± 2.24*	6.35 ± 0.93	25.66 ± 2.33*	1.28 ± 0.21*
4	-	13.09 ± 0.86	6.63 ± 0.57	21.35 ± 1.51	3.26 ± 0.30
	+	18.43 ± 2.15*	5.27 ± 1.07*	22.63 ± 2.12	0.45 ± 0.20*

*mean value significantly different from control group ($p \leq 0.05$)
[A]mean value and standard deviation
n=30 chicks (6 pooled samples) per treatment group

treated chickens was comparable to that in untreated control chickens (Table 2.4). Similarly, except in trial 3, the percentage of CD4, CD8 and CD3 T lymphocytes in T-2 toxin-treated chickens was comparable to that in untreated control chickens (Table 2.4).

Effect of T-2 toxin on B- and T-lymphocytes in spleen. At 9 days of age, the percentage of B lymphocytes in T-2 toxin-treated chickens was significantly lower than in untreated controls chickens (Table 2.5). The percentage of CD4 and CD3 T lymphocytes in T-2 toxin-treated chickens was significantly higher than in untreated controls. In trial 3, the percentage of CD8 T-lymphocytes was significantly higher in T-2 toxin-treated chicks than in untreated control chicks.

At 2 weeks post-treatment (22 days of age), the percentage of B lymphocytes in T-2 toxin-treated chickens was comparable to that of untreated chickens (Table 2.6). Except in trial 2, the percentages of CD4 and CD8 T-lymphocytes in the T-2 toxin-treated chickens were comparable to that in untreated chicks. No consistent effect of T-2 toxin on the percentage of CD3 T lymphocytes was noted.

Table 2.4. Effect of T-2 toxin on B- and T-lymphocyte subpopulations in peripheral blood of 21-day-old chickens treated with toxin for 7 consecutive days after hatch.

		Lymphocyte Subpopulations (%)			
		T-lymphocytes			B-lymphocytes
Trial No.	T-2 toxin	CD4	CD8	CD3	
1	-	10.30 ± 1.76	6.85 ± 1.62	20.37 ± 4.11	7.19 ± 2.03
	+	10.45 ± 1.40	6.38 ± 0.74	19.86 ± 2.76	6.95 ± 0.99
2	-	12.64 ± 1.39	7.12 ± 0.74	24.76 ± 2.79	6.27 ± 1.30
	+	12.43 ± 1.63	8.24 ± 1.06	25.16 ± 2.71	5.30 ± 1.38
3	-	12.43 ± 0.81	5.97 ± 0.79	22.77 ± 1.57	8.22 ± 0.51
	+	12.80 ± 1.17	7.29 ± 0.60*	24.63 ± 1.01*	6.26 ± 0.87*
4	-	14.04 ± 1.59	9.24 ± 1.55	23.42 ± 4.66	7.39 ± 0.29
	+	15.40 ± 1.03	9.86 ± 0.72	24.69 ± 1.36	6.99 ± 0.75

*mean value significantly different from control group ($p \leq 0.05$)
n=18 chicks (6 pooled samples) per treatment group

Table 2.5. Effect of T-2 toxin on B- and T-lymphocyte subpopulations in spleen of 9-day-old chickens treated with toxin for 7 consecutive days after hatch.

		Lymphocyte Subpopulations (%)			
		T-lymphocytes			B-lymphocytes
Trial No.	T-2 toxin	CD4	CD8	CD3	
2	-	23.04 ± 0.89	29.96 ± 0.88	74.02 ± 2.11	23.15 ± 2.21
	+	33.04 ± 1.63*	31.36 ± 3.12	78.94 ± 2.67*	8.64 ± 2.61*
3	-	22.38 ± 1.82	24.77 ± 2.22	58.99 ± 6.21	31.83 ± 4.53
	+	28.60 ± 0.66*	32.45 ± 2.27*	77.63 ± 4.15*	9.28 ± 1.54*
4	-	15.50 ± 1.49	11.27 ± 2.19	60.75 ± 3.68	22.06 ± 4.64
	+	21.28 ± 1.70*	13.94 ± 6.67	76.23 ± 1.33*	4.79 ± 1.01*

*mean value significantly different from control group ($p \leq 0.05$)
n=30 chicks (6 pooled samples) per treatment group

Table 2.6. Effect of T-2 toxin on B- and T-lymphocyte subpopulations in spleen of 22-day-old chickens treated with toxin for 7 consecutive days after hatch.

		Lymphocyte Subpopulations(%)			
		T-lymphocytes			B-lymphocytes
Trial No.	T-2 toxin	CD4	CD8	CD3	
1	-	18.20 ± 0.84	32.72 ± 3.18	71.31 ± 4.70	27.95 ± 3.48
	+	18.20 ± 0.56	29.57 ± 0.48*	64.95 ± 3.72*	30.38 ± 2.29
2	-	16.66 ± 1.60	26.51 ± 3.20	67.08 ± 5.58	28.80 ± 6.63
	+	19.90 ± 1.37*	28.45 ± 1.86	70.35 ± 4.76	27.30 ± 5.39
3	-	17.78 ± 3.63	39.76 ± 5.32	67.75 ± 2.07	29.07 ± 4.64
	+	19.21 ± 1.47	39.16 ± 2.66	71.63 ± 2.87*	28.27 ± 1.69
4	-	17.48 ± 1.45	33.87 ± 3.61	63.57 ± 4.47	25.64 ± 6.33
	+	17.03 ± 0.59	37.69 ± 2.13	65.49 ± 1.58	32.58 ± 2.83

*mean value significantly different from control group ($p \leq 0.05$)
n=18 chicks (6 pooled samples) per treatment group

Discussion

Data from this study indicate that exposure of day-old chicks to T-2 toxin may significantly reduce the percentage of both peripheral blood and splenic B-lymphocytes but only within 7 days of treatment. The data clearly suggest that repeated sublethal doses of T-2 toxin severely depleted B-lymphocytes, but not CD4, CD8 and CD3 T-lymphocytes. However, at 3 weeks of age (two weeks post-treatment), the gross appearance of the lymphoid organs, and the percentages of B- and T-lymphocytes in peripheral blood and spleen were comparable to that of untreated control chickens. Repeated exposure of farm and laboratory animals to low levels of T-2 toxin has been reported to produce immunosuppression characterized by inhibition of both humoral and cell-mediated immunity (Masuko et al., 1977; Lafarge-Frayssinet et al., 1979; Otokawa et al., 1979; Rosenstein et al., 1979, 1981; Mann et al., 1982, 1983; Taylor et al., 1985).

In this study, T-2 toxin significantly reduced the body weight of chicks within 7 days of treatment. This finding is consistent with results of subchronic and chronic exposure of chickens to T-2 toxin reported by Chi et al. (1977); Wyatt et al. (1972, 1973) and Hoerr et al. (1982a,b). The decrease in body weight associated with exposure to T-2 toxin has been attributed to reductions in feed consumption and weight gain. The decreased body weight observed in this

study was accompanied by atrophy of the bursa of Fabricius, spleen and thymus. This finding is also consistent with the findings previously reported by Hoerr et al. (1982a). The present study also confirms that the effect of T-2 toxin on lymphoid organs is transient with rapid regeneration and repopulation of the lymphoid organs upon termination of treatment of animals with the toxin. Hoerr et al. (1981) found that a single dose of T-2 toxin administered via crop gavage to 7-day-old chicks caused acute lymphoid necrosis and depletion followed by rapid repletion in the severely affected lymphoid organs by 3-7 days post-treatment.

Results from flow cytometric analysis conducted in the present study revealed that at 1 week post-treatment, depletion of both peripheral blood and splenic B-lymphocytes and a relative increase in CD4, CD3, T-lymphocyte subpopulations, suggesting that B-lymphocytes are highly susceptible to the toxic effects of the toxin. In contrast, Smith et al. (1994) reported the percentage of $CD45R^{+}$ B cells in the spleen of T-2 toxin-treated adult mice was not statistically different from untreated controls. However, a significant decrease in the total number of both B- and T-lymphocytes was noted. The depletion of B-lymphocytes in chickens treated with T-2 toxin is probably due to a direct effect of T-2 toxin on B-lymphocytes. Alternatively, T-2 toxin may have a cytotoxic effect on the progenitors of B

lymphocytes. Holladay et al. (1993) reported that unlike thymocytes, T-lymphocyte progenitors are highly sensitive targets for cytotoxic effects of T-2 toxin and are likely responsible for the thymic atrophy reported in mice. Further, Smith et al. (1994) reported an increase in percentages of double negative cells ($CD4^-8^-$) and decrease in percentage of double positive ($CD4^+8^+$) cells in the thymus of T-2 toxin-treated mice, suggesting that T-2 toxin may inhibit thymocyte maturation and differentiation. Results from the present study indicate that at 21-22 days of age, the percentages of both peripheral blood and splenic B- and T-lymphocytes of T-2 toxin-treated chickens were comparable to that of untreated controls. This finding correlated well with the findings of the gross examination of the lymphoid organs.

Analysis of data on both peripheral blood and splenic B-lymphocytes also confirmed that T-2 toxin may be associated with depression of humoral immunity (Jagadeesan et al. 1982). The reason for the increased proportions of CD3 and CD4 T-lymphocytes in the T-2 toxin-treated chickens or the inconsistent effect of the toxin on CD8 T-lymphocytes reported in the present study was not determined. Since the absolute numbers of B- and T-lymphocytes per unit sample was not determined, it is likely that T-2 toxin induced mild to moderate reductions in the T-lymphocyte subpopulations, as

suggested by mild to moderate lymphoid depletion of the thymus and T-lymphocyte regions of the spleen.

It is worth noting that the percentages of both peripheral blood and splenic B- and T-lymphocytes in untreated chickens were generally lower than those that had previously been reported for normal chickens (Chan et al., 1988; Hu et al., 1993; Rodenberg et al., 1994). These discrepancies in percentages of lymphocytes in normal chickens may be explained by experimental protocol, age of chicks, treatment as well as genetic factors. Hala et al. (1991, 1992) reported differences in percentages of CD4 and CD8 T- lymphocytes in both peripheral blood and spleen of inbred lines of congenic chickens that differ only in the MHC. It is therefore likely that ontogenetic or physiological development of individual chickens may influence the proportions of T-lymphocyte subpopulations.

Flow cytometric analysis of the blood and splenic lymphocytes suggests that treatment of chickens with T-2 toxin may result in significant depletion of B-lymphocytes within 7 days after treatment. Such depletion of B-lymphocytes may lead to reductions in the amount of immunoglobulins (IgG, IgM) which may adversely affect the immunologic and defense mechanisms of animals exposed to subclinical levels of T-2 toxin in the feed (Jagadeesan et al., 1982; Mann et al., 1983; Taylor et al., 1985).

CONCLUSIONS

The results from the research presented in this dissertation indicate that:

1) Treatment of RPRL line $15I_5$ X 7_1 white leghorn chickens with T-2 toxin at sublethal doses of 0.75, 1.0 and 1.25 mg/kg body weight for seven days after hatch may cause a significant dose-dependent decrease in body weight, as well as in relative weight of lymphoid organs, thymus, spleen, and bursa of Fabricius. However, at 1 and 2 weeks after the last treatment, the body weight and the relative weight of lymphoid organs were comparable to that of untreated controls. The decrease in relative weights of lymphoid organs correlated well with a dose-dependent mild to moderate or severe lymphoid depletion of the organs.

2) Treatment of chickens with T-2 toxin at a sublethal dose of 1.25 mg/kg body weight for seven days after hatch may:

   a) cause a reduction in HVT titres in vaccinated chicks within 7 days after hatch;

   b) shorten the incubation period for MD mortality in unvaccinated chickens.

3) Using the regimen described in this dissertation, treatment of chicks with T-2 toxin at a dose of 1.25 mg/kg

body weight for seven days did not influence the incidence of MD lesions/mortality in HVT-vaccinated chickens.

4) Using flow cytometric analysis, T-2 toxin at a sublethal dose of 1.25 mg/kg body weight administered to chicks for seven consecutive days after hatch may:

a) cause severe depletion of B-lymphocytes, and

b) a relative increase in CD4 and CD3 T-lymphocytes in peripheral blood and spleen at 8-9 days of age. However, at 21-22 days of age, the percentage of B- and T-lymphocytes was comparable to untreated chickens. The findings from flow cytometric analysis correlated with lesions noted in microscopic examination of the lymphoid organs.

APPPENDIX

APPENDIX

Introduction

Information regarding the toxicity of multiple doses of T-2 toxin in RPRL line $15I_5$ x 7_1 chickens is generally not available. The LD_{50} values for single (72-hr) and multiple (14 days) dose of T-2 toxin administered to 7-day-old male broiler chicks have been shown to be 4.0 and 2.90 mg/kg body weight, respectively (Hoerr et al. 1981). Therefore, in order to study the effects of T-2 toxin on vaccinal immunity to Marek's dissease and on B- and T-lymphocytes in 1-day-old white leghorn chicks, as described in chapters 1 and 2, respectively, it was necessary to conduct two preliminary trials to determine the lethal and sublethal (maximum tolerated) doses of T-2 toxin in white leghorn chickens treated with multiple doses of the toxin at hatch.

Trial 1

The objective of this trial is to determine the lethal dose of T-2 toxin in white leghorn chickens treated with single or multiple doses of the toxin at hatch.

Materials and Methods

T-2 toxin was obtained in crystalline form from Sigma Chemical Company (St. Louis, MO) and stored at -80^0 F until ready for use. F_1 progeny of line $15I_5$ males and 7_1 females

were used. In Trial 1, a total of 26, day-old chicks were divided into 2 replicates of 13 chicks each. Each replicate was divided into 4 treament groups. In each of groups 1, 2 and 3, three chicks were treated with T-2 toxin at a dose of 0.5, 1.5 and 4.5 mg/kg body weight. Four chicks in group 4 were maintained as untreated controls. Chicks were wing-banded and weighed everyday and the average weight for each group was used to calculate the dosages of T-2 toxin. T-2 toxin-treated and untreated chicks were housed in separate isolators.

T-2 toxin stock solution was prepared by adding 2.5 ml of 100% ethanol to a 25 mg of purified extract of toxin and stored in the freezer until ready for use. Phosphate buffered saline (PBS) was used as a diluent for T-2 toxin stock solution. For each dose group, T-2 toxin was added to the required volume of PBS and administered to day-old chicks via crop gavage. Untreated controls were treated with ethanol in PBS.

Results

Clinical signs and mortality

The severity of the clinical signs exhibited by chicks treated with T-2 toxin was dose-related. Chicks treated with T-2 toxin at a dose of 4.5 mg/kg body weight were depressed and developed moderate to severe straw-colored

watery diarrhea. All chicks died within 24-48 hrs after treatment (Table 3.1). Chicks treated with T-2 toxin at a dose 1.5 mg/kg body weight had mild to moderate straw-colored diarrhea; 83% of treated chicks died within 48-72 hrs after treatment. Although 33% of chicks treated with T-2 toxin at a dose of 0.5 mg/kg body weight died within 48-72 hrs after treatment, no clinical signs were noted. No clinical signs were noted in untreated control groups, but 13% of chicks died from unknown reasons.

Necropsy findings

Chicks treated with T-2 toxin at a dose of 4.5 mg/kg body weight developed moderate to severe subcutaneous edema around the crop and ventral abdomen which extended to the thighs. A large amount of straw-colored fluid with flecks of chalky-white material was present in the body cavities. The liver had severe multifocal to locally extensive subcapsular hematomas which extended in some areas into the parenchyma. Severe acute necrosis of lymphocytes characterized by pyknosis and karyorrhexis with sparing of the cortical lymphocytes within the medulla of the follicles of bursa of Fabricius was noted. Within the necrotic areas, numerous heterophils infiltrated the interfollicular connective tissue stroma (Figure 3.1). Severe acute random necrosis with moderate heterophilic infiltrate was noted in the cortex of thymus. Mild to moderate acute lymphocyte necrosis

Table 3.1. T-2 toxin-induced mortality in $15I_5 \times 7_1$ chicks 24-72 hrs after treatment with various doses of toxin at hatch.

Dose (mg/kg body weight)	T-2 toxin-induced mortality		
	Replicate 1	Replicate 2	Total (%)
Control	1/4*	0/4	1/8 (13)
0.5	2/3*	0/3	2/6 (33)
1.5	2/3	3/3	5/6 (83)
4.5	3/3	3/3	6/6 (100)

*No significant gross or microscopic lesions were noted.

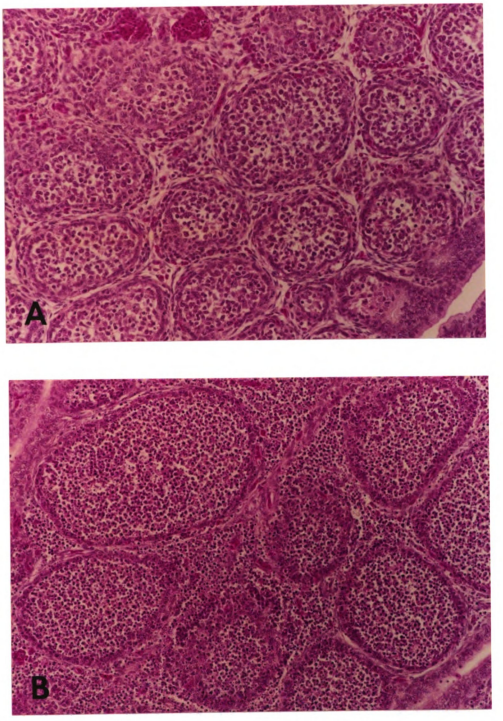

Figure 3.1. Photomicrographs of bursa of Fabricius from 2-day-old untreated control chick (A); and from a chick treated at hatch with T-2 toxin at a dose of 4.5 mg/kg body weight (B). Notice the severe extensive acute lymphoid necrosis within follicles and numerous heterophils within interfollicular connective tissue stroma (H&E X 242).

was noted randomly in the spleen. Severe extensive foci of hemorrhage with associated hepatocellular necrosis was noted in liver (Figure 3.2).

Chicks treated with T-2 toxin at a dose of 1.5 mg/kg body weight had mild to moderate acute necrosis of the lymphoid organs and liver had mild to moderate focally extensive hemorrhages. No significant gross or microscopic lesions were seen in either chicks treated with T-2 toxin at a dose of 0.5 mg/kg body or in untreated control chicks.

Trial 2

The objective of this trial was to determine the maximum (sublethal) tolerated dose.

Materials and Methods

Eighty, day-old chicks were wing-banded and divided into 2 replicates of 40 chicks each. Chickens in each replicate were divided into 4 treatment groups, each consisting of 10 chicks. In groups 1, 2 and 3, chicks were treated with T-2 toxin at hatch via crop gavage at a dose of 0.75, 1.00, 1.25 mg/kg body weight, respectively, for 7 consecutive days. T-2 toxin dose was based on the average weight of the chicks used in each treatment group. Chicks in group 4 were maintained as untreated controls. At 8, 15 and 22 days of age, 3 chicks from each group in replicate 1 were

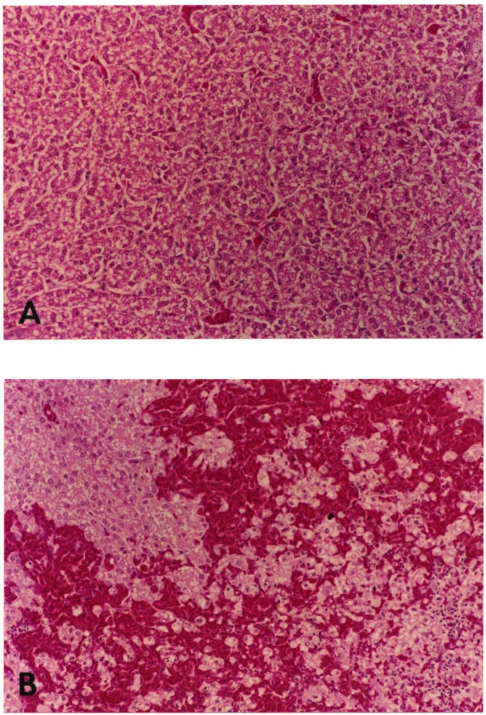

Figure 3.2. Photomicrographs of liver from 2-day-old untreated control chick (A); and from a chick treated at hatch with T-2 toxin at a dose of 4.5 mg/kg body weight (B). Notice the severe locally extensive area of hemorrhage with associated hepatocellar necrosis (H&E X 242).

weighed and sacrificed. The lymphoid organs were aseptically collected, weighed and placed in 10% neutral buffered formalin to be processed for histopathological evaluation. In addition, 6 chicks from various treatment groups in replicate 2 were bled at 8, 15, and 22 days of age for the determination of hematocrit value.

For statistical analysis ANOVA and SNK was done to determine the effects of the toxin on body weight, and on lymphoid organ weights using body weight as a covariate.

Results

Mortality of chicks within 24-48 hrs after treament is shown in Table 3.2. These deaths probably were due to nonspecific causes or accidental drowning from the treatment. At 7 days of age, (the end of the dosing period) there was a significant dose-related decrease in hematocrit, body weight, and the relative weights of the bursa of Fabricius, spleen and thymus (Table 3.3-3.7). However, at 15 and 22 days of age, body weights, hematocrit and relative weights of lymphoid organ in treated chicks were comparable to those in untreated chicks.

Examination of the bursa of Fabricius revealed moderate to severe depletion of lymphocytes within the medulla of the bursal follicles. The bursal follicles were reduced in size and there was an increased interfollicular fibrosis (Figure 3.3a,b). Mild to moderate depletion of lymphocytes were

Table 3.2. T-2 toxin-induced mortality in $15I_5$ X 7_1 chicks within 24-48 hrs after treatment with various doses of toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	T-2 toxin-induced mortality			
	Replicate 1	Replicate 2	Total	(%)
Control	0/10	1/10	1/20	(5)
0.75	2/10	1/10	3/20	(15)
1.00	1/10	0/10	1/20	(5)
1.25	2/10	0/10	2/20	(10)

Table 3.3. Hematocrit values of $15I_5 \times 7_1$ chicks at various ages after treatment with various doses of T-2 toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	N	Age of chicks -- Hematocrit[AB]		
		8 days	15 days	22 days
Control	6	30.83 ± 1.47[a]	29.50 ± 1.76	29.50 ± 1.76
0.75	6	31.83 ± 1.60[a]	30.50 ± 1.38	26.33 ± 12.97
1.00	6	29.67 ± 1.97[a]	31.67 ± 1.51	32.50 ± 3.08
1.25	6	27.67 ± 1.37[b]	31.67 ± 1.97	28.67 ± 4.37

[A]Means values within a column followed by different superscript letter are significantly different ($P<0.05$).
[B]Means and standard deviation.

Table 3.4. Body weight of $15I_5 \times 7_1$ chicks at various ages after treatment with various doses of T-2 toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	N	Age of chicks -- Body Weight[AB]		
		7 days	14 days	21 days
Control	3	74.87 ± 5.93[c]	117.27 ± 30.27	162.13 ± 27.19
0.75	3	64.40 ± 2.75[ac]	126.30 ± 11.52	181.93 ± 16.87
1.00	3	55.47 ± 6.35[ab]	125.63 ± 11.67	162.15 ± 9.75
1.25	3	52.37 ± 7.23[a]	120.83 ± 8.47	153.03 ± 20.75

[A]Means values within a column followed by different superscript letter are significantly different ($P<0.05$)
[B]Means and standard deviation

Table 3.5. Relative weight of bursa of Fabricius of $15I_5$ X 7_1 chicks at various ages after treatment with various doses of T-2 toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	N	Age of chicks -- Relative Weight[AB] of Bursa		
		7 days	14 days	21 days
Control	3	0.45 ± 0.05[c]	0.75 ± 0.30	1.22 ± 0.26
0.75	3	0.29 ± 0.05[a]	0.73 ± 0.14	1.17 ± 0.32
1.00	3	0.28 ± 0.03[a]	0.70 ± 0.14	1.15 ± 0.06
1.25	3	0.21 ± 0.04[b]	0.64 ± 0.11	0.90 ± 0.21

[A]Mean values within a column followed by different superscript letter are significantly different ($P<0.05$)
[B]Means (adjusted for body weight) and standard deviation.

Table 3.6. Relative weight of spleen of $15I_5$ X 7_1 chicks at various ages after treatment with various doses of T-2 toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	N	Age of chicks -- Relative Weight[AB] of Spleen		
		7 days	14 days	21 days
Control	3	0.22 ± 0.02[d]	0.32 ± 0.03	0.44 ± 0.14
0.75	3	0.18 ± 0.01[a]	0.36 ± 0.08	0.45 ± 0.06
1.00	3	0.17 ± 0.01[ac]	0.32 ± 0.04	0.43 ± 0.05
1.25	3	0.17 ± 0.02[bc]	0.29 ± 0.02	0.38 ± 0.09

[A]Mean values within a column followed by different superscript letter are significantly different ($P<0.05$)
[B]Means (adjusted for body weight) and standard deviation.

Table 3.7. Relative weight of thymus (right) of $15I_5$ X 7_1 chicks at various ages after treatment with various doses of T-2 toxin for 7 consecutive days after hatch.

Dose (mg/kg body weight)	N	Age of chicks -- Relative Weight[A,B] of Thymus (Right)		
		7 days	14 days	21 days
Control	3	0.43 ± 0.03[c]	0.50 ± 0.09[a]	0.53 ± 0.11
0.75	3	0.29 ± 0.03[a]	0.55 ± 0.03[ab]	0.72 ± 0.17
1.00	3	0.23 ± 0.01[ab]	0.57 ± 0.05[b]	0.64 ± 0.14
1.25	3	0.21 ± 0.05[b]	0.51 ± 0.02[ab]	0.64 ± 0.11

[A]Mean values within a column followed by different superscript letters are significantly different ($P<0.05$)
[B]Means (adjusted for body weight) and standard deviation.

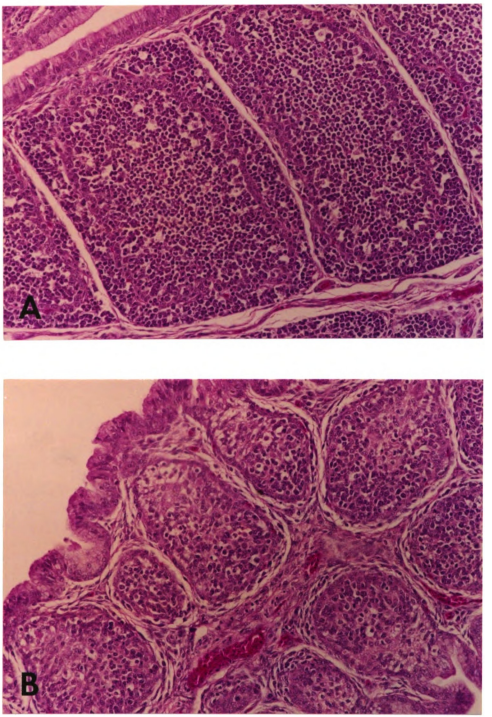

Figure 3.3. Photomicrographs of bursa of Fabricius from 8-day-old untreated control chick (A); and from a chick treated with T-2 toxin at a dose of 1.25 mg/kg body weight for 7 consecutive days after hatch (B). Notice the extensive depletion of lymphoid cells within follicles and increased interfollicular connective tissue (H&E X 242).

noted within the cortex of the thymus (Figure 3.4a,b) and white pulp of the spleen (Figure 3.5a,b).

The cellularity of the bone marrow from chicks treated with T-2 toxin at a dose of 1.25 mg/kg body weight was comparable to that of untreated controls (Figure 3.6a,b).

Discussion

Data from these trials indicate treatment of day-old white leghorn chicks with T-2 toxin at a dose of 1.5 and 4.5 mg/kg body weight may cause up to 100% mortality within 72 hrs after treatment. In contrast, the tolerated doses of 0.75 and 1.25 mg/kg body weight reduced both body weight and relative weights of the lymphoid organs, but only at 8 days of age. This is consistent with the findings by Wyatt et al. (1973), Boonchuvit et al. (1975). The data also suggest that of all lymphoid organs examined histologically, the bursa of Fabricius was the most severely affected. This finding is consistent with studies by Hoerr et al., 1981, 1982a,b.

Analysis of data obtained from examination of bone marrow suggests that the hematopoeitic cells of the bone marrow were not affected by the sublethal dose of T-2 toxin, compared with the negative effects of the toxin on the lymphoid organs (Chi et al., 1977a,b). While specific identification of the heterogeneous population of bone marrow cells was not done, the data suggest that these cells

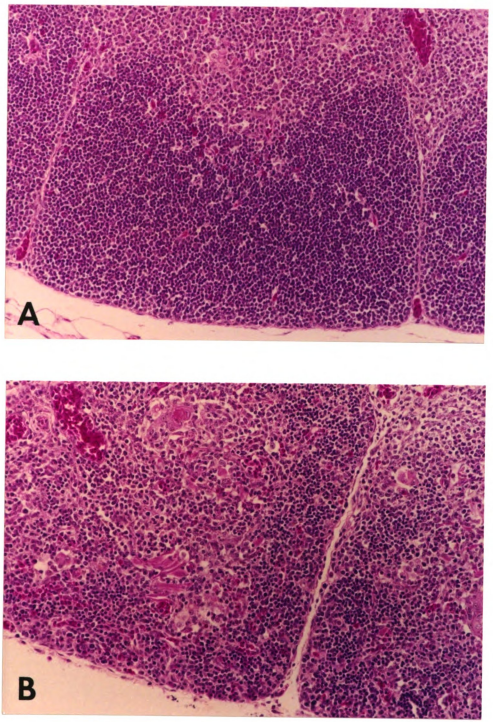

Figure 3.4. Photomicrographs of thymus from 8-day-old untreated control chick (A); and from a chick treated with T-2 toxin at a dose of 1.25 mg/kg body weight for 7 consecutive days after hatch (B). Notice the moderate depletion of cortical lymphocytes (H&E X 242).

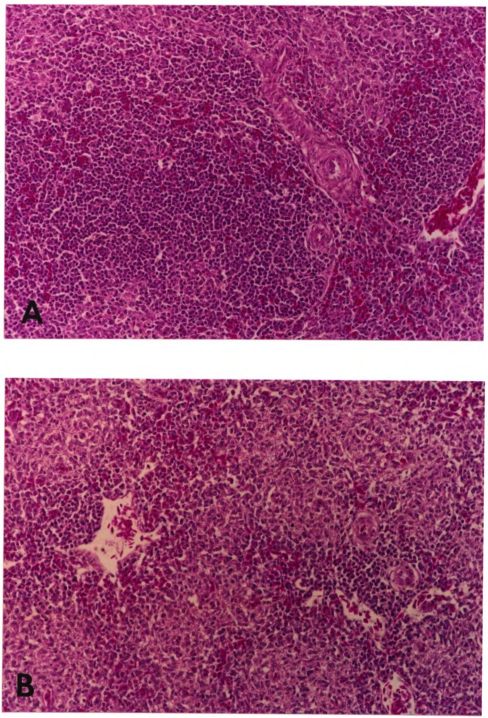

Figure 3.5. Photomicrographs of spleen from 8-day-old untreated control chick (A); and from a chick treated with T-2 toxin at a dose of 1.25 mg/kg body weight for 7 consecutive days after hatch (B). Notice moderate depletion of lymphocytes with reticoloendothelial cell hyperplasia (H&E X 242).

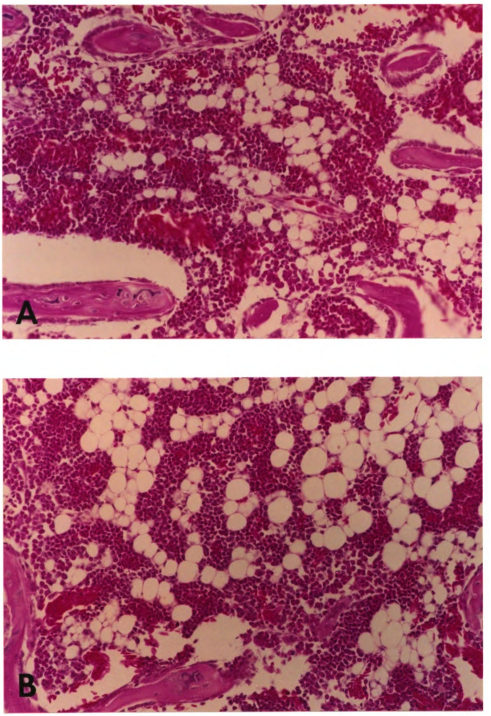

Figure 3.6. Photomicrographs of bone marrow from 8-day-old untreated control chick (A); and from a chick treated with T-2 toxin at a dose of 1.25 mg/kg body weight for 7 consecutive days after hatch (B). Note absence of loss of hematopoeitic cells and the similar cellularity compared to control chick (H&E X 242)

may allow the lymphoid organs to be repopulated within one week after termination of treatment with T-2 toxin. In addition, it appears that the sublethal dose of 1.25 mg/kg body weight used in this study was immunotoxic but probably not myelotoxic in chickens. Treatment with T-2 toxin at a dose of 1.25 mg/kg body weight did not affect the liver and kidney when examined histologically suggesting that this dose which is clearly immunotoxic, probably did not have other subclinical effects.

These adverse effects of T-2 toxin have been shown to be associated with the inhibitory effects of T-2 toxin on protein synthesis (Ueno et al., 1968; Bamburg, 1974; Cundliffe and Davis, 1977).

On the basis of the findings from these two trials, a maximum tolerated dose of 1.25 mg/kg body weight was selected for the studies described in chapters 1 and 2.

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