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VOMITOXIN-INDUCED MURINE IgA NEPHROPATHY AND ROLE OF CYTOKINE DYSREGULATION

By

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ABSTRACT

VOMITOXIN-INDUCED MURINE IgA NEPHROPATHY AND ROLE OF CYTOKINE DYSREGULATION

By

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Fusarium mycotoxins are commonly found in food and feed and have been shown to cause human and animal health problems. The objectives of this research were to assess the immunopathogenic parameters associated with one potential health effect, vomitoxin-induced IgA nephropathy, and to determine role of cytokine dysregulation in this novel toxin-induced dysregulation of IgA production. IgA nephropathy was induced in female B6C3F1 mice exposed to 25 ppm vomitoxin as evidenced by increased mesangial IgA deposition, serum IgA, circulating IgA immune complexes, IgA production in cultured splenic and Peyer's patch cells, $CD4^+$ population in spleen and Peyer's patches, and hematuria. These effects persisted as long as 16 weeks after cessation of vomitoxin exposure. EL4-IL2, a mouse thymoma cell line, was used as a model cell line for evaluating the effects of vomitoxin on T cells. The cell line was induced with the T cell mitogen, phorbol myristate acetate, in the presence and absence of vomitoxin. Superinduction of interleukin 2 (IL-2) and interleukin 5 (IL-5) was observed in cellular mRNA

from cells treated with 50-100 ng/ml vomitoxin for 12-24 and 48 hours, respectively. Supernatant IL-2 and IL-5 were also significantly increased in cells treated with 50 ng/ml vomitoxin for 12-48 hours and for 8 days, respectively. The results suggest that dietary exposure to vomitoxin induces persistent murine IgA nephropathy. Superinduction of IL-2 and IL-5 in T cells may potentially play important roles in the hyperproduction of IgA in vomitoxin-induced murine IgA nephropathy.

To my parents who have inspired me throughout my life.

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KEY TO ABBREVIATIONS:

AFC	antibody forming cell
AU	arbitrary unit
BSA	bovine serum albumin
C	complement
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
cDNA	complementary DNA
CMC	carboxymethyl cellulose
Con A	concanavalin A
CSF	colony stimulating factor
DMEM	Dulbecco's modified Eagle medium
DNP	dinitrophenol
DON	deoxynivalenol or vomitoxin
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GBM	glomerular basement membrane
GM-CSF	granulocyte/macrophage colony stimulating factor
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HS	herring sperm
IC	immune complex
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MALT	mucosal associated lymphoid tissue
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDA	potato dextrose agar
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PFC	plaque forming cell
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP	Peyer's patch
RIA	radioimmunoassay
SC	secretory component
TGF-β	transforming growth factor β
Th	T helper
TLC	thin layer chromatography
TNF	tumor necrosis factor
TPA	tetradecanoylphorbol acetate

Chapter One

VOMITOXIN-INDUCED IgA NEPHROPATHY IN THE FEMALE B6C3F1 MOUSE

1.0 INTRODUCTION

1.1 Vomitoxin

1.1.1 *Mycotoxins*

Secondary metabolism is restricted mostly to the lower forms of life and most secondary metabolites play no obvious role in the economy of the organism (Campbell, 1984). The enzymes involved in secondary metabolism are apparently of low specificity, which usually results in the production of particular chemical families of structurally related metabolites (Betina, 1989). Mycotoxins are examples of secondary fungal metabolites which can be toxic to humans and animals after exposure. Mycotoxins and mycotoxicoses received little attention until the early 1960s, when the aflatoxins were discovered.

Mycotoxins are significant because they can cause diseases in food-producing animals and moldy changes in crops with concurrent economic losses and because they can potentially impact human health (Bhat *et al.*, 1989; Cote *et al.*, 1984). Mycotoxin-producing fungi are ubiquitous in nature and fungal invasion of crops and foods can occur at any stage of a crop growth and food production. The occurrence of mycotoxins in food and feed depends on their formation by specific fungal strains and is affected by

environmental factors such as humidity and temperature (WHO, 1979). Mycotoxin contamination varies with geographical conditions, production, storage methods, and the type of food. Aromatic amino acids such as phenylalanine, tyrosine, and tryptophan are used in considerably high amounts by fungi in mycotoxin biosynthesis (Yamazaki, 1978). Organ specificity and degree of toxicity of different mycotoxins vary greatly, depending on both their structure and the experimental animal species.

1.1.2 *Trichothecenes*

The trichothecenes are recognized as one of the most important classes of mycotoxins in the world. They are a family of structurally closely related sesquiterpenoids, and biologically active secondary metabolites produced mainly by *Fusarium* and various other species of fungi such as *Trichothecium, Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium,* and *Stachybotrys* (Ichinoe and Kurata, 1983; Ueno, 1983). Trichothecenes have been reported to be associated mostly with wheat, barley, corn, safflower, and sorghum (Ueno, 1983). Trichothecin, the first known member of trichothecenes, was originally discovered as an antifungal antibiotic and a metabolite from *Trichothecium roseum* (Freeman and Morison, 1948).

Trichothecene mycotoxins produce symptoms such as nausea, emesis, feed refusal, skin irritation, diarrhoea, leukopenia, hemorrhaging, and reproductive problems (Betina, 1989; Pestka and Bondy, 1990; Ueno, 1987). Trichothecenes

are also cytotoxic, bind to eukaryotic ribosomes, and are potent protein synthesis inhibitors (Bamburg, 1983; Betina, 1989; Ueno, 1983) and are known to cause immunosuppression (Corrier, 1991). In whole animals, the trichothecenes target rapidly dividing cells such as bone marrow, thymus, spleen, lymph nodes, epithelium, testis, and ovary (Grove and Mortimer, 1969).

Trichothecenes possess the tetracyclic 12,13-epoxy-trichothec-9-ene skeleton. Compounds with 12,13-epoxy-trichothec-9-ene have marked cytotoxicity against HeLa cells in culture (Machida and Nozoe, 1972) and inhibit protein synthesis (Yoshizawa *et al.*, 1985). The opening of the epoxide ring by hydrogenation detoxifies these compounds. The presence of acetyl, hydroxyl, and hydrogen groups on the trichothecene nucleus modifies their biological activities such as lymphotoxicity (Forsell and Pestka, 1985). Sequential deacetylation or hydroxylation of T-2 toxin significantly reduces the immunotoxicity of its metabolites (Forsell *et al.*, 1985). A second epoxide at C7-8 reduces and at C9-10 can markedly increase activities such as anti-leukemic effect (Jarvis *et al.*, 1983).

1.1.3 *Biochemical effects*

Effects of trichothecenes on eukaryotic protein synthesis have been reviewed (Kiessling, 1986; Ueno, 1987). Three steps in protein synthesis are recognized, i.e. initiation, elongation, and termination. A methionine residue donated by a special initiating methionyl-tRNA

attaches to an AUG-initiation codon in the mRNA in the initiation step. An aminoacyl-tRNA complex specified by the next coding triplet in the mRNA binds to the 80S complex of ribosomal subunit in the elongation step. Peptide bonds are formed and catalyzed repeatedly by peptidyl transferase until the polypeptide chain is completed. An ending codon in the mRNA signals the termination step and the polypeptide is released from its terminal tRNA. Trichothecenes have high affinity for the ribosomes of eukaryotic cells, particularly for the 60S subunit (Wei *et al.*, 1974). The inhibitory effects of a particular trichothecene also depend on its structural features and concentration (Lafarge-Frayssinet *et al.*, 1979; Miller and Atkinson, 1986). Whether the trichothecene is an initiation inhibitor (I-type) or elongation-termination inhibitor (ET-type) can be determined by the presence or absence of certain substituents such as ester group at carbon positions such as C3, C4, and C15 (Kiessling, 1986). Vomitoxin is a ET-type inhibitor and inhibits the elongation process. It probably interacts with the peptidyl transferase center on the 60S ribosomal subunit and suppresses the formation of peptide bonds.

In mammalian cells, the trichothecenes also inhibit DNA and RNA synthesis. However, the inhibition of nucleic acid synthesis requires higher toxin concentrations and the degree of inhibition is far less than that of protein synthesis. It is thus generally considered as secondary

effect following impairment of protein synthesis (Ueno, 1985). However, in protozoa *Tetrahymena pyriformis*, low concentration of T-2 toxin inhibits the synthesis of protein, DNA, and RNA at the same level (Ueno, 1983).

T-2 toxin interacts with cell membranes and alters membrane function in L-6 myoblasts. The impairment of the uptake of calcium, glucose, leucine, tyrosine, and thymidine leads to abnormality of cell physiology and failure of protein and nucleic acid incorporation (Bunner and Morris, 1988).

1.1.4 *Immunological effects*

Acute toxicity of trichothecenes can cause severe damage of lymphoid organs and tissues (Grove and mortimer, 1969). Chronic toxicity of trichothecenes results in immune modulation. Immunosuppressive effects of trichothecenes that have been observed include progressive leukopenia, inhibition of antibody responses, prolongation of skin allograft survival (Rosenstein *et al.*, 1979), depressed delayed hypersensitivity (Pestka *et al.*, 1987; Tryphonas *et al.*, 1986), decreased blastogenic response (Lafarge-Frayssinet *et al.*, 1979; Pang *et al.*, 1987), and enhanced microbial infection (Corrier and Ziprin, 1987; Tai and Pestka, 1988a,b). Immunostimulatory effects of trichothecenes include enhanced resistance to microbial infection (Corrier and Ziprin, 1986), increased splenic cell proliferation (Hughes *et al.*, 1989), increased splenic spontaneous antibody-producing cells (Cooray and Lindahl-

Kiessling, 1987), and elevated serum IgA production (Forsell *et al.*, 1986). Many of these immunotoxic manifestations can be related to the malfunction of T cells.

1.1.5 *Vomitoxin*

Many trichothecene mycotoxins produced by *Fusarium* either *in vitro* or naturally cause emesis in a variety of laboratory animals. Vomitoxin was first identified from *Fusarium*-infected barley in Japan (Yoshizawa and Morooka, 1973). Its chemical structure was analyzed and designated as 4-deoxynivalenol (DON). It was simultaneously isolated from *Fusarium*-contaminated field corn that had caused vomiting in experimental pigs and given the name "vomitoxin" (Vesonder *et al.*, 1973). Vomitoxin is found in cereal grains worldwide (Tanaka *et al.*, 1988).

Lethal toxicity of vomitoxin has also been studied in many animals through different routes. LD_{50} are 70.0 mg/kg (i.p.) or 46.0 mg/ml (p.o.) in male mice, 76.7 mg/kg (i.p.) in female mice, and 27.0 mg/kg (s.c.) in duckling (Ueno, 1983). It should be noted that besides vomitoxin, T-2 toxin, HT-2 toxin, nivalenol, 3-acetyl-DON, 15-acetyl-DON, and diacetoxyscirpenol have been identified as natural contaminants in cereal grains (Karppanen *et al.*, 1985; Mirocha *et al.*, 1976).

Vomitoxin has been produced in culture by *F. graminearum* on rice and purified for toxicological studies. Recent approaches for purification include chromatograph or a charcoal-alumina column followed by separation on a Sephadex

LH20 column (Ehrlich and Lillehoj, 1984) or simply by water-saturated silica gel chromatography (Witt *et al.*, 1985).

Feed refusal and emesis are two most apparent symptoms experimentally caused by vomitoxin in animals. It was believed that feed refusal factors were toxic metabolites of *Fusarium* species (Vesonder *et al.*, 1977) and were revealed to be identical with vomiting factors in swine (Vesonder *et al.*, 1979). Doses of vomitoxin which induce emesis are 13.5 mg/kg (s.c.) in duckling, 0.1-0.2 mg/kg (i.v.) in dog, and 0.05 mg/kg (i.p.) or 0.1-0.2 mg/kg (p.o.) in swine (Ueno, 1983).

Vomitoxin administration by oral gavage or dietary exposure can increase susceptibility to *Listeria* infection, depress the delayed hypersensitivity response, and impair the murine splenic plaque forming cell (PFC) response to the sheep red blood cells (Pestka *et al.*, 1987; Tryphonas *et al.*, 1986).

Vomitoxin has a molecular weight of 296 and is poorly immunogenic (Casale *et al.*, 1988). There is no evidence of increased antigen penetration through epithelial wall of Peyer's patches or lamina propria after prolonged dietary vomitoxin exposure. Food and bacterial antigens may be responsible for initiating the immune response.

Pestka *et al.* (1989) reported that 25 ppm dietary exposure to vomitoxin induced elevation of serum immunoglobulin (Ig) A and IgA nephropathy in mice. The same effects were not found when the feed intake of control mice

was restricted to the level consumed by vomitoxin-treated mice, since feed refusal was commonly found in vomitoxin-treated animals. An increased ratio of polymeric to monomeric IgA was found in vomitoxin-treated mice (Pestka *et al.*, 1989). Male mice were found to be more prone to vomitoxin-induced IgA dysregulation and IgA nephropathy than female (Greene *et al.*, 1993). Recently, it was determined that hyperelevation of total and specific serum IgA for oral and self antigens occurred during vomitoxin feeding in mice (Rasooly and Pestka, 1992).

1.2 IgA Nephropathy

1.2.1 *History*

IgA nephropathy, known as Berger's disease, was first recognized in 1968-1969 by Jean Berger, a French pathologist (Berger, 1969), who employed fluorescein-conjugated antibodies against IgA for the first time in human renal biopsies. The result showed a strong positive reaction in 25% of the patients with renal disorders. Most of the patients had no systemic symptoms but suffered recurring macroscopic hematuria or persistent microscopic hematuria. IgA was the most predominant isotype found in mesangial deposition and often co-deposited with IgG and complement (C) 3. IgA nephropathy was subsequently found to be the most frequent (about 10%) single cause of end-stage renal failure requiring dialysis and the most common form of glomerulonephritis in the world (D'Amico 1987; D'Amico *et*

al., 1985).

1.2.2 *Classification*

IgA nephropathy is associated with other diseases, particularly those involving mucosal immunity and/or immune response of IgA isotype. It has been classified into two forms (Woodroffe *et al.*, 1982). Primary IgA nephropathy refers to isolated IgA nephropathy associated with systemic disease Henoch-Schönlein purpura. Secondary IgA nephropathy is caused by alcoholic liver disease, portal systemic shunts, celiac disease, dermatitis herpetiformis, IgA monoclonal gammopathy, mycosis fungoides, mucin-secreting and other carcinomas, leprosy, pulmonary hemosiderosis, ankylosing spondylitis and other seronegative spondyl-arthropathies, and cyclical neutropenia (Clarkson, 1987).

1.2.3 *Geographic, age, sex, and race distribution*

In many countries, IgA nephropathy is the single most common type of primary glomerulonephritis (D'Amico *et al.*, 1985). The incidence ranges from 20-25% in France, Italy, Spain, Finland, Scotland, Holland, Germany, Singapore, and Australia, whereas it is as high as 30-40% in Japan. The incidence appears to be lower in North America and the United Kingdom (Sissons *et al.*, 1975; Wyatt *et al.*, 1984). Although they may reflect renal variations in disease occurrence, the incidences are parallel to the frequency of routine urinalysis, the aggressiveness of renal biopsy policies, and the use of immunofluorescence antibodies to examine biopsy material, since different accepted

indications for renal biopsy are applied in different countries. The actual incidence of disease may be even higher than the 20-30% of biopsied populations.

The absence of dramatic clinical manifestations in many patients makes it impossible to determine the exact date of onset. Most studies show an obvious male predominance. The ratio of IgA nephropathy incidence in male and female varies from 1.2:1 to 15:1, but most of the ratios are approximately 2:1 (Power and Simpson, 1990). IgA nephropathy seems distinctly uncommon in American blacks (Jennette *et al.*, 1985), whereas American Indians appears to have a high incidence (Smith and Tung, 1985).

1.2.4 *Clinical and laboratory features*

In human IgA nephropathy, mesangial IgA deposition and hematuria occur almost always concomitantly (Emancipator and Lamm, 1989). Thirty to thirty-five percent patients with IgA nephropathy have macroscopic hematuria. Macroscopic hematuria often occurs in patients with infections of other mucosal surfaces, for instance, pharyngitis, tonsillitis, pneumonia, gastroenteritis, and urinary tract infections. It is frequently associated with systemic symptoms such as fever, fatigue, and abdominal pain (Walshe *et al.*, 1984), and generally disappears in one day to one week.

Microscopic hematuria also occurs in 30-35% patients with IgA nephropathy. It is often asymptomatic, persistent in the majority of cases, and accompanied by proteinuria at the late stage (Kincaid-Smith and Nicholls, 1983).

Unlike other systemic diseases, IgA nephropathy has diverse clinical features such as hematuria, proteinuria, edema, and hypertension, but very few of them are important in terms of diagnosis. In the course of the disease, there are usually very few clinical signs and symptoms. Sometimes mild microscopic hematuria with or without proteinuria may be the only signs in patients. Nearly half of patients are discovered during routine medical examinations. However, in severe cases, it often gives rise to severe hypertension, edema, and renal failure in the later stage of this disease.

Clinically, signs of unfavorable prognosis of IgA nephropathy include older age at the onset, being of the male sex, no history of macroscopic hematuria (Droz *et al.*, 1984), decreased glomerular filtration rate, malignant hypertension, severe microhematuria (Nicholls *et al.*, 1984), and heavy proteinuria (Kobayashi *et al.*, 1983). Pathologically, glomerular and interstitial sclerosis (D'Amico *et al.*, 1985), intra-capillary and extra-capillary proliferation, segmental thickening of glomerular basement membrane (GBM), and extension of immunofluorescent deposits to the peripheral capillary walls (D'Amico, 1984) are evidence of more frequent and faster deterioration of renal function.

Serum concentrations of IgA are increased in about 50% of patients (Clarkson *et al.*, 1977). The increase of serum IgA could be the combination of increased IgA production and decreased elimination by the liver (Peppard *et al.*, 1981).

Normal human plasma contains relatively high concentration of IgA but its role is still unclear. IgA is involved in host defense against microbial invasion and the clearance of dietary and environmental materials that may gain access across the various mucosal surfaces.

More IgA is synthesized than any other Ig in the human (McGhee *et al.*, 1989; Mestecky *et al.*, 1986). Although the function of serum IgA is less defined, one possible role is the nonphlogistic neutralization of toxins, viruses, and enzymes (Mestecky, 1988). IgA is the main active Ig at the mucus membrane and serves as an immunologic barrier to the penetration of macromolecules and microorganisms. In secretions, it exists as a dimer linked by a secretory component and a J chain.

In human, there are two IgA subclasses (IgA1 and IgA2). IgA1 is the main circulating IgA subclass in systemic compartment, while IgA2 subclass is of mucosal origin. IgA2 is present in fluids associated with mucosal surfaces, synthesized locally by plasma cells, and dimerized intracellularly with a cystine-rich polypeptide or J chain prior to secretion. It is highly resistant against proteolysis because of incorporating of a secretory component (SC) produced by local epithelial cells. Both subclasses are found as mesangial deposits in IgA nephropathy patients. Some studies show that IgA1 was predominant (André *et al.*, 1980), while others find IgA2 was chief subclass in mesangial deposition (Hall *et al.*, 1983). It is possible

that antigenic stimulation of a mucosa will cause local IgA2 secretion in the mucosa, but preferentially IgA1 synthesis in the associated lymphoid tissue. Murine IgA does not have subclasses, but concepts of systemic and mucosal origins from human IgA can be applied also to murine IgA.

IgA immune complex (IC) was initially separated by sucrose density gradient ultracentrifugation at specific pH values (Lopez-Trascass *et al.*, 1980). The testing method has been dramatically improved recently. It becomes more specific using enzyme-linked immunosorbent assay (ELISA) to analyze IgA in polyethylene glycol (PEG)-precipitated immune complexes.

High levels of circulating IgA-IC and increased ratio of polymeric to monomeric IgA appear to be associated with mesangial deposition of polymeric IgA-IC in patients with IgA nephropathy by many studies (Tomino *et al.*, 1984). It is believed that the immunoglobulins deposited in the glomeruli are the same as those in circulating complexes. Mesangial IC deposition causes renal histological changes since superoxide release by mesangial cells was found after incubation with IC (Sedor *et al.*, 1987). Most of the passive experimentally-induced IgA nephropathy models are associated with administration of IgA-IC or aggregated polymeric IgA (Emancipator, 1988). When polymeric and monomeric IC were prepared *in vitro* and were injected in mice, it was found that only polymeric IC induced murine IgA nephropathy (Rifai *et al.*, 1985). Polymeric rather than

monomeric IgA-IC correlates significantly with the persistence of microhematuria in several studies (Egido *et al.*, 1984b). It may be advantageous for IgA nephropathy patients to preferentially secrete mucosal polymeric IgA, because it is resistant to proteolytic enzymes produced by a number of microorganisms.

It is possible that the IgA deposited in the mesangium has a mucosal origin and the generation of IgA anti-IgA-IC is the cause of IgA nephropathy. Mucosal IgA is transported to and cleared by the liver where it can be bound to bile cholesterol and phosphatidylcholine. This form of IgA is protected from pancreatic enzyme hydrolysis and may be antigenic. After release from liver, it can bind with IgA to form IgA anti-IgA-IC which can then be deposited in the mesangium (Allardyce, 1987).

It is believed that IgA bearing cells are precursors of IgA secreting cells. They are switched from premature cells with μ heavy chain to mature IgA secreting B cells with α heavy chain after stimulation by antigens or mitogens. *In vitro* secretion of IgA in culture supernatant by blood mononuclear cells has been observed in patients with IgA nephropathy. Increased IgA production was found in both mitogen-stimulated (Egido *et al.*, 1982b) and unstimulated or spontaneous (Endoh, 1984) culture systems. The ratio of $CD4^+$ to $CD8^+$ cells is elevated in IgA nephropathy patients (D'Amico, 1987; Rothschild and Chatenoud, 1984).

1.2.5 *Histopathology*

IgA nephropathy is characterized by diffuse mesangial deposition of Ig with IgA as a predominant isotype. It is not unusual to see some fine granular staining in peripheral capillary loops of mesangium (Power and Simpson, 1990). The mesangium attached by glomerular capillaries is the central anatomic structure of the glomerulus. It is composed of mesangial cells, a small number of normally resident macrophages, and a matrix consisting of a mixture of muco-polysaccharides and glycoproteins. Collagen is usually absent and any presence of collagen in the matrix indicates a pathologic change. Cell culture studies suggest that the matrix is produced by the mesangial cells (Schienman *et al.*, 1976). Mesangial cells are of renal origin, resemble smooth muscle cells, and regulate the area of the GBM available for filtration by contraction. The rate of filtration controls the amount of macrophages entering the mesangium. Mesangial cells are capable of regulating regional immune reaction by producing interleukin (IL) 1, prostaglandins, and free oxygen radicals (Lovett *et al.*, 1983). A single layer of fenestrated endothelium and the GBM separate the mesangial region from the capillary lumen. This allows molecules from the circulation to slowly and persistently enter the mesangium.

The mesangium is enlarged by changes of cytoplasm and matrix in IgA nephropathy (McCoy *et al.*, 1974; Sinniah and Churg, 1983). The mesangial cells show abundant cytoplasm

with increased number of mitochondria and prominent endoplasmic reticulum in the acute and active stages of the disease. The thickened and serpiginous matrix caused by hyperplasia of collagen fibers and deposition of immunoproteins intrude into the mesangial cells and capillary loops, leading to disruption of the GBM, subsequent leaking of intraluminal material into the Bowman's capsule, and eventual glomerular destruction (Sinniah, 1987).

In the majority of cases, IgA nephropathy can be recognized only by immunofluorescent examination (Andres and Elwood, 1978). Minor mesangial cell hyperplasia with slightly increased mesangial matrix can be seen under light microscope. Some mesangial electron-dense deposits and slight subendothelial changes can be observed under electron microscope.

The most common co-deposits are IgG and C3, followed by IgM, C4, C1q, and fibrinogen (D'Amico, 1983). In about 65% of cases, the IgA and other proteins were confined to the mesangium. However, the deposits were also extended into the paramesangium-subendothelium along the peripheral capillary loops in the remaining 35% of the cases (Sinniah *et al.*, 1981).

The type of deposited Ig is not related to the clinical course (Sinniah and Ku, 1984; Vangelista *et al.*, 1984). The severity of deposits in glomerular wall relates to acute glomerular necrosis, hematuria, inflammation, and crescent formation. Persistent mesangial deposition is believed to

contribute to the progressive decline in renal function, development of hypertension, increasing mesangial expansion, and glomerular sclerosis (Sinniah, 1987).

The alternative pathway is thought to be the primary mechanism for complement activation since the presence of C3 and properdin was frequently reported, whereas C4 and C1q were rarely reported in IgA nephropathy cases (Evans *et al.*, 1973; Götze and Müller-Eberhard, 1971).

Resident macrophages are derived from bone marrow. Although their role in cellular immunity is unclear, they have the effect on mesangial cell proliferation. They bear Fc receptors and 50-60% of these cells bear the Ia antigen (Schreiner *et al.*, 1981).

1.2.6 *Animal models of IgA nephropathy*

There are three types of animal models of IgA nephropathy: (1) passive systems; (2) active systems; and (3) models of secondary IgA nephropathy (Emancipator *et al.*, 1987).

Passive systems were the first animal models developed for IgA nephropathy. These systems employ intravenous injection of IgA antibody and appropriate antigen, either simultaneously as IC or sequentially to allow IC to form in the circulation. They have provided evidence that IgA nephropathy may be IC in nature and are useful for studying mechanisms of IC deposition. Antigen-antibody complexes of the hapten-specific mouse IgA myeloma protein MOPC-315 and dinitrophenyl (DNP) hapten conjugated to bovine serum

albumin (BSA) carrier protein were employed in the first mouse model of IgA nephropathy (Rifai *et al.*, 1979). This intravenous injection resulted in deposition of IgA antibody and DNP-BSA in the mesangium. A similar model was induced by parenteral injection of dextran and IgA myeloma protein W3129 (Isaacs and Miller, 1983). Dextran, one of the polysaccharides, is known to block the uptake of IgA-IC and polymeric IgA by the reticuloendothelial system. Murine IgA nephropathy can also be induced by the intravenous injection of aggregated polymeric IgA (Egido *et al.*, 1982a). One major problem with passive animal models of IgA nephropathy is that the route of antigen stimulation, the source of antibody, and dynamics of the natural disease are unclear.

Active systems employ a known exogenous antigen with a specific route of stimulation. Excessive endogenous IgA produced by experimental animal in response to the prolonged antigenic stimulation can be found in the circulation and the mesangium. But interpretation of the experimental results depends on current knowledge of mucosal and systemic immune system. Oral administration of ovalbumin, bovine gamma globulin, and horse spleen ferritin can cause the formation of IgA-IC that are deposited in the mesangium (Emancipator *et al.*, 1983). When dextran is injected through intraperitoneal and intravenous immunization of aged mice, mesangial deposition of predominant IgA, along with associated IgM and C3 co-deposition, and hematuria is observed (Isaacs *et al.*, 1981). Various protein antigens

have also been used to directly stimulate on mucosal surfaces with the emphasis on oral immunization (Emancipator *et al.*, 1983). Here, preferential IgA responses to the immunizing proteins have been found with significant increases in serum IgA and IgA mesangial deposition. But co-deposition of IgG, IgM, or C3 and hematuria have not been observed in this mouse model. Horse spleen ferritin was introduced orally to two substrains of C3H mice: HeJ and HeB with the emphasis on the genetic background of experimental animal. Elevated serum IgA and mesangial IgA deposition with the co-deposition of IgG and IgM were found only in the high IgA-producing substrain C3H/HeJ, not in the low IgA-producing substrain C3H/HeB. Co-deposition of C3 and hematuria were not found in this model (Genin *et al.*, 1985).

Models of secondary IgA nephropathy have emphasized the relationship between glomerular IgA associated and systemic diseases, particularly hepatocellular and biliary diseases. All models of secondary IgA nephropathy reported so far were induced by chemical hepatic cirrhosis or bile duct ligation. It is believed that IgA-IC is cleared in the liver by both hepatocytes and Kupffer cells and transported through bile duct (Sancho *et al.*, 1984). IgA nephropathy was found when liver function was impaired by parasitic infection (Van Marck *et al.*, 1977), extrahepatic cholestasis caused by experimental partial ligation of the portal vein or bile duct (Melvin *et al.*, 1983), and hepatic cirrhosis caused by carbon tetrachloride (Gormly *et al.*, 1981; Woodroffe and

Lomax-Smith, 1984). Spontaneous increase of serum IgA and mesangial IgA deposition were also found in ddY mice in response to retrovirus-induced tumors (Imai *et al.*, 1985).

1.3 Rationale

It is possible that vomitoxin may play an etiologic role in human IgA nephropathy. Dietary vomitoxin exposure may serve as a probe for studying immunologic and pathologic mechanisms of IgA nephropathy. Furthermore, study of vomitoxin-induced immunotoxicity and nephrotoxicity may serve as a model for food toxicant interactions with cellular components of the gastrointestinal immune system.

The working hypothesis of this research was that vomitoxin induces IgA dysregulation and IgA nephropathy in mice. The capacity for vomitoxin to induce murine IgA nephropathy over time was examined and its persistence was evaluated in this study.

2.0 MATERIALS AND METHODS

2.1 General Experimental Design

The objective of this work was to test the hypothesis that vomitoxin induces IgA dysregulation and IgA nephropathy in mice. In the first experimental set, the capacity for vomitoxin to induce murine IgA nephropathy over time was tested. Two groups of female B6C3F1 mice were employed. Mice in the treatment group were fed 25 ppm vomitoxin, while mice in the control group were fed control diet. Serum IgA, IgG, IgA-IC, hematuria, and proteinuria were monitored at 4-week intervals as immunopathologic endpoints of murine IgA nephropathy. Mesangial deposition was measured using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgA, IgM, and C3 to stain kidney sections at the end of the experiment. Effects of vomitoxin on cellular surface markers of splenocytes and Peyer's patch (PP) lymphocytes were also examined with monoclonal antibodies against B cells, IgA^+ cells, T cells, $CD4^+$ cells, and $CD8^+$ cells at the termination of the experiment. Splenocytes and Peyer's patch lymphocytes were cultured in the presence or in the absence of mitogens and culture supernatant were assayed for Ig production by ELISA.

In the second set of experiments, the persistence of

vomitoxin-induced IgA nephropathy was evaluated. Three groups of female B6C3F1 mice were employed. Mice in the treatment group were fed 25 ppm vomitoxin in feed for 24 weeks. Mice in the withdrawal group were fed toxin feed for the first 8 weeks and then switched to control diet for the remaining 16 weeks of the experiment. Mice in the control group were fed control diet for the whole 24 weeks of the experimental period. Immunopathological changes, as described in the first experimental set, were monitored over the course of the experiment.

2.2 General Procedures

2.2.1 *Vomitoxin purification*

The procedure described by Witt *et al.* (1985) was used for vomitoxin purification. Thirty-nine grams of Potato dextrose agar (PDA) were boiled and dissolved in one liter of distilled water (final pH 5.6 ± 0.2 at 25°C), and then autoclaved (Wilmot Castle Co., Rochester, NY) at 121°C for 15 min. The medium was cooled, kept in 45-50°C water bath, and dispensed into petri dishes in a laminar flow hood. PDA plates were inoculated with a few grains of stock soil cultures of *Fusarium graminearum* R6576 (*Gibberella zeae* U5373; also designated as W-8), sealed with parafilm, and incubated at room temperature for seven days.

Carboxymethyl-cellulose (CMC) was used for spore preparation. The medium consisted of 1.0 g of NH_4NO_3, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of yeast extract, and

15.0 g of blended CMC in one liter of distilled water. Three plugs from growing edges of colonies were added to 100 ml autoclaved CMC medium in 500-ml Erlenmeyer flasks in a hood. Flasks were agitated at 250 rpm on Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at room temperature for four days. The suspension was filtered through 4 layers of sterile cheesecloth. The concentration of microconidia in the filtrate was determined by counting on a hemacytometer. A suspension with 10^6 microconidia was used to inoculate 350 g white rice previously autoclaved with 150 ml distilled water in 2800-ml Fernbach culture flasks. Flasks were incubated at 28°C for 15 days in the dark without shaking.

Following incubation, the contents of each flask contained dense white and pink pigmented mycelia. This was blended with 60% aqueous methanol for 30 sec, allowed to stand overnight at room temperature, and filtered through Whatman No.4 filter paper and a Buchner funnel. Methanol was then removed on a steam bath. The aqueous solution was saturated with sodium chloride overnight, filtered to remove precipitated material, and extracted three times with ethyl acetate with a volume ratio of 1:1 (ethyl acetate:water). Combined ethyl acetate extracts were evaporated by rotatory evaporation (Brinkmann Instruments, Westbury, NY) at 45°C. The residue was rinsed into a small beaker with a small amount of ethyl acetate, dried in a hood, redissolved in several milliliters of methylene chloride, and applied on a

silica gel column equilibrated with distilled water.

The effluent of the column was filtered by a piece of 5-mm filter paper. Fitted with a safety shield, Teflon end fittings, connectors, and 2-mm i.d. tubing, the 37-mm i.d. Michel-Miller glass chromatographic column was prepared by dry packing with 200/425 mesh silica (about 170 g). The column was then connected with automatic liquid fraction collector and a pump which is adapted with low flow fittings. Coupled to a flow rate of about 5 ml/min, the column was washed several times with methanol and methylene chloride prior to sample-loading, eluted with methylene chloride until pigment and 15-acetyl-DON ceased to appear in the effluent, and then eluted with distilled water to remove vomitoxin.

The above procedures were monitored by thin layer chromatography (TLC). Semiquantitative TLC was performed on precoated 20 × 10 cm^2 silica gel G plates (Redi-Plates, Fisher Scientific Co., Fairlawn, NJ) with vomitoxin standard, 15-acetyl-DON, and samples. Plates were developed with 100 ml toluene/ethyl acetate (1:3) in a glass tank, dried, sprayed with a 15% aluminum chloride solution (15 g $AlCl_3 \cdot 6H_2O$ in 85 ml of ethanol and 15 ml of water; Sigma Chemical Co., St. Louis, MO) in a fume hood, and incubated at 110°C for 5 min. Under those conditions, vomitoxin produces a blue fluorescent spot under longwave (365 nm) UV light at Rf value 0.3, while Rf value for 15-acetyl-DON was 0.5. The concentration of vomitoxin was estimated by visual

comparison to its standard in a UV viewing cabinet.

Water fractions containing vomitoxin were extracted with ethyl acetate (volume ratio of 1:1) until vomitoxin ceased to appear in the water layer, as indicated by TLC. Combined ethyl acetate extracts were concentrated on a rotatory evaporator, dried in a fume hood, redissolved in a minimum volume of ethyl acetate, which was then filtered into a small beaker through Whatman No.4 filter paper and sodium sulfate, and seeded with a few crystals of pure vomitoxin. Crystallization occurred in the beaker covered by aluminum foil at 4°C. The supernatant was carefully removed with a glass pipet and reapplied on silica gel column to recover remaining vomitoxin. Vomitoxin crystals were washed with minimal shaking with ice-cold ethyl acetate (or methanol) to remove residual pigment and quantitated by high performance liquid chromatography (HPLC).

An HPLC system equipped with a Model 2300 HPLC pump, V^4 variable wavelength absorbance detector (5-mm flow cell; ISCO Co., Lincoln, NE), RP-18 Spheri-10 HPLC analytical (22 cm × 4.6 mm i.d.), and guard (3 cm × 4.6 mm i.d.) cartridges (Brownlee Lab, Inc., Santa Clara, CA) was used to access purity and concentration of vomitoxin. Mobile phase was 20% methanol (v/v) in water with a flow rate of 2 ml/min. Vomitoxin was dissolved in mobile phase and detected at 224 nm and 0.05 a.u.f.s. Vomitoxin standard had a average retention time of 3.66 ± 0.09 min at room temperature.

2.2.2 *Safety*

Face masks, vinyl gloves, and glasses were used during vomitoxin purification and toxin feed preparations. Most steps of purification procedure were performed in a fume hood. Toxin-contaminated labware was detoxified by soaking overnight in 10% sodium hypochlorite solution (Thompson and Wannemacher, 1984) prior to washing.

2.2.3 *Experimental animals*

Female B6C3F1 (C57BL/6 × C3HeN) mice (8-9 weeks; Charles River, Boston, MA) were randomized and housed in pairs in protected environment cages (Nalgene, Rochester, NY). Cage units were made up of a transparent polycarbonate body with filter bonnet, stainless-steel wire lid, and raised floor above a layer of heat-treated hardwood chips. Mice were acclimated for seven days to housing, feed, a 12-hour light and dark cycle, and a negative pressure ventilated area prior to beginning feeding regimens.

2.2.4 *Toxin administration and feeding protocol*

Two hundred milligrams of vomitoxin were completely dissolved in 300 ml of 100% ethanol and then mixed with 200 g of powered semipurified AIN-76A diet (ICN Biochemical, Cleveland, OH) by stirring with a glass bar in a beaker to make 1000 ppm vomitoxin stock feed. Ethanol was evaporated in a hood. Fifty grams of vomitoxin stock feed was evenly diluted into 1950 g of AIN-76A diet in an automatic food mixer to form 25 ppm vomitoxin feed.

Mice in treatment group were fed 25 ppm toxin feed,

while mice in control group were fed unmodified diet (Forsell *et al.*, 1986). Mice in withdrawal group were fed 25 ppm toxin feed for eight weeks and then switched to control diet for the remained of the experimental period. The 25 ppm level of vomitoxin has been shown previously to have maximum effect on IgA hyperproduction (Pestka *et al.*, 1989). Distilled water was provided *ad lid*.

2.2.5 *Sample collection and preparation*

Approximately 400 μl of blood sample from each mouse was obtained by retro-orbital bleeding in a 0.5-ml centrifuge tube using capillary pipet from ether-anesthetized mice and refrigerated overnight. Serum samples were then collected by centrifugation at 450*g* for 10 min.

Urine samples were obtained by housing each mouse in a fabricated metabolism cage overnight. The cage consisted of a plastic funnel with a small outlet (1.5-cm i.d.) to collect urine, an enlarged body (10-cm i.d.) to house the mouse, a steel grid lid to prevent the escape of mice, and a steel grid at the neck of the funnel to establish a floor to allow mouse to stand and to separate urine from fecal pellets. Approximately 1 ml of urine from each mouse was collected in a small glass tube which was connected to the outlet of the cage. Urine samples were visually monitored for the contamination of feed to prevent false report of proteinuria.

Kidneys from CO_2-euthanized mice were attached to a small piece of cork with gum balsam, immediately frozen in

liquid nitrogen, and stored at -70°C. These were sectioned to 6 μm on a cryostat at -20°C, and mounted on a slide. Slides were stored at -20°C prior to staining.

2.3 Serum Evaluation

2.3.1 *Ig quantitation*

IgA and IgG levels were quantitated by ELISA at 4-week intervals. Wells of Immulon II Removawell microtiter strips (Dynatech Laboratories, Alexandria, VA) were coated by overnight incubation at 4°C with 50 μl of heavy-chain-specific goat anti-mouse IgA or IgG (Cappel, Malvern, PA) diluted 1:1000 in 0.1 M bicarbonate coating buffer (pH 9.6). Coated plates were washed three times with 0.1 M phosphate-buffered saline (PBS, pH 7.5) containing 0.2% Tween 20 (PBS-Tween). To reduce nonspecific binding, 0.3 ml of 1% (w/v) BSA in PBS was added to each well. Plates were then incubated at 37°C (Chicago Surgical and Electrical Co., Chicago, IL) for 30 min and washed four times with PBS-Tween. For Ig quantitation, standard mouse immunoglobulin reference serum (ICN Immunobiologicals, Lisle, IL) or samples were diluted in 1% BSA-PBS, and 50 μl was added to appropriate wells. Plates were covered and incubated at 37°C for 1 hr and then washed five more times. Fifty microliters of heavy-chain-specific goat anti-mouse IgA or IgG horseradish peroxidase (HRP; Cappel), diluted 1:1000 in 1% BSA-PBS, were added to each well. Plates were then incubated at 37°C for an additional 30 min. Absorbance of

bound peroxidase was measured at 405 nm on an ELISA plate reader (Biotek Instruments, Inc., Burlington, VT) or V max kinetic microplate reader (Molecular Devices Co., Menlo Park, CA). Ig levels were calculated using the ELISAnalysis program (Birminham, AL).

2.3.2 *Circulating IgA-IC quantitation*

Twenty-five microliters of each mouse serum sample and 975 μl of 3.5% PEG 6000 (Sigma) in 0.1 M borate buffer (pH 8.4) were mixed in 1.5-ml centrifuge tubes, incubated for 18 hr at 4°C, centrifuged at 2040*g* for 30 min at 4°C, and washed three times with 3.5% PEG. Pellets were dissolved in 500 μl of 1% BSA-PBS (Imai *et al.*, 1987; Digeon *et al.*, 1977). Circulating IgA-IC level was then quantitated by IgA ELISA as described above. The specificity of IgA-IC precipitated by PEG was determined by ELISA. The plate was coated with goat anti-mouse IgA or IgG and goat anti-mouse IgG HRP was used as secondary antibody.

2.4 *In vitro* Ig production

All cell culture media were purchased from Sigma unless otherwise noted and filter sterilized. RPMI-1640 medium was supplemented with 5×10^{-5} M 2-mercaptoethanol, 25 mM Hepes buffer, and 0.1 mM nonessential amino acids. Dulbecco's modified Eagle medium (DMEM) was supplemented with 1%(v/v) NCTC-135. Both RPMI and DMEM media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate.

Peyer's patches were pooled (5-6 mice/group) in RPMI-1640 medium (Sigma), teased apart with tissue forceps, and passed through an 85-mesh stainless-steel screen. Cells were suspended in Hanks medium, washed by centrifugation at 450*g* for 10 min, and resuspended in RPMI-1640 medium with 10% fetal calf serum (FCS; Gibco Laboratories, Grand Island, NY).

Spleens were teased apart with tissue forceps in RPMI-1640 medium, pooled (five to six mice for each experimental group), and centrifuged at 450*g* for 10 min. Erythrocytes were lysed by resuspending in 0.83% ammonium chloride for 2 min at room temperature. Cells were centrifuged again and resuspended in RPMI-1640 medium with 10% FCS. Cells were enumerated with a Neubauer hemacytometer (American Optical, Buffalo, NY). Cell viability was determined by trypan blue dye exclusion (Harlow and Lane, 1988).

Splenic and Peyer's patch lymphocytes (5×10^5 per well) were cultured in 96-well tissue culture plates with RPMI 1640 supplemented with 10% FCS in the presence and absence of the mitogens *Salmonella typhimurium* lipopolysaccharide (LPS; 25 μg/ml) or concanavalin A (Con A; 5 μg/ml) at 37°C and 8% CO_2. After seven days of incubation, supernatants were harvested and assayed for IgA and IgG by ELISA.

2.5 Immunofluorescent Staining of B and T Cells

Splenic or Peyer's patch cells were distributed into V-shape bottomed 96-well microtiter plates (2×10^6/well).

Supernatants were removed from each well by centrifugation at 450*g* for 10 min. FITC-conjugated goat antisera (Sigma) were diluted 1:100 in 0.01 M-PBS (pH 7.2), containing 1% BSA to prevent non-specific binding and 0.02% sodium azide to prevent capping, and 100-microliter aliquots were added to appropriate wells. The cells were incubated at 4°C for 45 min, washed twice in 1% BSA-PBS, and mounted in 45% glycerol in PBS. Total B cells were determined with FITC-conjugated IgG fraction goat anti-mouse Ig (IgA, IgG, IgM-heavy and IgM-light chain specific, Cappel). Membrane IgA^+, IgG^+, and IgM^+ cells were detected with affinity isolated α, γ, and μ chain specific FITC-conjugated goat antisera, respectively. Total T cells and $CD8^+$ cells (T cytotoxic/suppressor subset) were determined with FITC-conjugated monoclonal anti-Thy 1.2 (Clone HO13.4) and anti-Lyt-2 (Clone H53.6.72) antibodies, respectively, obtained from American Type Culture Collection (ATCC, Rockville, MD). $CD4^+$ cells (T helper subset) were determined by FITC-conjugated monoclonal anti-L3T4 antibody (Clone RL-172.4; Krusemeier and Snow, 1988) developed by A. Singer and kindly provided by E. C. Snow. T cell monoclonal antibodies were prepared from cell culture, conjugated to FITC by Roscoe Warner as described by Goding (1986), and diluted 1:40 in 1% BSA-PBS.

2.6 Specificity of FITC-labeled Goat Anti-mouse Antibodies

Specificity of FITC-labeled goat anti-mouse IgA, IgG, IgM (heavy chain specific, Sigma), and C3 (affinity purified

IgG fraction, Sigma) was verified by ELISA. Briefly, walls of microtiter plate wells were coated overnight at 4°C with 100 ml of murine IgA, IgG, IgM, C3, or BSA (10 mg/ml) in 0.1 M bicarbonate buffer (pH 9.6). Coated plates were washed three times with 0.01 M PBS-Tween (pH 7.2). Three hundred microliters of 1% BSA-PBS was added to each well to reduce non-specific binding. The plates were incubated at room temperature for 30 min and washed four times with PBS-Tween. Goat anti-mouse IgA-, IgG-, IgM-, and C3-FITC, and normal goat IgG-FITC were serially diluted in 1% BSA-PBS, and 50 µl were added to appropriate wells. Plates were sealed and incubated at 37°C for 1 hr and washed five more times. One hundred microliters of rabbit anti-goat IgG-HRP (1:1000) in 1% BSA-PBS were added to each well. Plates were incubated at 37°C for 30 min. Bound peroxidase was then determined as described previously in Section 2.3.1 (Pestka *et al.*, 1980).

2.7 Immunofluorescent Staining of Cryosections

Sections were stained with FITC-labeled goat anti-mouse IgA, IgG, IgM, and C3 by the procedure of Valenzuela and Deodhar (1981). Briefly, slides were defrosted at room temperature. Sections were encircled with a hydrophobic PAP pen (Research Products International, Mount Prospect, IL), soaked in PBS for 3 min, blocked in 1% BSA-PBS for 15 min, stained with FITC-labeled goat anti-mouse Ig and C3 (diluted 1:10 for IgA, 1:50 for IgG, IgM, and C3 in 1% BSA-PBS), incubated at room temperature in a humid chamber for 60 min.

Stained slides were then dipped rapidly into distilled water containers three successive times and washed three times with distilled water for ten minutes on an orbital shaker (American Rotator, Elgin, IL) rotating at 50 rpm. Slides were mounted on a drop of buffered glycerol saline (pH 9; DIFCO Laboratories, Detroit, MI). Sections were coded, randomized, and examined under a Nikon Labophot fluorescence microscope with ND2/4 and B2 filters.

2.8 Quantitative Assessment of Renal Deposition of Ig and C3

Glomerular fluorescence intensities which represented Ig isotypes or C3 were quantitated by the ACAS 470 Interactive Laser Cytometer (Meridian Instruments Inc., Okemos, MI). Briefly, 10 glomeruli of the same approximate size were randomly selected for each section using standard light optics, and the mean fluorescent emissions at 530 ± 15 nm in polygons of defined area were determined using the ACAS image analysis program. Excitation of the sections was at 488 nm. Sections with the highest fluorescence intensity were used to determine maximal color-coded value settings to prevent image saturation and provide optimal dynamic range. Each glomerulus selected was scanned only once to minimize and balance the effects of any potential photobleaching.

2.9 Urinalysis

Mouse urine samples (approximately 1 ml/mouse/16 hr) were collected in a metabolism cage at 4-week intervals and

centrifuged at 500*g* for 10 min. Erythrocytes in the sediment in 10 random microscopic fields (×45) were counted. Microhematuria indices of 1, 2, 3, 4, 5, and 6 were assigned for 0, 1-3, 4-10, 11-20, 21-30, and >30 erythrocytes per microscopic field, respectively. Proteinuria was tested by Ames 2820 Multistix (Miles Inc., Elkhart, IN) of reagent strips for urinalysis and graded as proteinuria index.

2.10 Statistical Analysis

Student's *t* test, Mann-Whitney *U* test, χ^2 test, least significant difference (lsd) test, Student-Newman-Keul's (SNK) test, completely random analysis of variance (one-way ANOVA), Pearson's correlation coefficient test, Spearman's rank correlation coefficient test, and multiple regression test were applied for comparison among data from control, treatment, and withdrawal groups using the Microcomputer Statistical Program (MSTATC, Crop and Soil Science, Michigan State University, MI).

3.0 RESULTS

3.1 Vomitoxin-induced IgA Nephropathy

In the initial study, experimental mice were divided into two groups (six mice per group). Mice in the treatment group were fed 25 ppm vomitoxin, while mice in the control group were fed control diet. Serum and urine samples were collected and analyzed at 4-week intervals. Mice were CO_2-euthanized at 4, 8, and 12 weeks and the following were measured: (1) mesangial Ig and C3 deposition; (2) effects of vomitoxin on surface antigens of T and B cells; and (3) *in vitro* Ig production by isolated and cultured splenocytes and Peyer's patch lymphocytes.

3.1.1 *General effects of vomitoxin on experimental mice*

Control mice appeared to be normal. However, feed refusal, reduced weight gain, and ruffled fur were observed in vomitoxin-treated mice. Acute clinical signs of vomitoxin feeding such as emesis, bloody diarrhea, and sudden death were not observed.

3.1.2 *Serum IgA, IgG, and IgA-IC*

Serum IgA, IgG, and circulating IgA-IC were examined at 4-week intervals to evaluate the isotype-specific effects of

Table 1. Serum IgA, IgG, and IgA-IC during 12-Week Dietary Vomitoxin Exposure

Weeks (μg/ml)	Treatment	IgA (μg/ml)	IgG (μg/ml)	IgA-IC
4	Control	573 ± 35	3740 ± 342	3.8 ± 0.4
	Treatment	902 ± 121*	3250 ± 309	7.4 ± 0.9**
8	Control	439 ± 38	2369 ± 280	3.1 ± 0.2
	Treatment	967 ± 157*	2290 ± 269	6.4 ± 1.3*
12	Control	541 ± 15	2000 ± 254	2.3 ± 0.3
	Treatment	3800 ± 748**	2950 ± 643	8.8 ± 2.1

Data are mean ± SE (six mice per group). * $p<0.05$ and ** $p<0.01$, comparison between control and treatment groups of the same feeding week. Results are representative of two separate experiments.

dietary exposure to vomitoxin. Serum IgA level increased significantly by 1.6-, 2.2-, and 7.0-fold in the treatment group compared to their matching controls at Weeks 4, 8, and 12, respectively. No changes in serum IgG level were found. Circulating IgA-IC level were also elevated by 1.9-, 2.1-, and 3.8-fold in the treatment group compared to their corresponding controls at Week 4, 8, and 12, respectively (Table 1). IgG was not significantly co-precipitated in PEG-precipitated IgA-IC (data not shown).

3.1.3 *Urinalysis*

Hematuria is a key characteristic of IgA nephropathy. Microscopic hematuria was found in urinary samples of vomitoxin-treated mice collected at 4-week intervals. The incidence occurred as early as Week 4 of vomitoxin feeding. The severity of hematuria increased as vomitoxin feeding continued (Fig. 1).

3.1.4 *Accumulation of mesangial Ig and C3*

Mesangial IgA deposition was measured as endpoint of IgA nephropathy. Co-deposition of IgG, IgM, and C3 is also commonly found in human IgA nephropathy. The specificities of FITC goat anti-mouse (affinity-purified IgG) antibodies were validated by an ELISA in which antigens were coated onto the polystyrene solid phase and then sequentially incubated with FITC-labeled antisera, peroxidase-labeled

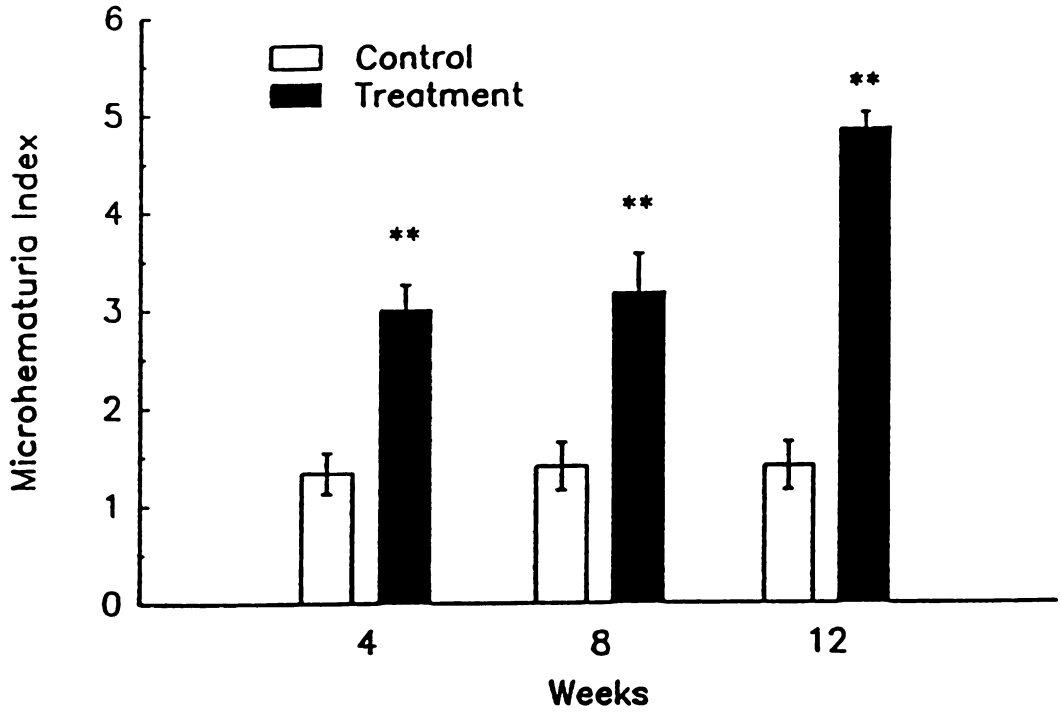


Figure 1. Microscopic hematuria induced by dietary vomitoxin exposure. Urine was collected at intervals and centrifuged, and erythrocytes were quantitated microscopically. Hematuria indices were graded, based on erythrocytes/field as follows: 1 (0), 2 (1-3), 3 (4-10), 4 (11-20), 5 (21-30), and 6 (>30). Data are means ± SE (n=6). ** Significant difference between control and treatment groups at the 0.01 level. Results are representatives of two separate experiments.

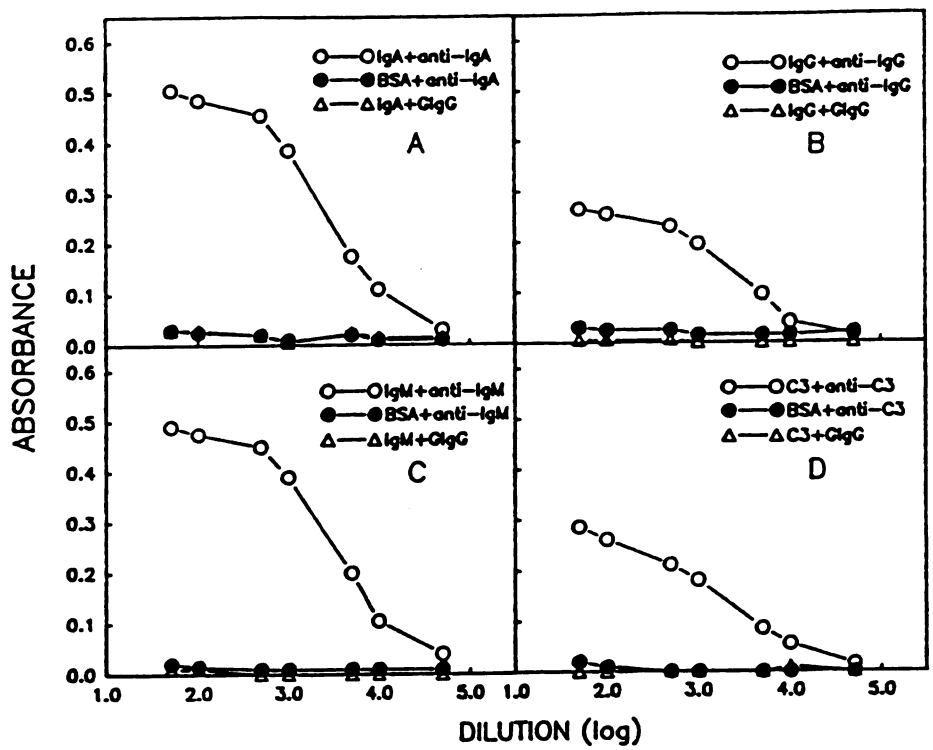


Figure 2. Specificity of FITC antibody preparations. Microwell plates were coated with specific antigen and reacted with specific antibody (O), coated with BSA and reacted with specific antigen (●), or coated with specific antigen and reacted with normal goat IgG-FITC (▲). Bound IgG was determined with rabbit anti-goat IgG peroxidase conjugate. ELISA absorbance values were associated with immuno-reaction of goat anti-mouse IgA-FITC (a), goat anti-mouse IgG-FITC (B), goat anti-mouse IgM-FITC (C), and goat anti-mouse C3-FITC (D) preparations.

rabbit anti-goat IgG, and peroxidase substrate (Fig. 2). It was revealed that the IgA, IgG, IgM, and C3 antisera reacted with their specific antigens as compared to the solid phase of BSA. Non-specific binding of equivalent concentrations of FITC-labeled goat IgG against the above antigens was not observed.

Accumulation of mesangial IgA deposition was observed by immunofluorescent microscopy. Compared to the control groups, mesangial IgA deposition was clearly noticeable in the treatment groups after only four weeks of vomitoxin exposure, but it was most pronounced at Weeks 8 and 12 (Fig. 3). Using interactive laser cytometry image analysis system, increased fluorescence intensities due to mesangial deposition of IgA were verified and quantitated (Fig. 4). Significant elevation of fluorescence intensities was found by 1.7-, 2.0-, and 3.0-fold in the treatment groups at 4, 8, and 12 weeks, respectively, compared to the control groups at the same weeks (Fig. 5).

Mesangial immunofluorescence was detectable neither in the control nor in the treatment groups when FITC-labeled normal goat IgG was applied to kidney sections (Figs. 6a,b). In the contrast, glomerular IgG and IgM deposits were present in both control and treatment groups after 12 weeks (Figs. 6c-f). Glomerular C3 deposition was detectable primarily in Bowman's capsule rather than in the mesangium of both control and treatment groups (Figs. 6g,h).

When quantitatively assessed by image analysis, there

Figure 3. Mesangial deposition detected by immuno-fluorescence microscopy. Glomeruli from mice fed basic diet for 12 weeks (A), or 25 ppm vomitoxin for 4 weeks (B), 8 weeks (C), and 12 weeks (D).

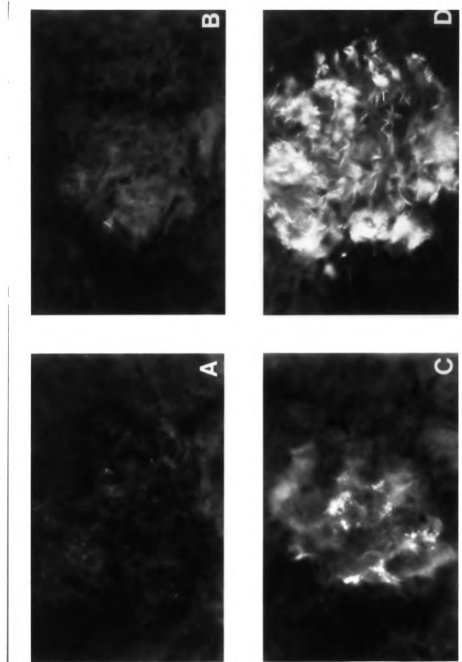
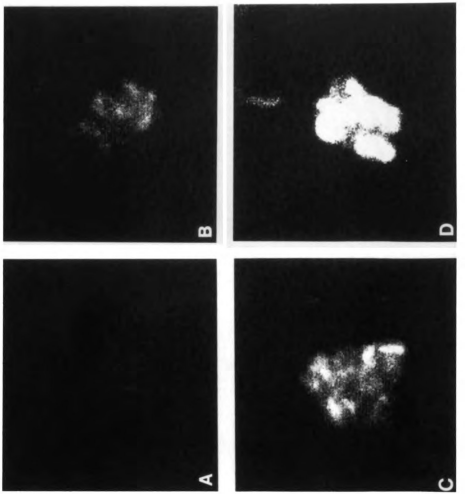

A
B
C
D

Figure 4. Mesangial IgA deposition detected by interactive laser cytometer image analysis. Glomeruli from a mice fed basic diet for 12 weeks (a), or 25 ppm vomitoxin for 4 weeks (b), 8 weeks (c), and 12 weeks (d).


B
D
A
C

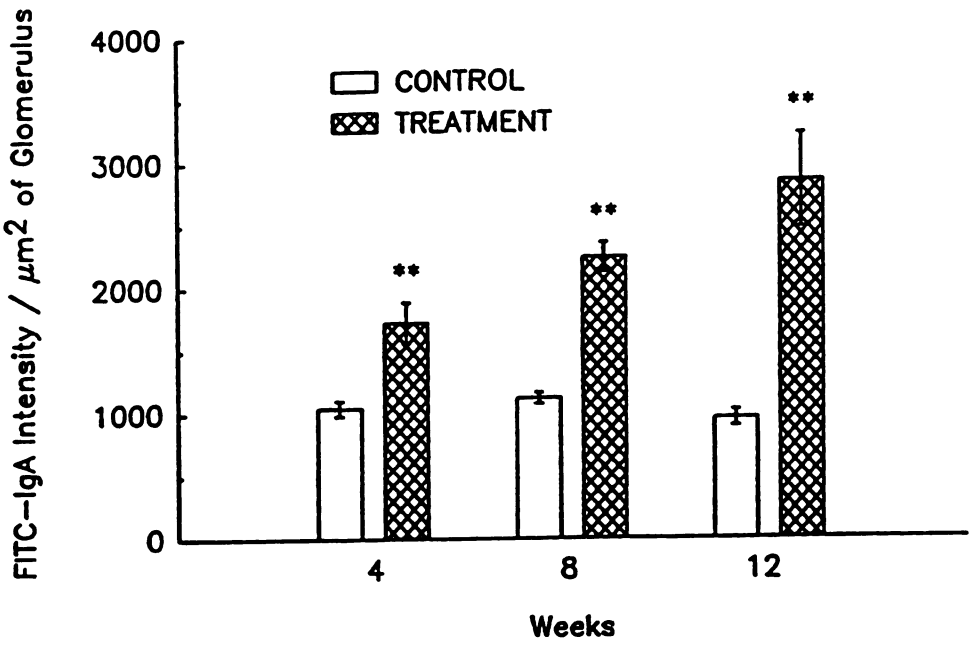
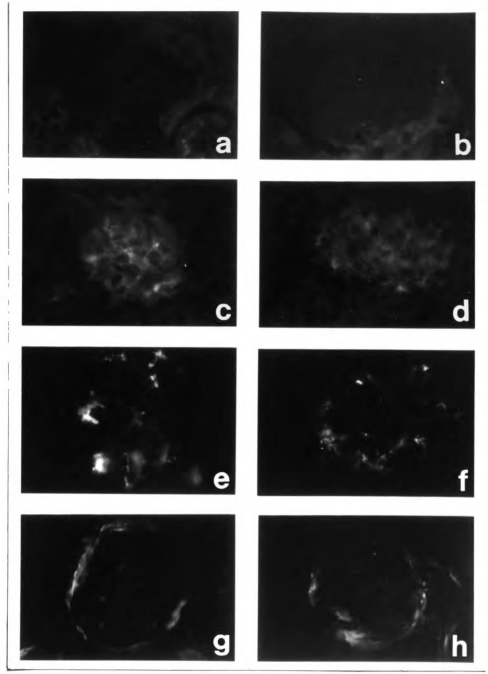


Figure 5. Quantitation of glomerular IgA accumulation by interactive laser cytometer image analysis. Fluorescence emission is expressed as arbitrary units (AU). Data are means ± SE (n=6). ** Significant difference ($p<0.01$) between control and treatment groups of the same week. Results are representative of two separate experiments.

figure 6. Glomerular immunofluorescence associated with IgG, IgM, and C3. Glomeruli of mice fed basic diet (a, c, e, g) or 25 ppm vomitoxin (b, d, f, h) for 12 weeks. Sections were stained with normal goat IgG-FITC (a, b), goat anti-mouse IgG-FITC (c, d), goat anti-mouse IgM-FITC (e, f), and goat anti-mouse C3-FITC (g, h).


a
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was significantly less fluorescence associated with IgM and IgG at 12 weeks in the treatment groups, whereas differences in glomerular C3 deposition were not observable between control and treatment groups (Fig. 7).

3.1.5 *Effects on B and T cell profiles*

To evaluate the effects of vomitoxin on B and T cell profiles, the percentages of IgA, IgG, and IgM cells in B cell population and $CD4^+$ and $CD8^+$ cells in T cell population of spleens and Peyer's patches in vomitoxin-treated mice were compared to that in control mice.

Vomitoxin treatment caused a slight increase in percentages of total B and T cells in Peyer's patches (Table 2) in comparison with the controls. Although vomitoxin exposure had no effect on total B cells in the spleens, there was a distinct (30-50%) increase in the percentage of total T cells in the spleens of toxin-treated mice in comparison with the controls (Table 3).

The percentage of membrane IgA^+ cells in the Peyer's patches of vomitoxin-treated mice was 2-fold higher than that of controls at Week 4, 8, and 12 (Fig. 8). Similarly, the percentage of membrane IgA^+ cells in the spleen of the treatment group was 1.6- to 1.8-fold higher than that found in the control group (Fig. 9). The percentage of membrane IgA^+ cells from both Peyer's patches and spleens of toxin-treated mice were significantly different from their respective controls when the mean values from three separate

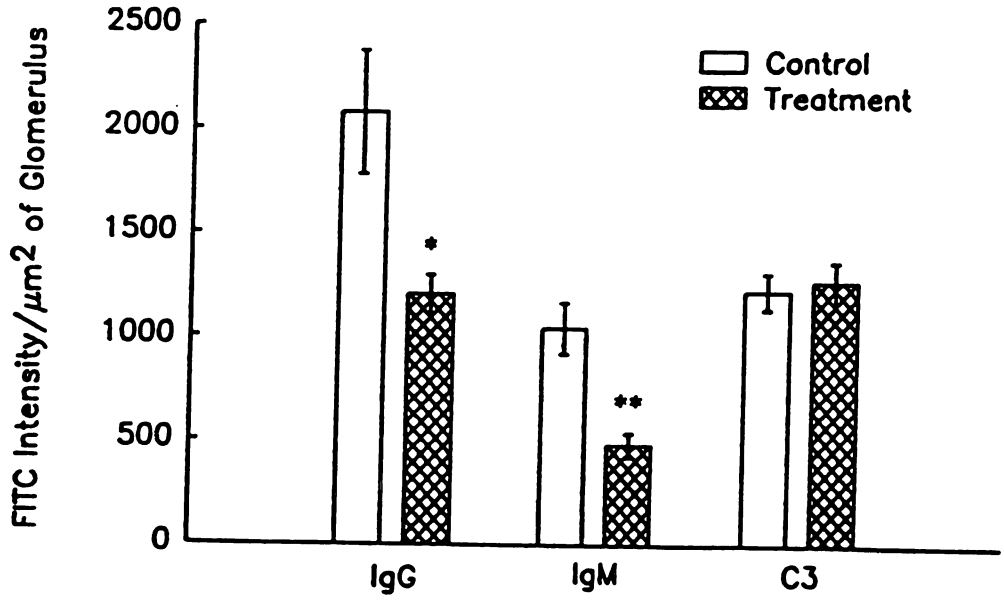


Figure 7. Quantitation of glomerular IgG, IgM, and C3 after 12-week dietary vomitoxin exposure by interactive laser cytometer image analysis. Fluorescence emission is expressed as arbitrary units (AU). Data are means ± SE (n=12). * $p<0.05$ and ** $p<0.01$, significant differences between control and treatment groups. Results are representative of two separate experiments.

Table 2. Effect of Vomitoxin on the Percentage of B and T Cells in PP

Numbers of Experiment	Weeks	B Cells (%)		T Cells (%)	
		Control	Treatment	Control	Treatment
1	4	44.0±0.3	50.9±0.9	20.2±0.2	21.0±0.4
2		44.8±2.6	55.9±3.9	ND	ND
3	8	50.7±0.5	50.9±0.9	19.6±0.3	20.1±0.9
4		51.2±1.4	51.2±1.1	18.3±0.3	19.9±0.3
5	12	51.1±0.8	53.3±0.8	20.7±0.8	23.6±1.0
6		50.8±0.7	53.8±1.0	19.9±0.5	23.4±0.6
	[a]Mean ± SD	48.8±1.4	52.7±0.8*	19.7±0.4	21.6±0.8*

[a] Means of cumulative experimental trials.
* $p<0.05$ Significant difference from the corresponding control values (Mann-Whitney *U* test). Mice were fed AIN-76 semi-purified diet containing 25 ppm vomitoxin (treatment) or the AIN-76 diet alone (control) and were euthanized at specified time intervals. Pooled Peyer's patch cell suspensions were prepared and the percentage of B and T cells were determined by immunofluorescence microscopy. Data are means ± SD (n=5-6) of triplicate determinations. ND = not determined.

Table 3. Effect of Vomitoxin on the Percentage of Splenic B and T Cells

Numbers of Experiment	Weeks	B Cells (%)		T Cells (%)	
		Control	Treatment	Control	Treatment
1	4	48.6±1.2	49.3±0.4	31.5±0.4	42.9±0.3
2		48.9±0.6	48.7±2.0	30.7±1.3	41.6±0.9
3	8	48.7±0.5	51.3±0.7	25.7±0.5	37.0±0.4
4		49.3±3.0	51.9±1.9	25.6±0.5	36.3±0.5
5	12	52.6±0.8	52.0±1.4	27.0±0.8	41.0±0.3
6		51.8±1.9	51.4±0.7	26.8±1.3	40.3±0.9
	[a]Mean ± SD	50.0±0.7	50.8±0.6	27.9±1.0	39.9±1.1**

[a] Means of cumulative experimental trials.
** $p<0.01$ Significant difference from the corresponding control values (Mann-Whitney *U* test). Mice were fed AIN-76 semi-purified diet containing 25 ppm vomitoxin (treatment) or the AIN-76 diet alone (control) and were euthanized at specified time intervals. Pooled splenic cell suspensions were prepared and the percentage of B and T cells were determined by immunofluorescence microscopy. Data are means ± SD (n=5-6) of triplicate determinations. ND = not determined.

experimental trials at 4, 8, and 12 weeks were compared. A slight decrease in the percentage of membrane IgG^+ cells in the treatment group compared to that in the control group was found in Peyer's patches at 12 weeks and in spleens at 8 and 12 weeks. A slight increase in the percentage of membrane IgM^+ cells in the treatment group was observed at 4 weeks in both Peyer's patches and spleens, but not later in the experiment (Fig. 8 and 9).

A trend towards an increase in $CD4^+$ cells was detected in the Peyer's patches of vomitoxin-treated mice (Table 4). This effect was more evident in the spleen, with an increased percentage of $CD4^+$ cells in toxin-treated mice by 30-50% at 4, 8, and 12 weeks (Table 5). There was only a slight increase in $CD8^+$ cells in the spleen and none in Peyer's patches. The relative effect of vomitoxin on these two T cell populations was also reflected in an increased $CD4^+$:$CD8^+$ ratios in both Peyer's patches and the spleen (Tables 4 and 5).

3.1.6 *In vitro Ig production*

To assess the effects of dietary vomitoxin on induction of IgA production at the gut mucosal level and systemic compartment, Peyer's patch and splenic lymphocytes from treated and control mice were cultured and supernatant IgA and IgG levels were quantified. The mitogens, LPS and Con A, were added to some cultures to identify specific effects on B and T cell subsets, respectively.

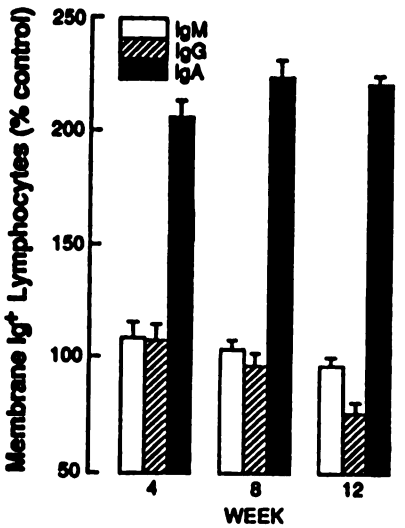


Fig. 8. Effect of dietary vomitoxin on the B cell profile in Peyer's patches. Mice were fed 25 ppm vomitoxin or control diet for specified time intervals. Pooled Peyer's patch cell suspensions (five or six mice/experimental group) were prepared and the percentages of IgM^+ (□), IgG^+ (▨) and IgA^+ (■) lymphocytes were determined, in triplicate, by immunofluorescence microscopy with FITC-labelled affinity purified antibody. Membrane Ig^+ lymphocytes reflect: (% Ig isotype vomitoxin treated ± SD)/(% Ig isotype control) × 100. The results presented are representative of three separate experimental trials.

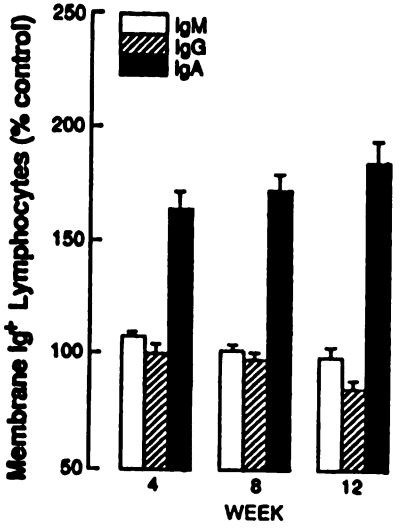


Fig. 9. Effect of dietary vomitoxin on the B cell profile [IgM^+ (□), IgG^+ (▨) and IgA^+ (■) lymphocytes] in the spleen. Experimental conditions and results are as reported in Fig. 2 except that spleen cells were used.

Table 4. Effect of Vomitoxin on the Percentage of $CD4^+$ and $CD8^+$ T Cell Populations in Murine Peyer's Patches

Exp.		$CD4^+$ (%)		$CD8^+$ (%)		$CD4^+$: $CD8^+$	
#	wks	Control	Treatment	Control	Treatment	Control	Treatment
1	4	15.0±0.2	15.6±0.3	4.7±0.3	4.6±0.3	3.19	3.39
2		14.7±1.0	13.4±1.2	4.8±0.7	4.5±0.9	3.08	3.01
3	8	13.8±0.3	15.4±0.3	4.3±0.4	3.9±0.4	3.21	3.94
4		13.6±0.4	15.7±0.3	4.3±0.1	4.0±0.4	3.14	3.97
5	12	14.7±1.3	17.9±0.5	4.5±0.6	5.3±0.9	3.26	3.37
6		14.7±1.2	17.8±1.2	4.7±0.4	5.1±0.3	3.15	3.49
[a]Mean ± SD		14.4±0.2	16.0±0.7*	4.6±0.1	4.6±0.2	3.17±0.03	3.53±0.10*

[a] Means of cumulative experimental trials.
* $p<0.05$ Significant difference from the corresponding control values (Mann-Whitney *U* test). Mice were fed AIN-76 semi-purified diet containing 25 ppm vomitoxin (treatment) or the AIN-76 diet alone (control) and were euthanized at specified time intervals. Pooled Peyer's patch cell suspensions were prepared and the percentage of $CD4^+$ and $CD8^+$ cells were analyzed by immunofluorescence microscopy with FITC-labeled anti-L3T4 and anti-Lyt-2 monoclonal antibodies, respectively. Data are means ± SD (n=5-6) of triplicate determinations.

Table 5. Effect of Vomitoxin on the Percentage of $CD4^+$ and $CD8^+$ T Cell Populations in Murine Spleens

Exp.		$CD4^+$ (%)		$CD8^+$ (%)		$CD4^+$: $CD8^+$	
#	wks	Control	Treatment	Control	Treatment	Control	Treatment
1	4	20.9±0.3	31.0±2.1	9.6±0.3	11.0±0.2	2.17	2.82
2		21.2±3.4	29.5±1.6	10.4±0.9	12.9±3.8	2.04	2.42
3	8	16.6±0.4	26.2±0.3	8.6±0.3	8.8±0.4	1.94	2.98
4		16.2±0.4	26.0±0.3	8.7±0.2	9.5±0.2	1.86	2.74
5	12	16.6±0.5	31.8±0.7	8.8±0.7	10.2±1.1	1.89	3.14
6		16.4±0.4	30.2±1.7	9.2±0.5	10.2±0.4	1.79	2.97
[a]Mean ± SD		18.0±1.0	29.1±1.0**	9.2±0.3	10.4±0.6*	1.95±0.1	2.85±1.0**

[a] Means of cumulative experimental trials.
* $p<0.05$ and ** $p<0.01$ Significant difference from the corresponding control values (Mann-Whitney *U* test). Experimental conditions and determination of results as described in Table 4, except that spleen cells were used.

IgA secretion by LPS- and Con A-stimulated and unstimulated Peyer's patch cultures from vomitoxin-treated mice was significantly increased. The highest IgA secretion was found in Con A-stimulated cultures from toxin-treated mice and was 5.3-, 6.2-, and 4.2-fold higher than that from their corresponding controls at 4, 8, and 12 weeks (Table 6).

IgG secretion in Peyer's patch cultures was increased in LPS- and Con A-stimulated cultures at 4 and 12 weeks and in unstimulated cultures at 8 and 12 weeks, respectively. However, levels of supernatant IgG secretion were 8- to 20-fold lower than that of IgA secretion in mitogen-stimulated and unstimulated cultures from both treated and control mice. As found with IgA, IgG secretion was also highest in Con A-stimulated Peyer's patch cultures (Table 6).

IgA secretion in splenic cultures from vomitoxin-treated mice was significantly elevated in LPS-stimulated cultures at 4 and 8 weeks and in unstimulated cultures at 8 and 12 weeks (Table 7). IgG secretion was decreased in splenic cultures from vomitoxin-treated mice compared to their corresponding controls at 4 weeks, but Con A-stimulated and unstimulated cultures from treated mice had significantly increased levels of IgG secretion at 12 weeks (Table 7).

3.2 Persistent Effects after Withdrawal of Dietary Vomitoxin

Experimental mice were divided into three groups. Mice in the treatment group were fed 25 ppm vomitoxin feed for 24

Table 6. Effect of Dietary Vomitoxin on Ig Secretion in Murine Peyer's Patches Cultures

		IgA (μg/ml)			IgG (μg/ml)		
wks	Mito-gens	Control	Treatment	% To Control	Control	Treatment	% To Control
4	LPS	4.8±0.80	7.5±1.10	(156)**	1.4±0.23	0.9±0.08	(64)**
	Con A	5.8±2.29	30.8±10.1	(531)**	0.6±0.19	1.8±0.49	(300)**
	HOH	0.5±0.11	8.1±4.29	(1620)**	0.2±0.03	0.4±0.14	(200)
8	LPS	6.4±1.10	14.8±2.00	(231)*	1.8±0.31	1.9±0.24	(106)
	Con A	7.9±2.51	48.8±15.4	(617)*	1.9±0.67	3.1±0.67	(163)
	HOH	0.9±0.14	11.3±2.46	(1410)*	0.2±0.03	0.6±0.10	(300)*
12	LPS	8.7±0.63	13.3±2.62	(153)*	1.4±0.29	0.9±0.88	(64)
	Con A	9.8±3.13	41.0±10.4	(418)*	0.9±0.28	3.7±1.60	(411)
	HOH	1.5±0.18	18.2±5.60	(1210)*	0.1±0.01	1.2±0.49	(1200)**

Mice were fed diet containing 25 ppm vomitoxin or control diet for the specified time intervals. Pooled Peyer's patch cell suspensions (five to six mice/group) were cultured with and without mitogen.
Ig secretion was measured by ELISA after 7 days. Data are means ± SE of quadruplicate cultures for 3 experimental trials (n=12). * and ** indicate values that are significantly different from control at 0.05 and 0.01 levels, respectively.

Table 7. Effect of Dietary Vomitoxin on Ig Secretion in Murine Spleen Cultures

		IgA (μg/ml)			IgG (μg/ml)		
wks	Mito-gens	Control	Treatment	% To Control	Control	Treatment	% To Control
4	LPS	1.0±0.18	1.6±0.16	(160)*	1.9±0.16	1.5±0.20	(79)
	Con A	0.6±0.17	0.5±0.08	(83)	0.3±0.05	0.3±0.05	(100)
	HOH	0.2±0.05	0.1±0.02	(50)	0.3±0.04	0.1±0.03	(33)*
8	LPS	1.5±1.12	2.8±0.21	(186)*	3.7±0.54	3.0±0.28	(81)
	Con A	1.2±0.15	2.2±0.50	(183)	0.9±0.29	1.1±0.41	(122)
	HOH	0.6±0.13	1.2±0.23	(200)**	0.2±0.06	0.7±0.26	(350)
12	LPS	2.0±0.29	2.2±0.28	(110)	2.8±0.38	2.1±0.28	(75)
	Con A	0.9±0.11	1.1±0.14	(122)	0.2±0.03	0.3±0.04	(150)**
	HOH	0.5±0.08	1.3±0.27	(260)**	0.1±0.02	0.4±0.10	(400)**

Experiment was performed as described in Table 6 except that spleen cells were used.

weeks. Mice in the withdrawal group were fed the toxin feed for the first 8 weeks and then switched to control diet for an additional 16 weeks. Mice in the control group were fed control diet for the whole 24 weeks of the experimental period. The immunological and clinical changes related to IgA nephropathy were monitored.

3.2.1 *General effects of vomitoxin*

Feed refusal and ruffled fur were observed in vomitoxin-treated mice, while acute clinical signs were not observed. Body weights of mice in all experimental groups were monitored throughout the 24-week feeding period at 2-week intervals. Mean body weight of mice in the treatment group remained at 21.6 ± 1.0 g (mean ± SD) level throughout the experiment. However, mean body weight of mice in the withdrawal group was substantially recovered after cessation of vomitoxin exposure and increased at a slightly faster rate of 0.88 g/wk compared to 0.71 g/wk of the control group (Fig. 10). Mean body weights of mice at the end of the experiment in the control and withdrawal groups were 36.9 ± 3.7 and 33.9 ± 2.4 g, respectively.

Mortality of experimental mice was also observed after a prolonged course of vomitoxin feeding. Whereas 92 and 100% mice in the control and withdrawal groups survived the whole 24-week feeding period, 100, 83, 67, and 50% mice in the treatment group remained alive at Week 18, 20, 22, and 24, respectively.

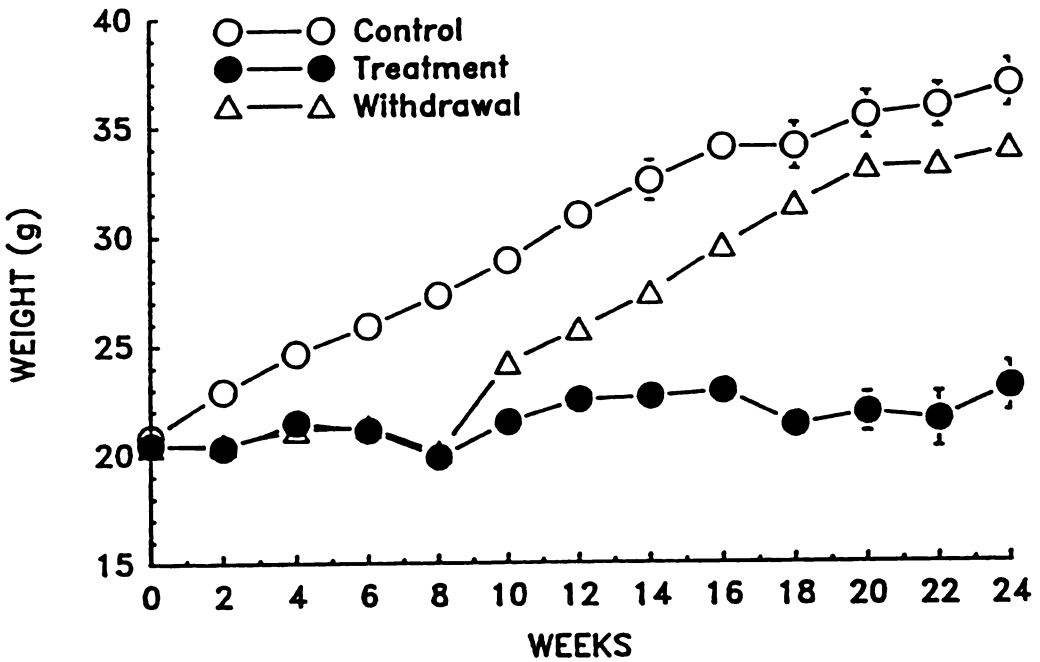


Figure 10. Effect of dietary vomitoxin exposure and withdrawal on body weight of B6C3F1 mice. Data are means ± SE (n=12). Arrow indicates when toxic diet was replaced by control diet in the withdrawal group. Completely random ANOVA and Student-Neuman-Keul's test were applied. After Week 2, control mice were significantly different from withdrawal mice and treatment mice. Withdrawal mice differed significantly from control and treatment mice at Week 10 and thereafter.

3.2.2 *Serum Ig and IgA-IC*

Serum IgA, IgG, and circulating IgA-IC were examined at 4-week intervals throughout the experimental period. Serum IgA in the treatment group increased steadily in the first 12 weeks of the experiment and remained at levels 11-18 fold higher than control level during the remaining 12 weeks. Serum IgA in the withdrawal group was also elevated after 8 weeks of vomitoxin exposure and remained persistently higher than that in the control group at the end of the experiment, but was significantly lower than that in the treatment group at Week 8 and thereafter (Fig. 11, top).

Circulating IgA-IC level also continuously increased in the treatment group and was persistently elevated after cessation of vomitoxin exposure in the withdrawal group up to Week 20, but returned to control level at Week 24 (Fig. 11, bottom). Serum IgG levels in all experimental groups were not affected consistently over the course of the feeding period (data not shown).

3.2.3 *Urinalysis*

Microhematuria is a hallmark of human IgA nephropathy and often accompanied by proteinuria at the later stage of this disease. Both clinical indices were measured as endpoints for vomitoxin-induced murine IgA nephropathy.

The microhematuria indices increased markedly in toxin-treated mice and were significantly greater than baseline control values after only 4 weeks. Microhematuria in the

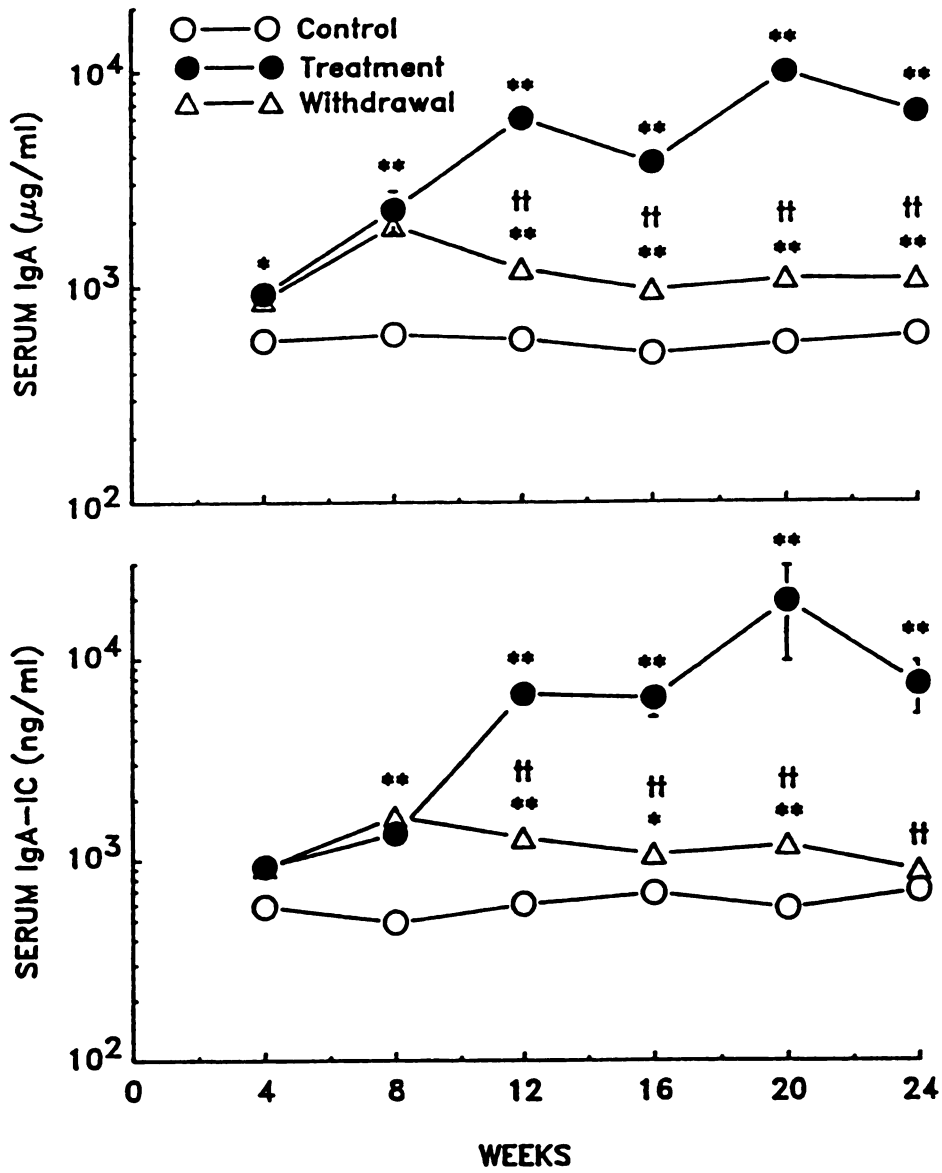


Figure 11. Effect of dietary vomitoxin exposure and withdrawal on serum IgA and circulating IgA-IC. Circulating IgA-IC were precipitated by polyethylene glycol. IgA levels were detected by ELISA. Arrow indicates when toxin feed was replaced by control diet in the withdrawal group. Data are means ± SE (n=12). Completely random ANOVA and lsd test were applied. * and ** Significant difference compared to the control groups of the same week at the 0.05 and 0.01 level, respectively. ‡ Significant difference compared to the withdrawal group of 8 weeks at the 0.01 level.

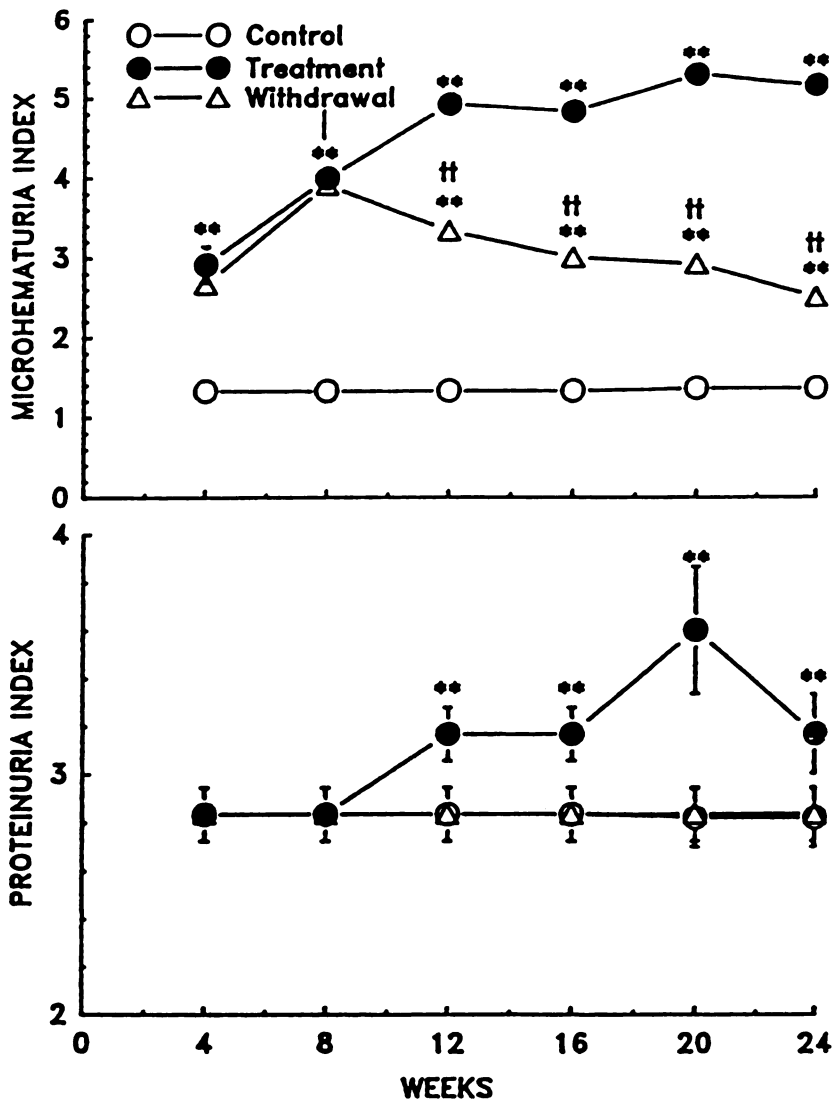


Figure 12. Effect of dietary vomitoxin exposure and withdrawal on microhematuria and proteinuria. Urine was collected at intervals and centrifuged, and erythrocytes were quantitated microscopically. Hematuria indices were graded, based on erythrocytes /field as follows: 1 (0), 2 (1-3), 3(4-10), 4(11-20), 5 (21-30), and 6 (>30). Proteinuria indices were graded accordingly as follows: 1 (negative), 2 (trace), 3 (30 mg/dl), 4 (100 mg/dl), and 5 (300 mg/dl). Arrow indicates when toxin diet was replaced by control diet in the withdrawal group. Data are means ± SE (n=12). χ^2 test was applied. ** Significant difference compared to the control groups of the same week at the 0.01 level. ‡ Significant difference compared to the withdrawal group of 8 weeks at the 0.01 level.

withdrawal group remained higher than that in the control group up to Week 24, but was lower than the treatment value of Week 8 (Fig. 12, top).

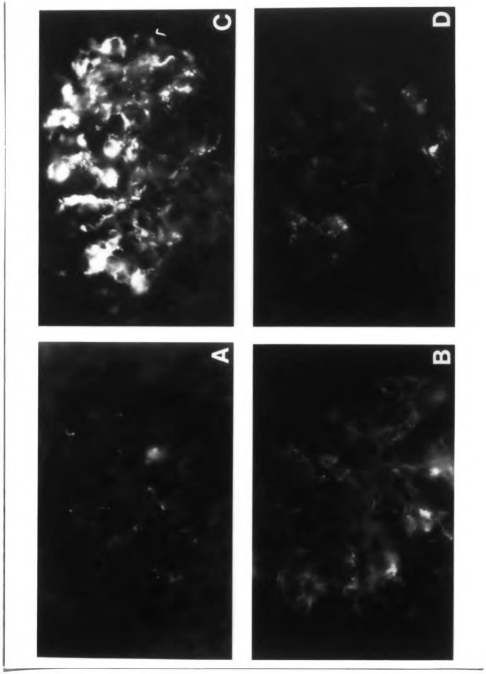
The proteinuria index in the treatment group was significantly increased at Week 12 of vomitoxin feeding, peaked at Week 20, and remained significantly elevated up to Week 24. However, this difference was not observed throughout the experiment between the withdrawal and control groups (Fig. 12, bottom).

3.2.4 *Mesangial Ig and C3 depositions after dietary vomitoxin exposure and withdrawal*

Mesangial Ig and C3 accumulation was examined in all experimental groups using immunofluorescence microscope as well as laser cytometer image analysis. A large degree of mesangial IgA deposition was found using immunofluorescence microscopy in the treatment group after 24 weeks of vomitoxin exposure compared to that in the control group of the same week. An increase in IgA fluorescence intensity was observed in the treatment group after 8 weeks of vomitoxin feeding and noticeably persisted in the withdrawal group after cessation of vomitoxin exposure for 16 weeks (Fig. 13).

Mesangial IgA deposition was quantitatively analyzed by interactive laser cytometer image analysis system and was verified to persist at Weeks 16 and 24 in the withdrawal group, but was significantly lower than that in the

figure 13. Mesangial IgA deposition after dietary vomitoxin exposure and withdrawal. Kidney sections were prepared from female B6C3F1 mice of each experimental group and stained with FITC-labeled anti-IgA conjugate. (A) 24-week control, (B) 8-week treatment, (C) 24-week treatment, (D) 24-week withdrawal groups.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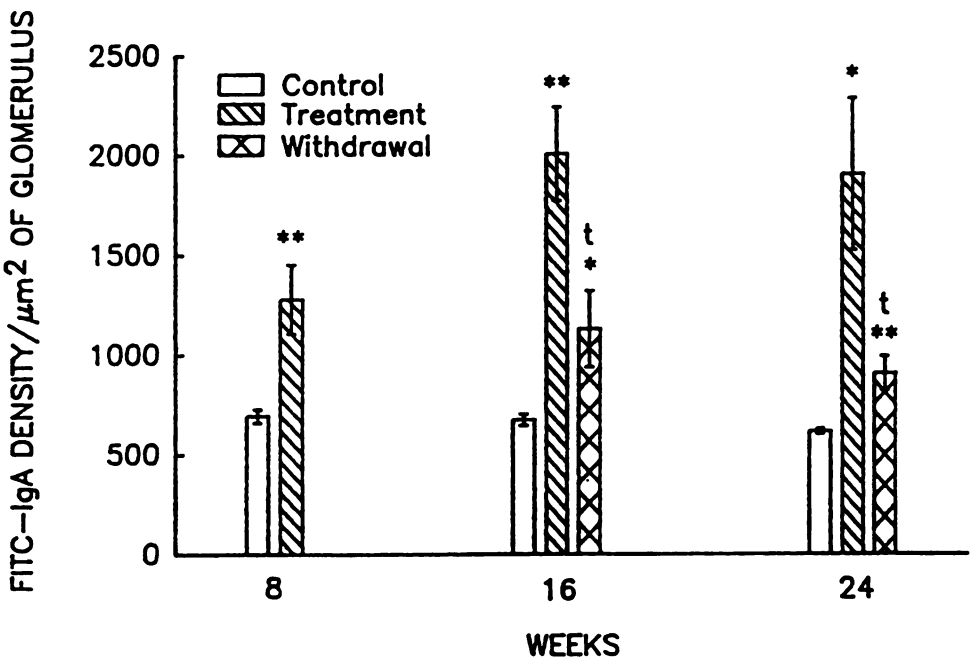


Figure 14. Quantitation of mesangial IgA deposition by interactive laser cytometer image analysis. Fluorescence emission is expressed as arbitrary units. Data are arbitrary units of means ± SE (n=12). Student's *t* test, completely random ANOVA, and lsd test were applied. * $p<0.05$ and ** $p<0.01$, Significant difference compared to the control groups of the same week. t $p<0.05$, comparison between 16-week or 20-week withdrawal group and 8-week treatment group.

treatment group of Week 8 (Fig. 14).

Mesangial IgG and IgM depositions were observed under immunofluorescence microscope in both control and treatment groups after 24 weeks of the experiment, but the intensity of IgA deposition was noticeably lower in the treatment group than that in the control group at Week 24. C3 was observable at the same fluorescence intensity mainly in Bowman's capsule instead of mesangium of both control and treatment groups (Fig. 15).

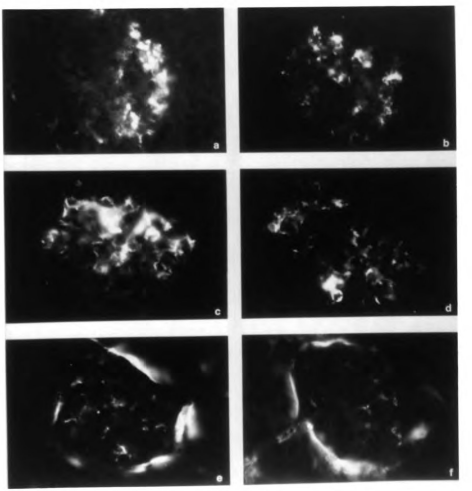
Mesangial deposition of IgG, IgM, and C3 was detectable in the control groups and tended to increase with age. Quantitated by interactive laser cytometer image analysis system, significantly less IgG deposition at 24 weeks and less IgM deposition at 8, 16, and 24 weeks in the treatment group were found, whereas differences in mesangial C3 deposition were not detectable between control and treatment groups throughout the 24 weeks of the experiment (Fig. 16).

3.2.5 *Effects on B and T cell profiles*

Surface antigens were used to study the effects of vomitoxin exposure and withdrawal on B and T cell profiles. Cell recovery from Peyer's patch lymphocytes of vomitoxin-treated mice was nearly 3-fold of that from controls after 16 and 24 weeks of vomitoxin feeding. There were no differences in cell recovery from Peyer's patches between control and withdrawal groups (Table 8).

Percentages of membrane IgA^+ and $CD4^+$ cells in Peyer's

Figure 15. Mesangial IgM, IgG, and C3 deposition after dietary vomitoxin exposure. Kidney sections were prepared from mice of control and treatment groups at Week 24 and stained with FITC-labeled anti-IgM, anti-IgG, and anti-C3 conjugates. (a) IgM control, (b) IgM treatment, (c) IgG control, (d) IgG treatment, (e) C3 control, and (f) C3 treatment.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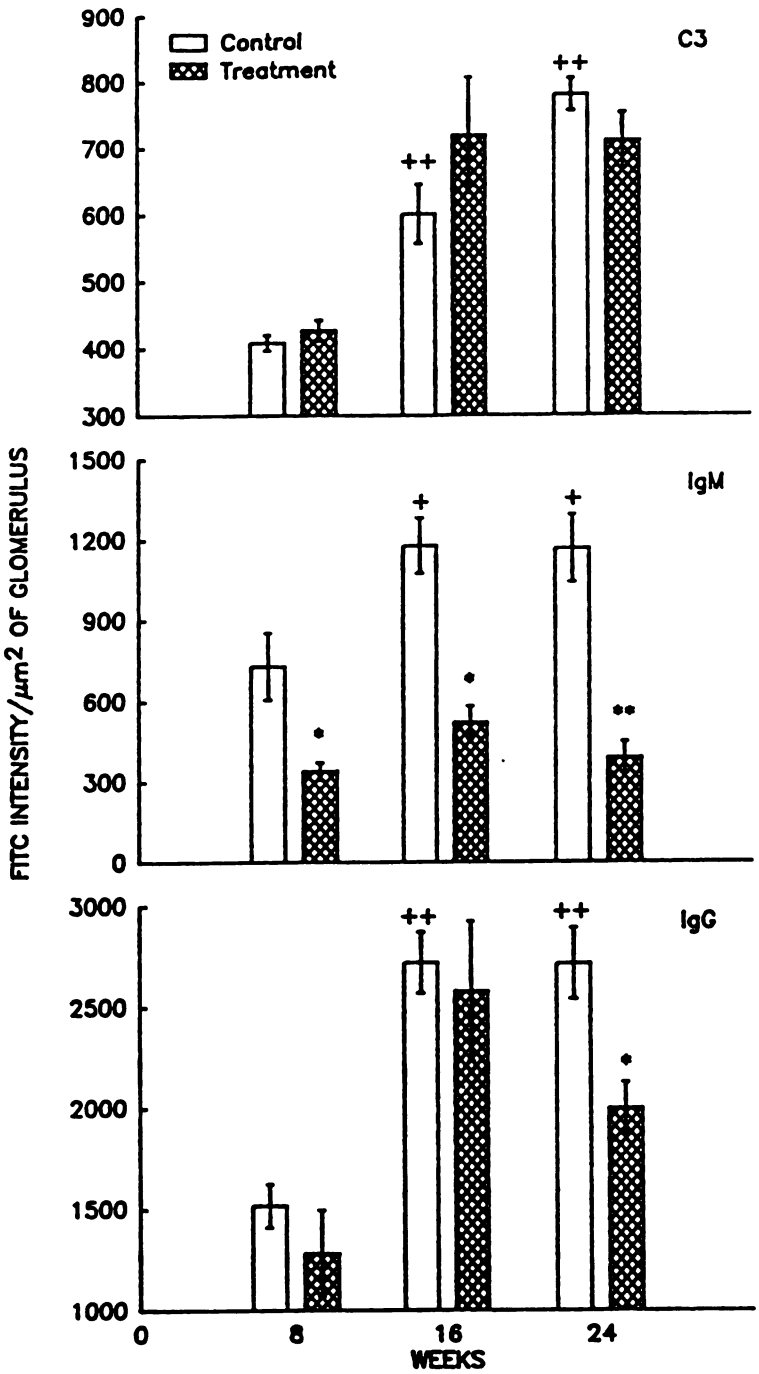

Figure 16. Quantitation of mesangial C3, IgM, and IgG deposition by interactive laser cytometer image analysis. Fluorescence emission is expressed as arbitrary units. Data are arbitrary units of means ± SE (n=12). Student's *t* test, completely random ANOVA, and lsd test were applied. * and ** Significant difference between control and treatment groups of the same week at the 0.05 and 0.01 levels, respectively. + and ++ Significant difference compared to the control groups of 8 weeks at the 0.05 and 0.01 levels, respectively.

patches increased significantly after 16 and 24 weeks of vomitoxin exposure. The increase in membrane IgA^+ cells persisted after cessation of vomitoxin feeding for 8 and 16 weeks, but the effect on $CD4^+$ cells was reversed (Table 8).

Cell recovery from splenic lymphocytes was lower in the withdrawal and treatment groups as compared to the control groups after 16 and 24 weeks, respectively (Table 9).

Percentages of membrane IgA^+ and $CD4^+$ cells in the spleen elevated significantly after 16 and 24 weeks of vomitoxin exposure, but returned to the control levels after cessation of vomitoxin exposure for 8 and 16 weeks (Table 9).

3.2.6 *In vitro Ig production*

There was a 2-10 fold increase in IgA secretion by unstimulated and LPS-stimulated cultures obtained from the treatment and withdrawal groups compared to their corresponding controls at Weeks 16 and 24. However, IgA output from the withdrawal cultures was consistently less than that from the treatment cultures. Increased IgG production was also prominent in unstimulated and LPS-stimulated cultures, but the relative increase was much lower than that observed for IgA (Table 10).

Table 8. Effects of Dietary Vomitoxin Exposure and Withdrawal on Peyer's Patch Lymphocyte Population[a]

Dietary regimen	Cell Number ($\times 10^6$)	% IgA$^+$	% CD4$^+$
16 wk of control diet	3.6 ± 0.4	5.6 ± 0.2	14.8 ± 0.6
16 wk of 25 ppm toxin	11.2 ± 1.9[c]	9.8 ± 0.5[c]	18.1 ± 0.8[c]
8 wk of 25 ppm toxin + 8 wk of control diet	5.3 ± 0.1[b]	8.0 ± 0.3[b,c]	14.4 ± 0.9[b]
24 wk of control diet	6.1 ± 1.4	5.7 ± 0.2	14.2 ± 0.6
24 wk of 25 ppm toxin	17.2 ± 2.5[c]	13.2 ± 0.8[c]	26.7 ± 1.2[c]
8 wk of 25 ppm toxin +16 wk of control diet	6.9 ± 1.1[b]	7.5 ± 0.3[b,c]	14.2 ± 0.5[b]

[a] Results are means ± SE determined from pooled cells (2 mice/pool) prepared from 12 mice except 25 ppm/24 week group, which had 6 mice. Statistical analysis performed using completely random ANOVA and Student-Neuman-Keul's test. Percentage data was transformed by arc sin square root.
[b] Significantly different from treatment ($p<0.01$).
[c] Significantly different from control ($p<0.01$).

Table 9. Effects of Dietary Vomitoxin Exposure and Withdrawal on Splenic Lymphocyte Population[a]

Dietary regimen	Cell Number ($\times 10^6$)	% IgA$^+$	% CD4$^+$
16 wk of control diet	123 ± 9	5.0 ± 0.3	15.9 ± 2.8
16 wk of 25 ppm toxin	105 ± 10	8.0 ± 0.2[d]	26.2 ± 4.3[d]
8 wk of 25 ppm toxin + 8 wk of control diet	81 ± 11[c]	4.9 ± 0.2[b]	15.8 ± 2.1[b]
24 wk of control diet	138 ± 13	4.9 ± 0.5	12.6 ± 0.6
24 wk of 25 ppm toxin	95 ± 23[c]	8.8 ± 0.1[d]	26.2 ± 1.3[d]
8 wk of 25 ppm toxin +16 wk of control diet	159 ± 5[b]	5.3 ± 0.3[b]	12.5 ± 0.5[b]

[a] Results are means ± SE. Experimental conditions and statistical analysis as described in Table 8.
[b] Significantly different from treatment ($p<0.01$).
[c,d] Significantly different from control ($p<0.05$ and $p<0.01$).

Table 10. Effects of Dietary Vomitoxin Exposure and Withdrawal on Ig Production in Spleen Culture [a]

Dietary regimen	Unstimulated		LPS-stimulated	
	IgA	IgG	IgA	IgG
16 wk of control diet	530± 89	1070±223	940±752	2380±153
16 wk of 25 ppm toxin	5410±707[e]	1990±306[d]	2090±128[e]	3230±224[d]
8 wk of 25 ppm toxin + 8 wk of control diet	2530±347[b,e]	2530±347[e]	1550± 82[c,e]	2580±173[b]
24 wk of control diet	100± 30	120± 56	700± 70	1000±109
24 wk of 25 ppm toxin	340± 68[e]	150± 73	3540±840[e]	1060±136
8 wk of 25 ppm toxin +16 wk of control diet	230± 79[b]	146± 73	1250±130[c,e]	1450± 91[c,e]

[a] Results are means ± SE (ng/ml). Experimental conditions as described in Table 8. Statistical analysis using log transformation, completely random ANOVA, and Student-Neuman-Keul's test.
[b,c] Significantly different from treatment ($p<0.05$ and $p<0.01$).
[d,e] Significantly different from control ($p<0.05$ and $p<0.01$).

4.0 DISCUSSION

Main features of vomitoxin-induced IgA nephropathy include: (1) increased serum IgA and IgA-IC; (2) early occurrence of hematuria and in later stages, proteinuria; (3) accumulation of mesangial IgA; (4) elevated total B cells in Peyer's patches and total B and T cells in both spleen and Peyer's patches; (5) increased percentages of splenic and Peyer's patch membrane IgA^+ cells and $CD4^+$ cells; (6) increased $CD4^+$:$CD8^+$ ratio in both spleen and Peyer's patches; and (7) increased spontaneous and mitogen-stimulated IgA secretion from *in vitro* culture of splenic and Peyer's patch cells. A major finding of this study was that these immunologic and pathologic changes persisted 16 weeks after cessation of dietary vomitoxin exposure.

Consistent with previous reports (Forsell *et al.*, 1986), vomitoxin-treated mice manifested signs of typical chronic toxicity, such as ruffled fur, feed refusal, and decreased weight gain, after prolonged vomitoxin feeding. Some mortality in the treatment group occurred after 20 weeks of vomitoxin exposure. It is possible that renal failure contributes to mortality after progressive renal dysfunction caused by prolonged vomitoxin feeding. It is notable that signs of general toxicity including ruffled fur, feed

refusal, and decreased weight gain, which often appeared in vomitoxin-treated mice, gradually disappeared in the withdrawal mice. Clearly, general toxicity in vomitoxin-treated mice was completely distinct from more persistent immunopathologic parameters.

Serum IgA is elevated in 50-70% of patients with IgA nephropathy (D'Amico, 1983) and was similarly elevated in experimental mice following dietary exposure to vomitoxin. This is consistent with earlier findings (Forsell *et al.*, 1986). One previous pair-fed study showed that no elevation of serum IgA was found in control mice fed the same amount of feed as consumed by vomitoxin-treated group (Pestka *et al.*, 1989). Therefore, elevation of serum IgA and IgA nephropathy induced by dietary vomitoxin are not simply a result of the nutritional deprivation.

It should be noted that both hyperproduction and impaired catabolism of IgA can lead to high concentration of serum IgA. The catabolism of IgA macromolecule in the liver is not impaired in patients with IgA nephropathy (Rifai *et al.*, 1989). However, further study is needed to reveal if the catabolism of IgA macromolecule is impaired in this vomitoxin-induced murine IgA nephropathy model.

As noted previously, serum IgG level generally remained unchanged or somewhat decreased (Forsell *et al.*, 1986; Pestka *et al.*, 1989). Reduced mesangial IgG deposition observed in this study may reflect the decrease in overall serum IgG level. Oral tolerance and heavy chain class

switching could be contributory to this observation. It has been shown that oral challenge with a variety of antigens results in mucosal immunity accompanied by partial or profound systemic unresponsiveness (Mattingly, 1984). The possible mechanisms of oral tolerance are (1) B cell clonal deletion; (2) antibody forming cell (AFC) blockade; (3) T-helper cell deletion; (4) T-suppressor cell generation (Roitt *et al.*, 1987). The increased number of $CD8^+$ cells in vomitoxin-treated mice in spleen suggests that the likely generation of T-suppressor cells might be responsible for partial unresponsiveness of IgG. Class switching, a process which is heavily regulated by helper T cells and their cytokines, can be another possible mechanism. A lymphocyte clone can switch class while maintaining the same variable region to ensure the same antigen-binding specificity. Thus it is feasible that vomitoxin enhanced class-switching from IgG to IgA.

It is widely suspected that human IgA nephropathy is an immune complex disease (Hebert, 1988). Elevation of IgA-IC observed in this study was consistent with this possibility. The strong association between circulating IgA-IC and the severity of IgA nephropathy has been well documented (Clarkson *et al.*, 1984; Egido *et al.*, 1984a; Emancipator and Lamm, 1989). The severity generally parallels the mesangial IgA deposition and the level of circulating IgA-IC. The chronic feature of IgA-IC observed in this experiment is suggestive of continuing antigenic stimulation of mucosal

associated lymphoid tissue (MALT).

Although macrohematuria is difficult to monitor in animal models, microhematuria was detected in the very early stage of vomitoxin-induced IgA nephropathy. This is consistent with findings in human IgA nephropathy as well as many other animal models of IgA nephropathy. Proteinuria appeared at Week 12 and peaked after Week 20 of vomitoxin feeding. At Week 24, the proteinuria index dropped considerably because of the death of 50% of mice in the treatment group, among which most of them were presumably died of renal failure. In human IgA nephropathy, persistent proteinuria exceeding 0.5 g/day is rarely observed less than five years from the onset of the symptoms, which suggests that proteinuria is a late stage manifestation of the disease (Kincaid-Smith and Nichholls, 1983). The presence of IgA mesangial deposition can proceed the appearance of proteinuria by several months (Berger *et al.*, 1975).

This study was the first to apply laser cytometer image analysis to quantitatively measure mesangial Ig deposition in experimental IgA nephropathy. The instrument provided a more sensitive and quantitative measurement of fluorescence intensity than conventional immunofluorescence microscope. Glomeruli can be found under regular light and then examined for fluorescence after laser excitation thus allowing unbiased measurement between control and treatment groups. Significantly higher amount of mesangial IgA deposition determined in this study was consistent with a previous

finding in vomitoxin feeding study (Pestka *et al.*, 1989). Increased mesangial IgA deposition along with hematuria reflect a successful induction of experimental murine IgA nephropathy by dietary exposure to vomitoxin.

IgG, IgM, and C3 were detected in the mesangium or Bowman capsule of both control and vomitoxin-treated mice, with no apparent differences among the groups. When quantitatively assessed by image analysis, both mesangial IgG and IgM deposition were significantly decreased, while C3 remained unchanged. In two other animal models for IgA nephropathy, the presence of mesangial IgG and IgM was similarly noted in both control and treated animals (Genin *et al.*, 1986; Sato *et al.*, 1986). However, decreased mesangial IgG or IgM is not completely consistent with the postulated role for these isotypes in the human IgA nephropathy and has not been reported before. The lack of mesangial IgG and IgM co-deposition may reflect the fact that there is reduced production of IgG and IgM due to vomitoxin exposure with the possible mechanism of class-switching.

Generally, animal models developed so far are representative of human IgA nephropathy except some models lack hematuria and/or C3 co-deposition (Emancipator *et al.*, 1987; Jessen *et al.*, 1987). Vomitoxin-induced model also lacks C3 deposition. Since intense C3 deposition occurred mainly in Bowman capsule area, which has been reported previously (Emancipator *et al.*, 1987), image analysis of the

glomerular deposits might not accurately measure the average fluorescent intensity for C3 deposition in the mesangium.

Part of this discrepancy between human IgA nephropathy and some animal models including vomitoxin-induced model may be due to facts that: (1) The diagnosis of IgA nephropathy is made solely by renal biopsy, a procedure that is performed selectively on patients with urinary abnormalities suggestive of glomerulonephritis, whereas all experimental animals are subject to morphological examinations. (2) IgA in mouse is a poor complement-fixing antibody. C3b generated by the alternative pathway does not bind well to the IgA-IC (Pfaffenbach *et al.*, 1982). In DNP-BSA model, C3 was absorbed by DNP, which is capable of fixing C3 with the mechanism of substitution of BSA. (3) It is possible that there was insufficient time to induce mesangial deposition of C3. One murine IgA nephropathy model did not have C3 mesangial deposition until 30 weeks (Sato *et al.*, 1986). Thus, the nature of antigens involved may be important in causing C3 deposition and other pathologic changes in IgA nephropathy.

Elevated $CD4^+/CD8^+$ ratio and IgA-bearing cells are found in the peripheral blood of patients with IgA nephropathy (D'Amico, 1987). However, it is controversial whether or not the increase of IgA-bearing cells occurred in peripheral blood is a diagnostic marker of the disease (Feehally *et al.*, 1984; Fiorini *et al.*, 1982). Initiation of the immune response in the gut is regulated in the Peyer's patch level

where B cell activation, switching, proliferation, and differentiation occur and where regulatory T cells and accessory cells are available (McGhee *et al.*, 1989). IgA-bearing cells were significantly increased in both Peyer's patches and the spleen from vomitoxin-treated mice. It is feasible that IgA bearing-cells in the spleen may arise from the intestinal mucosa, Peyer's patches in this case, via the circulation. Size and histologic changes in Peyer's patches after vomitoxin treatment indicated enhanced germinal center development, while secretory IgA in the intestinal lumen was not increased as demonstrated by measurements of IgA with intestinal washing (Pestka *et al.*, 1990). It is possible that net migration of B and T cells from Peyer's patches into the mesenteric lymph nodes and systemic compartment was increased.

Increased spontaneous and mitogen-stimulated production of IgA was observed in culture supernatant of splenocytes and Peyer's patch cells. Ig secretion by cell cultures served as a surrogate for Ig production *in vivo*. Results strongly suggest that dietary vomitoxin acts primarily at the Peyer's patch level where it promotes proliferation of IgA-secreting progenitors and terminal differentiation of IgA-secreting cells. In cell culture studies, it is quite difficult to compare the results from different experiments because of heterogeneity in sources of the cell population used, in activation stages of cells when cultures were started, and in culture conditions such as concentration of

CO_2, temperature, and humidity.

The increased number of IgA-bearing cells and IgA production in vomitoxin-treated mice might be due to the possibility of increased activity of helper T cell or decreased activity of putative suppressor T cell. T cells in human peripheral blood with Fc receptors for IgA (Tα cells) are significantly increased in patients with IgA nephropathy (Lum *et al.*, 1979; Sakai *et al.*, 1982). Tα cells were found to enhance IgA secretion and switching (Sakai *et al.*, 1989). Often studies have shown a decreased activity of IgA-specific suppressor T cells in patients with IgA nephropathy (Egido *et al.*, 1983). An increased ratio of $CD4^+/CD8^+$ cells has been found in many studies (Rothschild and Chatenoud, 1984) including this one. The potential for vomitoxin-induced dysregulation of cytokines produced by $CD4^+$ cells is addressed in Chapter Two.

In human IgA nephropathy, once the disease has reached a stage at which it can be diagnosed, the histologic lesions persist indefinitely (Kincaid-Smith, 1985). Similarly, most of the pathologic changes in vomitoxin-induced murine IgA nephropathy persisted 16 weeks after cessation of vomitoxin exposure. It is critical to note that upon cessation of vomitoxin exposure, most pathologic parameters including serum IgA, IgA-IC, hematuria, and mesangial IgA deposition did not continuously increase but remained significantly elevated over control values for 16 weeks.

Vomitoxin has a relatively short half-life in sheep

(Prelusky *et al.*, 1986) and it is unlikely to persist in mice. Since serum IgA also has a short half-life (24 hr) in mice (Delacroix *et al.*, 1985), persistent elevation of serum IgA suggested a continuous expansion of IgA-secreting B cell population in withdrawal mice. This notion is supported by the elevated IgA-bearing cells in Peyer's patches from withdrawal mice and by increased IgA production in spleen of withdrawal mice, which are indicative of increased numbers of B cells that are partially or fully differentiated to IgA secretion. The inability of serum IgA or IgA-bearing cells to increase further after withdrawal of dietary vomitoxin exposure may be highly dependent on the inability of the experimental animals to maintain elevated $CD4^+$ population. However, the maintenance of elevated level of IgA-bearing cells may not need further help from $CD4^+$ cells.

In order to better understand the relationships among immunologic and pathologic parameters of IgA nephropathy, correlations among these measured endpoints were calculated and statistically analyzed (Table 11). Serum IgA level was significantly correlated to circulating IgA-IC, hematuria, and proteinuria. Circulating IgA-IC level was significantly correlated to hematuria. Microhematuria was significantly correlated to proteinuria. Mesangial IgA deposition was significantly correlated to serum IgA, circulating IgA-IC, hematuria, and proteinuria. Pearson's correlation coefficients (r) were used for analysis among serum IgA, IgA-IC, and mesangial IgA deposition. Spearman's rank

correlation coefficients (r_s) were applied for correlations involving hematuria or proteinuria.

Multiple regression analysis showed that hematuria was only significantly correlated to serum IgA level ($y=0.704x_1+0.242x_2+0.259x_3-0.173x_4-3.955$) and mesangial IgA deposition was only significantly correlated to circulating IgA-IC level ($y=0.657x_1+0.661x_2-0.662x_3+0.221x_4-4552.5$). The significance of partial regression coefficients (b) was tested. Results are summarized in Table 12.

Neither serum IgA nor IgA-IC level was statistically correlated to IgA production in either spleen or Peyer's patch culture supernatant (data not shown).

Notably, many immunologic and pathologic endpoints are significantly correlated to each other according to simple correlation analysis. It is unlikely that every significant correlation represents a strong biological association. However, some of these correlations have been reported in the literature with somewhat conflicting conclusions. For example, the correlation between macrohematuria and pathologic findings has several different interpretations. Some results showed severe glomerular changes in patients who had a history of macrohematuria (Bennett and Kincaid-Smith, 1983), while others found only minor lesions in these patients, compared to those without a history of macrohematuria (D'Amico *et al.*, 1985; Shirai *et al.*, 1978). Another study found no correlation between the severity of glomerular lesions and previous gross hematuria history

Table 11. Correlations among Immunologic and Pathologic Endpoints (1)

	X[a]				
Y	Serum IgA	IgA-IC	Hematuria	Proteinuria	Mes. IgA
Serum IgA	N/A				
IgA-IC	0.681**	N/A			
Hematuria	0.895**	0.743**	N/A		
Proteinuria	0.651**	0.449	0.786**	N/A	
Mes. IgA	0.636**	0.723**	0.577*	0.535*	N/A

* $p<0.05$; ** $p<0.01$
[a] Spearman's rank correlation coefficient (r_s) for correlations involving hematuria and proteinuria. Pearson's correlation coefficient (r) for correlations among serum IgA, IgA-IC, and mesangial IgA.

Table 12. Correlations among Immunologic and Pathologic Endpoints (2)

	X[a]					
Y	Serum IgA	IgA-IC	Hematuria	Proteinuria	Mes. IgA	Intercept
Serum IgA	N/A	0.019	0.801**	0.011	0.195	1.897
IgA-IC	0.045	N/A	0.669	-0.447	0.476*	2.045
Hematuria	0.704**	0.242	N/A	0.259	-0.173	-3.955
Proteinuria	0.036	-0.588	0.941	N/A	0.209	2.322
Mes. IgA	0.657	0.661*	-0.662	0.221	N/A	-4552.5

* $p<0.05$; ** $p<0.01$
[a] Partial regression coefficient (b) of multiple regression

(Droz *et al.*, 1984).

When multiple regression analysis was applied in this study to sort out the strongest associations among all parameters analyzed, microhematuria was significantly correlated with pathologic findings, especially serum IgA (Table 11 and 12). These findings are very important because no correlation between IgA levels and any clinical, histologic, and immunologic features has been reported to date. However, the biological relation of serum IgA and microhematuria is unclear. It is possible that mesangial IgA deposition is the sole Ig deposition in this model. Serum IgA level may affect mesangial IgA deposition as well as signs of glomerulonephritis such as hematuria.

It is widely recognized that, in both children and adults, the level of proteinuria is closely correlated with the severity and extent of the glomerular lesions such as focal glomerular sclerosis and interstitial fibrosis (D'Amico *et al.*, 1985; Croker *et al.*, 1983). In this study, proteinuria appeared to be correlated with serum IgA, microhematuria, and mesangial IgA deposition (Table 11). But these correlations were not significant according to multiple regression analysis (Table 12).

Consistent with the results of many studies (Emancipator and Lamm, 1989; Tomino *et al.*, 1984), The deposition of mesangial IgA was significantly correlated to circulating IgA-IC level (Table 11). Multiple regression analysis also strongly suggested that high level of IgA-IC is the leading

contributor to the mesangial IgA deposition (Table 12). It is clearly demonstrated that this experimentally-induced IgA nephropathy and IgA deposited in the mesangium, like those observed in human IgA nephropathy and many other animal models, are immune complex in nature. This report is the first to statistically confirm the relationship between circulating IgA-IC and mesangial IgA deposition among all immunologic and pathologic indicators analyzed by multiple regression.

5.0 SUMMARY AND FUTURE RESEARCH NEEDS

Although the etiology of human IgA nephropathy is still unknown, factors such as diet, prior mucosal infection, enterotoxin produced by bacteria, and genetic predisposition have been suspected to be contributory (D'Amico, 1987; Coppo *et al.*, 1986; Coppo *et al.*, 1990). Vomitoxin levels are unregulated in most countries including the U.S. even though it appears to be a contaminant in retail food products at levels sufficient to increase serum IgA levels in mice (Abouzied *et al.*, 1991). It is possible that vomitoxin may play an etiologic role in human IgA nephropathy.

IgA nephropathy has been characterized and recognized as a distinct clinical and pathologic entity only in the past two decades. Many questions still remain to be answered: (1) Although a number of experimental IgA nephropathy models were induced by the continued mucosal administration of antigen, no such direct evidence exists in human; (2) Pharmaco-kinetics of vomitoxin and catabolism of IgA macromolecule in the body are still unclear; (3) Roles of macrophage influx and dysregulation of cytokine production need to be studied; (4) It is desirable to further examine the risk that vomitoxin and other trichothecenes in diet may play an etiologic role in human IgA nephropathy.

Chapter Two

ROLE OF CYTOKINE DYSREGULATION

1.0 INTRODUCTION

1.1 Definition of Cytokine

Cytokines are a group of soluble protein mediators produced by cells during the effector phases of natural and specific immunity that regulate and facilitate cell-to-cell communication of immune response. Cytokines can affect the same cell that secretes the cytokine (autocrine action) and/or affect adjacent or distant cell (paracrine action).

1.2 Sources of Cytokines

Cytokines can be produced by variety of cells. One of the most important classes of producers is the $CD4^+$ T helper (Th) cell. At least two major subsets of $CD4^+$ cells have been recognized as Th1 and Th2 on the basis of cytokine production (Mosmann *et al.*, 1986). In addition to cytokines secreted by both subsets of $CD4^+$ cells such as IL-3, tumor necrosis factor (TNF) α, and granulocyte/macrophage colony stimulating factor (GM-CSF), Th1 cells are capable of producing IL-2, interferon (IFN) γ, and lymphotoxin, while Th2 cells produce IL-4, IL-5, IL-6, IL-9, and IL-10 (Abehsira-Amar *et al.*, 1992). Two other subsets of $CD4^+$ cells have also been recognized as Th0 and T null (Fan *et*

al., 1990; Mosmann and Coffman, 1989). Th0 cells are considered as precursors of Th1 and Th2 and produce both IL-2 and IL-4, while T null cells do not produce cytokines.

1.3 Quantitation of Cytokines

It was initially very difficult to study T cell function because of the small amount of cytokines produced by a T cell. However, many bioassays and monoclonal antibodies against various cytokines have been developed during the past a few years, which makes the functional study much easier.

Bioassays are often performed using cloned cell lines, which may grow indefinitely *in vitro*. Many cell lines have an absolute dependence on a particular cytokine, which makes cytokine bioassay possible. Cloned cell lines provide a homogeneous cell population with the same potential to respond to biologically active cytokine(s). However, bioassays require exacting tissue culture techniques for measuring cellular biological activities in response to the presence of cytokines. Variations in results often occur from experiment to experiment (Wadhwa *et al.*, 1991).

Immunological assays such as radioimmunoassays (RIA) or ELISA can also be used to quantitate cytokines. The results indicate the level of cytokines that are present in cultured cells. But these cytokines are not necessarily biologically viable. Both assays are rapid, easy to perform, and very sensitive. However, both assays require high affinity

monoclonal antibodies against specific cytokines; possible cross-reactivity may occur due to structural similarity of cytokines. Furthermore, radioactive ^{125}I and carcinogenic substrate used in RIA and ELISA, respectively, constitute health hazards (Meager, 1991).

In Western blot analysis, cytokines with structural homology are separated based on their molecular weight on polyacrylamide gel electrophoresis and transferred to nylon membrane. Individual protein bands can be recognized by hybridization with monoclonal antibodies.

1.4 Quantitation of Cytokine-specific mRNA

Northern analysis can be used to detect cytokine mRNA in tissues or cells. It is useful for measuring the amount of mRNA transcribed and to compare with protein level of cytokines translated from mRNA (Naylor and Balkwill, 1991). Recently, the polymerase chain reaction (PCR) has been employed as a powerful method to amplify and to quantitate small amounts of cytokine mRNA in a small number of cells (Ballagi-Pordány *et al.*, 1991; Carding *et al.*, 1992; Kanangat *et al.*, 1992; Mohler and Butler, 1991).

1.5 Cytokine Gene Regulation

Most cytokine genes have been cloned, sequenced, and analyzed (Taniguchi, 1988). Their transcription is often transiently activated in the producer cells by extracellular stimuli and most of the cytokine specific mRNAs are usually

undetectable unless the cells are induced by appropriate mitogenic agents such as phorbol myristate acetate (PMA). Although gene expression can be controlled at many levels, expression of cytokines is regulated primarily at the transcriptional level by the action of transcriptional factors (Hudson, 1991). Among cytokines, expression and regulation of IL-2 and IFN genes have been most extensively studied.

IL-2 gene expression is regulated at transcriptional level. Well-conserved DNA sequences within the 5'-flanking region of both human and mouse IL-2 genes have been found (Fuse *et al.*, 1984). These genes play important roles in regulating gene expression and have been localized by fusing excised putative regulatory sequences from the IL-2 gene to the chloramphenicol acetyltransferase (CAT) gene and by monitoring CAT activity in T cell or non-T cell clones constructed with fused CAT genes. The regulatory sequences within the 5'-flanking region which induce maximal mRNA transcription in tetradecanoylphorbol acetate (TPA)-induced EL-4 cells are located between positions -319 and -264 with respect to the IL-2 gene cap site (Fuse *et al.*, 1984). In human IL-2 gene, regulatory sequences were found to be divided into three core elements from position -326 to -52 on the 5'-boundary (Durand *et al.*, 1987; Taniguchi *et al.*, 1986). Regulatory sequences were also observed in 3' non-translated region of a rearranged IL-2 gene in the gibbon leukemia T cell line (Chen *et al.*, 1985). Studies on IFN-γ

regulatory sequences reveal that IFN-γ gene is also regulated primarily on the transcriptional level similar to the IL-2 gene (Krönke *et al.*, 1985).

The expression of cytokine genes is also regulated at the posttranscriptional level (Taniguchi, 1988). Cytokine mRNA has a relatively short half-life. For example, the half-life of IL-2 mRNA is less than 2 hr in the TPA-induced EL-4 cell line (Fujita *et al.*, 1986). Therefore, selective mRNA degradation or stabilization is also important to the maintenance of cytokine mRNA and cytokine levels.

IL-2 and IFN-β mRNAs are superinduced by cycloheximide leading to extensive production of IL-2 and IFN-β (Efrat and Kaempfer, 1984; Nir *et al.*, 1984). A possible model for this shutoff mechanism is that cycloheximide inhibits the *de novo* synthesis of a short-lived protein repressor that is responsible for controlling the mRNA level. The shutoff mechanism is restored rapidly upon removal of cycloheximide. LPS reportedly induces GM-CSF mRNA in macrophages by stabilizing mRNA (Thorens *et al.*, 1987). Presumably, LPS induces the synthesis of a protein(s) that averts mRNA degradation.

Highly conserved AU-rich sequences within the 3' nontranslated regions of mRNA have been found to be contributory to the instability of cytokine mRNA (Caput *et al.*, 1986; Shaw and Kamen, 1986). Synthetic AT-rich sequence was inserted into the 3' noncoding region of the rabbit β-globin gene. The expressed mRNA becomes unstable

compared to mRNA transcribed from a control DNA with the same length. An unstable protein that interacts with the above sequences appears to be another factor that controls the cytokine level and is readily inhibited by cycloheximide as part of the superinduction for IL-2 and IFN-β.

Alternative splicing of primary cytokine mRNA precursor into ineffective cytokine mRNA was also proposed as a control mechanism for the regulation of the cytokine gene expression, although its biological significance is far from understanding (Clark and Kamen, 1987; Nagata *et al.*, 1986).

It is believed that regulatory sequences interact with multiple factors to achieve maximum expression. Although the inhibitory mechanisms are unknown, cytokine gene expression is apparently regulated by inhibitors such as glucocorticoid hormones and cyclosporin A. The expression of most lymphokine genes including IL-2, IFN-γ, IL-3, and GM-CSF genes can be suppressed by dexamethasone (Culpepper and Lee, 1987). However, cyclosporin A appears to inhibit only certain cytokines on enhancer region of the IL-2 gene. For example, cyclosporin A inhibits the expression of IL-2, IFN-γ, IL-3, and IL-4 genes, but not GM-CSF gene (Bickel *et al.*, 1987; Elliott *et al.*, 1984). On the other hand, AP-1 is one of several nuclear factors that may interact with the regulatory sequences on IL-2 gene (Angel *et al.*, 1987). AP-1 is composed of the protein products of the *c-jun* and *c-fos* oncogenies though the "leucine zipper" and binds to TPA responsive elements at positions -185 to -176 on IL-2 gene

(Edwards and Mahadevan, 1992). The expression level of a cytokine can also be regulated by other cytokines (cytokine-induced cytokine gene expression), but their regulatory mechanisms are still unclear (Grabstein *et al.*, 1986; Kohase *et al.*, 1987).

1.6 Cytokines Involved in Regulation of IgA Production

Regulation of IgA synthesis and immune response by T cells and cytokines has been extensively reviewed by McGhee *et al.* (1989). Cytokines involved in IgA regulation are summarized in Table 13. CH12LX cells are murine B cell lymphoma and consist of 98-99% of $mIgM^+$ cells and 1-2% of $mIgA^+$ cells (Haughton *et al.*, 1986; Kunimoto *et al.*, 1988). When incubated with LPS, IL-4 significantly increases the number of $mIgA^+$ cells in CH12LX population, but IL-5 does not. When CH12LX cells are treated with both IL-4 and IL-5, the number of $mIgA^+$ cells and the secretion of IgA are elevated (Kunimoto *et al.*, 1988). Studies using $mIgM^+$ I.29μ B lymphoma cell line revealed that LPS induced Cα mRNA germline transcripts through DNA recombination, while IL-4 increased germline Cϵ mRNA transcripts in LPS-treated I.29μ cells and increased Cα transcripts in LPS-triggered splenic B cells (Stavnezer *et al.*, 1985; Stavnezer *et al.*, 1990). It was suggested that (1) IL-4 induced the differentiation of $mIgM^+$ cells to $mIgA^+$ cells and IL-5 drove terminal differentiation of $mIgA^+$ cells; or (2) LPS induced switching of small numbers of $mIgM^+$ cells to $mIgA^+$ cells and IL-4 and

Table 13. Summarization of Cytokines Involved in IgA Regulation

Cytokines	Effects on IgA production	References
IL-1	B and T cells in Peyer' patches	Cowdery *et al.*, 88
IL-2	Enhance switching effect of TGF-β	Coffman *et al.*, 89
IL-4	Increase $mIgA^+$ cells in CH12LX	Kunimoto *et al.*, 88
IL-5	B cells in spleen and PP cells	Murray *et al.*, 87
	LPS-stimulated $mIgA^+$ and $mIgA^-$ cells	Harriman *et al.*, 88 Leman & Coffman, 88
	Enhance switching effect of TGF-β	Coffman *et al.*, 89
IL-6	$mIgA^+$ B cells of Peyer's patches	Beagley *et al.*, 89
TGF-β	Switch $mIgA^-$ to $mIgA^+$ cells	Lebman *et al.*, 90

IL-5 enhanced this clonal expansion.

It has been shown that IL-5 selectively increases IgA secretion in mice. An IL-4- and IL-5-producing autoreactive T-cell line derived from mouse Peyer's patches or its culture supernatant enhances secretion of both IgG1 and IgA (Murray *et al.*, 1987). Purified IL-5 enhances only IgA synthesis when incubated with splenic LPS-triggered B cell blasts. Other studies have shown that supernatant from Th2 clones increases IgA secretion in LPS-stimulated splenic B cells (Coffman *et al.*, 1987; Bond *et al.*, 1987). The purified IgA-enhancing factor (IgA-EF) from cell culture supernatant of Th2 clones was subjected to amino acid sequence and proved to be consistent with murine IL-5 complementary DNA (cDNA; Azuma *et al.*, 1986; Kinashi *et al.*, 1986). IL-5 enhances proliferation and Ig production of B cells activated by antigen or mitogen (Yokota *et al.*, 1988). IL-5 also enhances IgA production only in LPS-treated $mIgA^+$ B cells and increases the number of IgA-secreting cells (Harriman *et al.*, 1988). However, one study has shown that IL-5 enhances IgA secretion in LPS-stimulated $mIgA^-$ B cells (Lebman and Coffman, 1988). Most of the evidence so far suggest that LPS may switch B cell to IgA-producing B cells and IL-5 induces terminal differentiation of IgA-committed B cells to secrete IgA (McGhee *et al.*, 1989).

Transforming growth factor (TGF)-β inhibits Ig synthesis in LPS-stimulated murine B cells, while it increases IgA synthesis in the same culture system (Coffman *et al.*, 1989).

The inhibition of Ig synthesis can be partially reversed and IgA synthesis further increased by IL-2 or IL-5, although IL-2 appears to be more effective than IL-5 (Coffman *et al.*, 1989). TGF-β maybe a switch factor for IgA production since it enhances IgA production by the $mIgA^-$ cells in Peyer's patches (Coffman *et al.*, 1989; Lebman *et al.*, 1990). IL-5 can also augment the effect caused by TGF-β on IgA synthesis of LPS-stimulated B cells, but fails to cause these cells to switch to IgA secreting cells (Sonoda *et al.*, 1989).

Recombinant IL-6 (rIL-6) induces IgA secretion in $mIgA^+$ B cells at the level of 2-4 fold higher than rIL-5 does in a dose-dependent fashion and in both large blast and small resting B cells of Peyer's patches (Beagley *et al.*, 1989). This suggests that IL-6 be more effective than IL-5 in promoting terminal differentiation for cells committed to IgA.

Several studies have shown that Th1 clones alone fail to initiate specific B cell responses (Boom *et al.*, 1988; Killar *et al.*, 1987). However, it is evident that cytokines produced by Th1 clones such as IL-2 and IFN-γ (Leibson *et al.*, 1984; Snapper *et al.*, 1988) can act synergistically with other cytokines such as IL-4 and IL-5 in enhancing production of IgA and other Ig (Lebman and Coffman, 1988; Stevens *et al.*, 1988; Giedlin *et al.*, 1986).

IL-1 may also be involved indirectly in IgA synthesis regulation. Human IL-1α increased IgA secretion in whole Peyer's patch cell cultures with both Peyer's patch B and T

cells; substitution of splenic B or T cells would not work (Cowdery *et al.*, 1988). It is conceivable that IL-1 selectively activated Th2 population to secrete IL-5 and IL-6 which were responsible for the upregulation of IgA.

1.7 Cytokines in Glomerulonephritis

It has been suggested from both *in vitro* and *in vivo* studies that cytokines are mediators of glomerulonephritis (Emancipator and Sedor, 1992). The macrophage is a possible major source of intraglomerular cytokines in glomerulonephritis and systemic immune disorder such as lupus nephritis, especially in diseases featuring leukocytic infiltration (Boswell *et al.*, 1988; Emancipator and Sedor, 1992). Increased IL-1 and TNF transcripts were found in cultured glomerular macrophages, not mesangial cells (Boswell *et al.*, 1988).

The mesangial cell is apparently the major source of intraglomerular cytokines within glomeruli in non-infiltrative glomerulonephritis. Among given cytokines, IL-1 has been heavily studied. Proliferating human and rat mesangial cells produce IL-1 in culture (Lovett and Larsen, 1988; Werber *et al.*, 1987), which can be enhanced by LPS, complement, and the combination of platelet-derived growth factor (PDGF) and epidermal growth factor (Lovett *et al.*, 1986; 1987; 1988). Some studies show that cultured mesangial cells release TNF, IL-6, and IL-8 in response to LPS (Baud *et al.*, 1988; Horii *et al.*, 1989; Kusner *et al.*,

1991). *In vivo*, increased IL-1 mRNA in renal cortex was found in cationized bovine gammaglobulin-induced rat membranous nephropathy (Werber *et al.*, 1987). Increased urinary excretion of IL-1 and TNF was observed in the chronic phase of renal disorders and in the progressive phase of renal failure (Noble *et al.*, 1990). Rifai *et al.* found that infusion of IL-1 or IL-6 increased urinary abnormalities, glomerular proliferation, matrix expansion, and thrombosis in animal model of IgA nephropathy induced by IgA anti-phosphorylcholine antibody and phophorycholine-substituted bovine albumin. Mesangial cells also express receptors for some cytokines in culture (Mené *et al.*, 1989).

PDGF is an autocrine growth factor for mesangial cells. Recently, increased expression of PDGF B-chain mRNA in renal cortex and mesangium was reported in a mouse model of IgA nephropathy (Gesualdo *et al.*, 1991; Iida *et al.*, 1991). The amount of mRNA correlated with the severity of clinical signs such as hematuria and proteinuria and with mesangial cell proliferation and matrix expansion. PDGF is likely an important mediator in glomerulonephritis.

Another important cytokine in glomerulonephritis is TGF-β which induces synthesis and secretion of PDGF and proteoglycan in mesangial cell culture (Border *et al.*, 1990a; Jaffer *et al.*, 1989; Silver *et al.*, 1989). The increase of both TGF-β protein and mRNA was found in glomerulonephritis induced by antibody-mediated mesangial cell damage and the associated increase in proteoglycan and fibronectin

synthesis was inhibitable by anti-TGF-β (Border *et al.*, 1990b; Okuda *et al.*, 1990). TGF-β activity in renal cortex is also increased in anti-GBM nephritis and inhibited by anti-TGF-β (Coimbra *et al.*, 1991). TGF-β mediates the replication rate and synthesis of collagen and fibronectin in cultures of mesangial, endothelial, and epithelial cell lines which are derived from mouse glomeruli and bear TGF-β receptor (MacKay *et al.*, 1989).

1.8 Rationale

Vomitoxin-induced cytokine dysregulation in a cloned cell line such as EL-4 cell line may serve as a model for study of the mechanism of cytokine superinduction and provide evidence for roles of cytokine in vomitoxin-induced IgA dysregulation described in the previous chapter. Cytokine superinduction refers to enhanced cytokine production by stimuli in the presence of strong cytokine inducer such as PMA.

The working hypothesis of this study was that *in vitro* exposure to vomitoxin alters production of cytokines that regulate murine IgA production.

2.0 MATERIALS AND METHODS

2.1 General Experimental Design

The objective of this work was to test the hypothesis that *in vitro* exposure to vomitoxin alters production of cytokines that regulate murine IgA production. The mouse thymoma cell line EL4-IL2 was employed because of its potential capability to produce IL-2, IL-4, IL-5, and IL-6, all of which can contribute to the hyperproduction of IgA. In two experiments, the effect of vomitoxin on induction of IL-2 and IL-5 mRNA by vomitoxin was assessed by Northern analysis of RNA extracts from EL4-IL2 cells treated with the T cell mitogen PMA. Culture supernatants were also assessed for IL-2 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay and for IL-5 by ELISA.

2.2 Preparation of cDNA Probes

2.2.1 *cDNA clones*

cDNA clones for murine IL-2 (pMIL-2-14) and IL-5 (pSP6K-mTRF23) were kindly provided by Drs. Akira Tominaga and Kiyoshi Takatsu (Kumamoto University Medical School, Japan). The detailed cloning methods for IL-2 and IL-5 cDNA were described by Kashima *et al.* (1985) and Kinashi *et al.* (1986), respectively. cDNA clone for rat glyceraldehyde-3-

phosphate-dehydrogenase (GAPDH) was kindly provided by Dr. Richard C. Schwartz (Department of Microbiology and Public Health, Michigan State University). The detailed cloning method was described by Piechaczyk *et al.* (1984). IL-2 cDNA was constructed at *Eco*R1 site of plasmid pBR322, a 586 bp fragment from *Pvu*II/*Acc*I digest was used as IL-2 cDNA probe. IL-5 cDNA was inserted at *Bam*HI site of plasmid pUC18, a 461 bp fragment from *Sac*I/*Acc*I digest was used as IL-5 cDNA probe. rGAPDH cDNA prepared by the double-tailing procedure was annealed to oligo-dG tailed plasmid pBR322, a 1 Kb fragment from *Pst*I digest was used as the GAPDH cDNA probe.

2.2.2 *Preparation of competent cells*

One clone of Strain DH5α of *Escherichia coli* (GIBCO BRL, Gaithersburg, MD) was inoculated into 10 ml of autoclaved L-broth (LB; 1% Bacto [DIFCO] tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, and pH 7.4) in a sterile glass tube, which was then incubated in a rotary incubator (New Brunswick) with very gentle rotation at 37°C overnight. The entire LB culture was inoculated into one liter of LB in a sterile 4-liter flask, which was then incubated at 37°C with vigorous shaking on a Gyrotory Shaker until an OD_{600} between 0.5-1.0 was attained (approximately 4 hr). The flask was chilled on ice for 15-30 min. Cell pellets were obtained by centrifugation in a refrigerated Sorvall GSA rotor (Du Pont, Wilmington, DE) at 4,000*g* for 15 min at 4°C and resuspended in a total of one liter cold sterile water. Centrifugation was repeated and pellets were resuspended in a total of 0.5

liter of 4°C sterile water. Centrifugation was repeated and the pellets were resuspended in 20 ml of 4°C 10% glycerol (Mallinckrodt, Paris, KY). The centrifugation was repeated once more and the pellet was resuspended to a final volume of 2-3 ml of 4°C 10% glycerol, yielding an approximate cell concentration of 1-3 × 10^{10} cells/ml. Cell suspension (100 μl aliquots) could be frozen on dry ice in acetone and stored at -70°C for at least six months (Dower *et al.*, 1988; Taketo, 1988).

2.2.3 *Electroporation*

DH5α cells were gently thawed at room temperature and placed on ice. In a cold 0.5-ml sterile microcentrifuge tube, 40 μl of the cell suspension were well mixed with about 1 ng in 1-2 μl of plasmid DNA solution constructed with appropriate cDNA in sterile TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA). The mixture was held on ice for 0.5-1 min and transferred to a ice-cold 0.1-cm electroporation cuvette. Electroporation was conducted in a Gene Pulser apparatus (BIO-RAD, Richmond, CA) at 25 μF, 1.65 kV, and 200 Ω , which produced a time constant of 4-5 msec and a field strength of 12.5 kV/cm. One microliter of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was immediately added to the cuvette and cells were quickly resuspended. The suspension was transferred to a 15-ml sterile tube and incubated in a rotating incubator at 225 rpm at 37°C for 1 hr. One hundred microliters of the above

solution were spread on LB plates containing ampicillin (selective for IL-2 and IL-5) or tetracycline (selective for rat GAPDH). LB plate consists of autoclaved 1.5% Bacto agar in LB with ampicillin added to 50 μg/ml or tetracycline added to 15 μg/ml. The plate was then sealed with parafilm (American National Can, Greenwich, CT) and incubated at 37°C for 12-24 hr. Single colonies were selected from growth plate and streaked on another plate with appropriate antibiotics. The plate was incubated at 37°C for 12-24 hr to yield isolated colonies (Fiedler and Wirth, 1988).

2.2.4 *Minipreparations of cDNA*

A sterile LB medium tube (5 ml) with appropriate antibiotics was inoculated with a single colony from the above plate and incubated at 37°C overnight with vigorous shaking. A pellet was obtained by centrifuging 1.5 ml of the above culture solution at 14,000*g* in a sterile micro-centrifuge tube for 1 min at 4°C and resuspended by vortexing in 100 μl of ice-cold GET solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, and pH 8.0). Two hundred microliters of freshly prepared 0.2 N NaOH with 1% SDS were added, mixed by rapidly inverting the tube 2-3 times, and stored on ice for 5 min. One hundred and fifty microliters of ice-cold potassium acetate solution (60 ml 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml H_2O, and pH 4.8) were added, vortexed gently in an inverted position for 10 sec, and stored on ice for 5 min. The supernatant was obtained by centrifugation at 10,000*g* for 5 min at 4°C,

transferred to a fresh and sterile 1.5-ml microcentrifuge tube. An equal volume of phenol/chloroform solution was added and mixed by vortexing. A transparent layer of supernatant was obtained by centrifugation at 14,000*g* for 2 min at 4°C and transferred to a fresh and sterile 1.5-ml microcentrifuge tube. Two volumes of ethanol (Quantum Chemical Co., Tuscola, IL) were added, mixed by vortexing, and stored at room temperature for 2 min. A pellet was obtained by centrifugation at 14,000*g* for 5 min at room temperature, washed in 1 ml of 70% ethanol by vortexing briefly. The pellet was again obtained by centrifugation at 10,000*g* for 2 min, air-dried at room temperature, and resuspended in TE containing DNAse-free pancreatic RNAse (20 μg/ml). The DNA was used to restriction digestion and subsequent gel electrophoresis (Sambrook *et al.*, 1989).

2.2.5 *Restriction digestion and gel electrophoresis*

DNA was digested by appropriate restriction enzyme(s) (Boehringer Mannheim Co., Indianapolis, IN) in corresponding restriction buffer(s) (Boehringer Mannheim) at 37°C for 1-2 hr/each digestion. In digested DNA sample, 6× loading buffer (0.25% Bromphenol Blue [BPB], 40% [w/v] sucrose, 6 mM EDTA, 60 mM Tris, and stored at 4°C) was added in one sixth of the digestion volume. The DNA sample and a λ-*Hind*III marker (200 ng) for molecular weight were loaded on 1% ultrapure agarose (GIBCO BRL) gel with TAE (0.04 M Tris-acetate and 0.001 M EDTA) as running buffer and ethidium bromide (0.5 μg/ml, Sigma) as DNA staining reagent. The

molecular weight and restriction pattern of the digested DNA fragments were observed under UV light (302 nm; Ultra-violet Products Inc., San Gabriel, CA).

2.2.6 *Large-scale preparation of plasmid DNA*

A single bacterial colony from Section 2.2.2 was inoculated into a sterile glass tube containing 10 ml of autoclaved TB medium (Terrific Broth: 1.2% bacto-tryptone, 2.4% bacto-yeast extract, 0.4% glycerol, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4) with appropriate antibiotics. The tube was incubated at 37°C overnight with vigorous shaking. One hundred microliters of the culture were inoculated into 25 ml of autoclaved sterile TB in a 100-ml sterile flask and incubated at 37°C with vigorous shaking until OD_{600} reached approximately 0.6. All 25 ml of the culture were inoculated into 500 ml of autoclaved TB with appropriate antibiotics (prewarmed to 37°C) in a 2-liter sterile flask. The flask was then incubated at 37°C for 15-18 hr with vigorous shaking (300 rpm).

The pellet was obtained by centrifugation at 2,600*g* for 15 min at 4°C in a Sorvall GSA rotor (Du Pont), resuspended in 18 ml of autoclaved Solution I (50 mM glucose, 25 mM Tris-Cl [pH 8.0], 10 mM EDTA [pH 8.0]). Two milliliters of freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris-Cl [pH 8.0]), 40 ml of freshly prepared Solution II (0.2 N NaOH [freshly diluted from a 10 N stock] and 1% SDS) were added, mixed by gently inverting several times, and stored at room temperature for 5-10 min. Twenty milliliters of

ice-cold potassium acetate solution were added, mixed by shaking several times until two distinguishable liquid phases no longer existed, and stored on ice for 10 min. A flocculent white precipitate was formed.

The supernatant was harvested by centrifugation at 27,000*g* for 20 min at 4°C in a SS-34 rotor (Du Pont), transferred to a fresh sterile SS-34 tube, mixed with 0.6 volume of isopropanol, and stored at room temperature for 10 min. The nucleic acid pellet was obtained by centrifugation at 3,000*g* for 15 min at room temperature, rinsed with 70% ethanol, and dissolved in 8 ml of TE in each microcentrifuge tube by incubating in 45°C water bath for 50-60 min.

Eight grams of solid CsCl (Boehringer Mannheim Co.) were added (exactly 1 g/ml), mixed, and dissolved by incubating in 30°C water bath. Every 10 ml of the DNA/CsCl solution was mixed with 0.8 ml of ethidium bromide (10 mg/ml). The final density of the solution was confirmed to be 1.55 g/ml by weighing on an analytical balance. The solution was transferred to 11.5-ml ULTRACRIMP tubes (Du Pont) which were carefully balanced, properly sealed by ULTRUCRIMP tube sealer (Du Pont), and ultracentrifuged at 150,600*g* for 36-48 hr at 20°C in a Sorvall T-865.1 rotor (RC60, Sorvall Instruments, Du Pont). Sterile hypodermic needles (21 gauge, Becton Dickinson, Rutherford, NJ) and 3-ml plastic syringe (Becton Dickinson) were used to collect closed circular plasmid DNA from CsCl gradients under long wave UV light (366 nm). An equal volume of isoamyl alcohol (SIGMA)

was added to extract ethidium bromide from the collected DNA solution. The solution was mixed by vortexing, centrifuged at 1,400*g* for 1-2 min at room temperature, and transferred to a fresh sterile 15-ml tube. The extraction was repeated until all the pink color disappeared from both the aqueous phase and the organic phase. The DNA solution was diluted in three volumes of TE, precipitated in four volumes of ethanol overnight, and centrifuged at 4°C on an SS34 rotor at 10,000*g* for 15 min. The precipitated DNA was dissolved in approximately 500 μl of TE and stored at -20°C. OD_{260}/OD_{280} fell into the range of 1.8-2.0 and the DNA concentration was calculated by the following equation: 50 × OD_{260} × dilution factors (μg/ml) (Sambrook *et al.*, 1989).

2.2.7 *Gel purification of cDNA*

The above DNA was digested by appropriate restriction enzymes in corresponding restriction buffers at 37°C for 1-2 hr and purified on 0.8% ultrapure agarose gel with TAE as running buffer and λ-*Hind*III (GIBCO BRL) as marker for molecular weight. Gels were then stained with ethidium bromide (0.5 μg/ml) in distilled water for 20 min with gentle shaking. The appropriate band was excised using a razor under UV light (366 nm) and placed into sterile 1.5-ml microcentrifuge tubes.

Gel in the tube was dissolved in three volumes of Prep-A-Gene binding buffer (Bio-Rad) in a 37-55°C water bath. Prep-A-Gene binding matrix (5 μl/μg DNA to be purified, Bio-Rad) was added, mixed by manually inverting tubes for 5 min,

incubated at room temperature for 10 min, and washed 3 times with binding buffer and 3 times with washing buffer (1 ml each time) by vortexing and centrifuging at 14,000*g* for 30 sec. cDNA was eluted twice from binding matrix by 20 μl of TE in 37-50°C for 5 min each time.

The cDNA concentration in TE was determined by gel electrophoresis. cDNA sample was loaded on 1% agarose gel with 6× loading buffer along with 200 ng λ-*Hind*III marker and 0.5 μg/ml ethidium bromide. The intensities of cDNA and λ-*Hind*III marker bands were compared and cDNA concentration was calculated accordingly (Sambrook *et al.*, 1989).

2.3 EL4-IL2 Cell Culture and Mitogen PMA

EL4-IL2 (ATCC) is a murine cytokine-producing thymoma cell line that produces IL-2, IL-4, IL-5, and IL-6. Cells were cultured in 75 cm^2 culture flask (Corning Inc., Corning, NY) at 37°C with 5% CO_2 concentration and maintained in 10% DMEM. Culture medium was replaced twice per week to maintain a cell density between 1-2 × 10^6 cells/ml. Cells were cultured in 20 ml of 1% RPMI 1640 (without 2-mercapto-ethanol) per flask.

Cytokine production was induced by adding 10 ng/ml PMA (Lasek *et al.*, 1989). Stock PMA with was prepared by dissolving 1 mg of PMA in ethanol to a concentration of 500 μg/ml in a glass vial covered with aluminum foil and stored at -20°C. (Warning: Plastic container should not be used for the storage of concentrated PMA and PMA is sensitive to

light!) A 1:1,000 dilution was made in culture medium immediately prior to the experiment. Twenty microliters of diluted PMA were added per milliliter of culture medium. The final concentration in cell culture medium was 10 ng/ml.

2.4 RNA Extraction from Cell Culture

Cells and supernatant in 20 ml culture medium were harvested by centrifugation at 450*g* for 10 min. The cell pellet was homogenized in 1 ml RNA STAT-60, a RNA isolation reagent (TEL-TEST "B", Friendswood, TX), using 22G needle (Becton Dickinson) and 1-ml syringe (Becton Dickinson). The cell homogenate was incubated at room temperature for 5 min and mixed with 0.2 ml of chloroform (J. T. Baker Inc., Phillipsburg, NJ) by vigorously vortexing. The mixture was incubated at room temperature for 2-3 min, centrifuged at 4°C at 12,000*g* for 15 min. The upper colorless aqueous phase was transferred to a free sterile 1.5-ml tube with screw-cap, mixed with 0.5 ml of isopropanol (J. T. Baker) by vortexing, and incubated at room temperature for 5-10 min. A white RNA pellet was obtained after centrifugation at 12,000*g* for 10 min at 4°C and washed with 1 ml of 75% ethanol by vortexing briefly. The RNA was pelleted again by centrifugation at 7,500*g* for 5 min at 4°C, air-dried, and dissolved in 50 μl sterile DEPC-treated distilled water in a water bath of 55-60°C for 10-15 min. RNA concentration was determined in a spectrophotometer using the equation: 40 × OD_{260} × dilution factor. RNA extracts were stored at -80°C

(Chomczynski and Sacchi, 1987; Kedzierski and Porter, 1991; Sambrook *et al.*, 1989).

2.5 Northern Analysis

Two sets of RNA samples were prepared by mixing 10 μg of total RNA in 7.5 μl distilled water with 25.8 μl of fresh prepared Premix solution (12.9% of 10× MOPS [SIGMA], 22.6% of 37% formaldehyde [J. T. Baker], and 64.5% of formamide [Boehringer Mannheim]) by vortexing and incubated in 55°C water bath for 15 min. The second set included a 0.24-9.49 Kb RNA Ladder (GIBCO BRL) for ethidium bromide staining. Each RNA sample was mixed with 6.7 μl of formaldehyde loading buffer (0.25% BPB, 0.25% xylene cyanol, 15% Ficoll 400, and stored at room temperature) by vortexing and loaded on a 1.2% ultrapure agarose gel with 1% formaldehyde and 1× morpholinopropanesulfonic acid (MOPS; 0.4 M MOPS [pH 7.0], 100 mM sodium acetate [J. T. Baker], 10 mM EDTA [pH 8.0, Boehringer Mannheim]) as running buffer. Gel was run on duplicate sets of RNA samples in a DNA Sub Cell (Bio-Rad) at a constant voltage of 75 V for 3.5 hr in a fume hood.

The duplicate set of the gel was excised out, rinsed several times with distilled water, stained with 0.5 μg/ml ethidium bromide in distilled water with shaking for 45 min, destained in fresh distilled water overnight at 4°C, and observed under UV light. Loading equivalence was verified by comparing the staining intensity of 18S and 28S ribosomal RNA of each sample and the migration of RNA ladder molecular

weight standards.

The remaining portion of the gel was rinsed several times with distilled water and washed with 10× SSC (8.8% sodium chloride, 4.4% sodium citrate, and pH 7.0) on a shaker for 45 min. A piece of pre-wetted nitrocellulose membrane (Stratagene, La Jolla, CA) was placed on the gel which was lying on a piece of chromatography paper (Whatman 3 mm, Whatman International Ltd., Maidstone, England) which was wider than the gel and long enough to extend into both chambers from the platform of an electrophoresis apparatus. Four layers of chromatography paper and a stack of paper tower were placed on top of the nitrocellulose. Parafilm was placed along the edges of the nitrocellulose and smaller sizes of chromatography paper and paper tower were used to prevent overhanging nitrocellulose membrane. Both chambers were filled with 20× SSC. A glass plate and a relatively heavy object were pressed on top of paper towers. Transfer of RNA from agarose gel to nitrocellulose membrane was carried out overnight.

The nitrocellulose membrane was oriented, carefully removed from the gel, air-dried, wrapped into an envelop of chromatograph paper, and baked in an oven (Precision Scientific Co., Chicago, IL) at 80°C for 2 hr under vacuum. The baked nitrocellulose membrane was pre-wetted and placed into a long incubation tube which contained 25 ml of prehybridization buffer (50% formamide, 5× Denhardt's reagent [0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, and

0.1% BSA], 0.1% SDS, 5× SSPE, 100 μg/ml Herring Sperm (HS) DNA [Boehringer Mannheim; denatured for 10 min at 100°C and placed on ice prior to use]). The tube was incubated in a Hybridization Incubator 310 (Robins Scientific, Sunnyvalc, CA) with rotation at 42°C for at least 2 hr.

Twenty-five nanograms of cDNA probe in a 1.5-ml sterile tube with screw cap were denatured by heating at 100°C for 10 min and subsequent cooling on ice. cDNA solution was mixed with 1 μl of dATP, 1 μl of dGTP, 1 μl of dTTP, 2 μl of reaction mixture, 5 μl of [α^{32}P] labeled dCTP (NEN Research Products, Boston, MA), 1 μl of Klenow enzyme (Random Primed DNA Labeling Kit, Boehringer Mannheim), and incubated in a 37°C water bath for 30 min. Labeled cDNA was separated on a Nuctrap Push Columns (Stratagene) by mixing the cDNA solution with 50 μl TE, passing through the column which was washed by another 70 μl TE , and finally collecting labeled cDNA in a fresh 1.5-ml sterile tube with screw cap. One microliter labeled cDNA solution was mixed with 5 ml of cocktail and tested on a scintillating counter (Packard Instrument Co., Downers Grove, IL). Specific activities were generally greater than 1 × 10^9 dpm/μg DNA.

Labeled cDNA was denatured by heating at 100°C for 10 min and subsequent cooling on ice prior to adding into an incubation tube in which prehybridization buffer was replaced by hybridization buffer (50% formamide, 2.5× Denhardt's reagent, 0.1% SDS, 5× SSPE, 100 μg/ml denatured HS DNA). The tube was then incubated in hybridization

chamber at 42°C overnight.

The nitrocellulose blot was washed twice in 250 ml of 6× SSPE with 0.1% SDS at room temperature for 15 min using an American Rotator V and twice in 250 ml of 0.1× SSPE with 0.1% SDS at 65°C in a Cyrotory water bath shaker (New Brunswick Scientific, Edison, NJ) for 15 min. The blot was wrapped with Saran wrap and exposed to Kodak XOMAT-AR film (Eastman KODAK Co., Rochester, NY) at -80°C for 1-7 days.

Probes on the blot were removed by washing in boiled 0.1% SDS and the blot was re-used. Band intensity was determined by an image analyzing system. A series of bands generated by consecutive 2-fold dilutions of RNA and probed with a [^{32}P] labeled PCR product were used as standard curve (Sambrook *et al.*, 1989).

2.6 MTT Assay for IL-2

IL-2 production in EL4-IL2 culture supernatant was detected by MTT bioassay (Mosmann *et al.*, 1983). CTLL-2 cell line (ATCC) is a cytotoxic T cell line and IL-2 dependent. Cells were maintained in 10% RPMI 1640 with 1 unit/ml recombinant human IL-2 (ICN Biochemicals, Costa Mesa, CA) in 75-cm^2 tissue culture flask. Culture medium was replaced twice per week to ensure that cell number did not exceed 1 × 10^5 cells/ml or cells would rapidly deplete and lose viability.

Cells used for the experiment were always cultured at least two days after they were split. Cells were harvested

by vigorously swirling flasks to obtain the adherent cells and centrifuging at 450*g* for 10 min. Cells were washed twice with Hanks medium (SIGMA) and cell number was adjusted to 5 × 10^5 cells/ml in RPMI 1640. Both standard and sample supernatant (200 μl) were mixed with lyophilized anti-IL-4 antibody (25 μl) produced by the cloned cell line 11B11 (ACTT) prior to assay. Fifty microliters of the above cells were added to a 96-well plate (Corning), mixed with 50 μl either IL-2 standard or sample supernatant, and incubated at 37°C overnight (about 20 hr). Ten microliters of 5 mg/ml MTT were added into each well and the plate was incubated for an additional 4-8 hr at 37°C. Culture supernatant was carefully discarded. One hundred microliters of acidified isopropanol (0.04 N HCl) were added to each well and mixed with cells by pipeting to dissolve formazan crystals. The plate was read on V max kinetic microplate reader (Molecular Devices) at 570-630 nm. Data were processed by built-in four-parameter program.

2.7 ELISA for IL-5

IL-5 production in EL4-IL2 culture supernatant was quantitated by ELISA. Wells of Immulon II Removawell microtiter strips (Dynatech Laboratories) were coated by overnight incubation at 4°C with 100 μl of IL-5 capture antibody (Phar Mingen, San Diego, CA) diluted in 0.01 M PBS. Coated plate was washed six times with 0.1 M PBS containing 0.2% Tween 20. To reduce nonspecific binding, 300 μl of 1%

(w/v) BSA in PBS was added to each well. Plate was then incubated at 37°C for 30 min and washed six times with PBS-Tween. Standard IL-5 was diluted in 1% BSA-PBS, and 50 µl of IL-5 standard or samples were added to appropriate wells. Plate was covered and incubated at 37°C for 1 hr and then washed six times. One hundred microliters of biotinylated IL-5 detection antibody (Phar Mingen) diluted in 1% BSA-PBS to 2.0 µg/ml were added to each well. Plate was incubated at room temperature for 1 hr and washed six times. Seventy-five microliters of streptavidin-HRP (Phar Mingen) diluted in 1% BSA-PBS to 1.5 µg/ml were added to each well. Plate was incubated at room temperature for 1 hr and washed ten times. One hundred microliters of ABTS substrate (1 mg/ml in 44 mM dibasic sodium phosphate, 28 mM citric acid, and 0.003% hydroxide peroxide) were added to each well. Absorbance of bound peroxidase was measured at 405 nm on a V max kinetic microplate reader (Molecular Devices). Data were analyzed by built-in four-parameter program (Mosmann *et al.*, 1990; Schumacher *et al.*, 1988).

2.8 Statistical analysis

Pearson's correlation coefficient, linear regression, random blocked ANOVA, and lsd test were applied using the Microcomputer Statistical Program (MSTATC).

3.0 RESULTS

3.1 Effect of Vomitoxin on Induction of IL-2 and IL-5 mRNA in EL4-IL2 Cells

Induction of IL-2 and IL-5 mRNA was examined at 24 and 48 hr, respectively, to evaluate the effect of stimulation by PMA on the induction of cytokine mRNA by vomitoxin *in vitro*. PMA increased both IL-2 and IL-5 mRNA, but not GAPDH mRNA (Fig. 17). In the presence of PMA, vomitoxin (50 ng/ml) superinduced IL-2 mRNA after 24 hr incubation and IL-5 mRNA after 48 hr incubation, while no GAPDH mRNA induction by vomitoxin was found.

The effect of time on IL-2 and IL-5 mRNA induction in the presence of PMA and 50 ng/ml vomitoxin was assessed. IL-2 mRNA was barely seen at 4 hr, superinduced by vomitoxin at 8 and 12 hr, and diminished after 24 and 48 hr of incubation (Fig. 18). IL-5 mRNA gradually increased from 4 to 12 hr, decreased at 24 hr, and increased again at 48 hr. IL-5 mRNA was superinduced by vomitoxin at 12, 24, and 48 hr. No changes were observed in GAPDH mRNA.

A dilution series of 18S RNA intensity was measured to obtain a standard curve for evaluating fold increase of autoradiography intensities of IL-2, IL-5, and GAPDH mRNA (Fig. 19). When normalized against the intensity of GAPDH

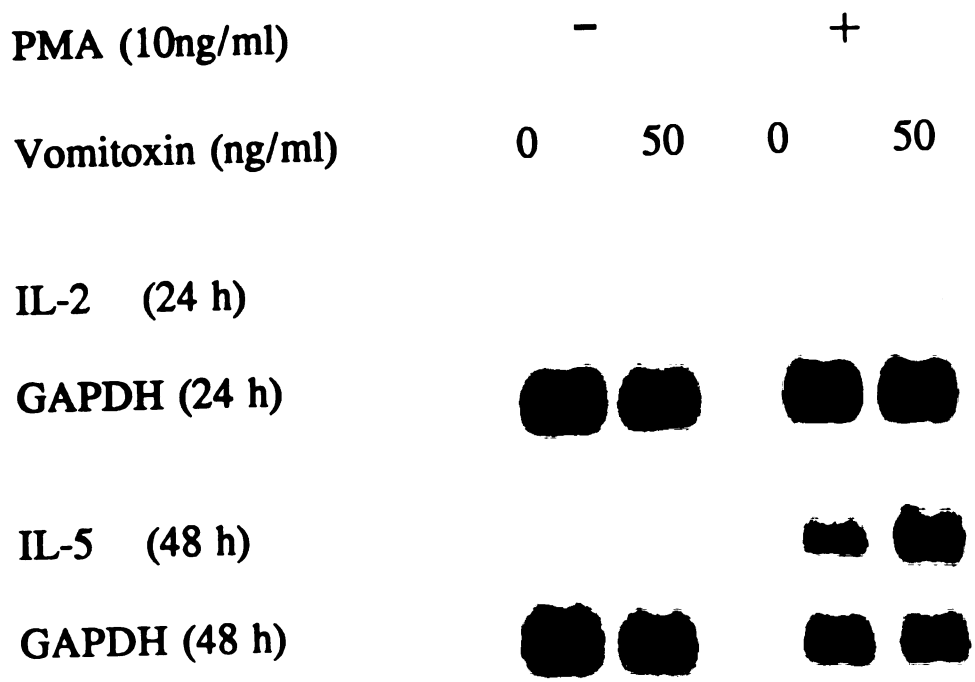

Figure 17. Effect of PMA and vomitoxin on IL-2 and IL-5 mRNA induction. EL4-IL2 cells were treated with or without 10 ng/ml PMA and with or without 50 ng/ml vomitoxin for 24 or 48 hr. 10 μg of cellular RNA extracted were hybridized subsequently in Northern analysis by murine IL-2, IL-5, and rat GAPDH cDNA probes labeled with [^{32}P]. Results are representative of two separate experiments.

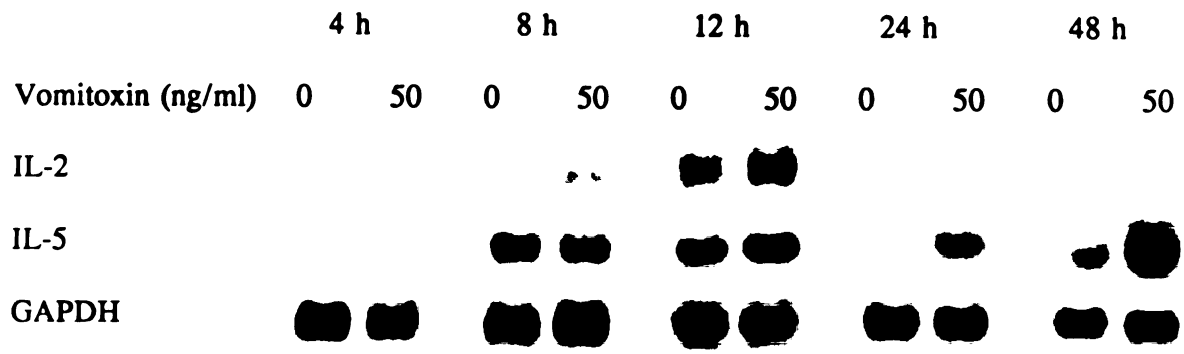

Figure 18. Time course of IL-2 and IL-5 mRNA induction in the presence and absence of vomitoxin. EL4-IL2 cells were treated with 10 ng/ml PMA and 0 or 50 ng/ml vomitoxin and incubated 4, 8, 12, 24, and 48 hr. 10 μg of cellular RNA extracted were hybridized subsequently in Northern analysis by murine IL-2, IL-5, and rat GAPDH cDNA probes labeled with [^{32}P]. Results are representative of two separate experiments.

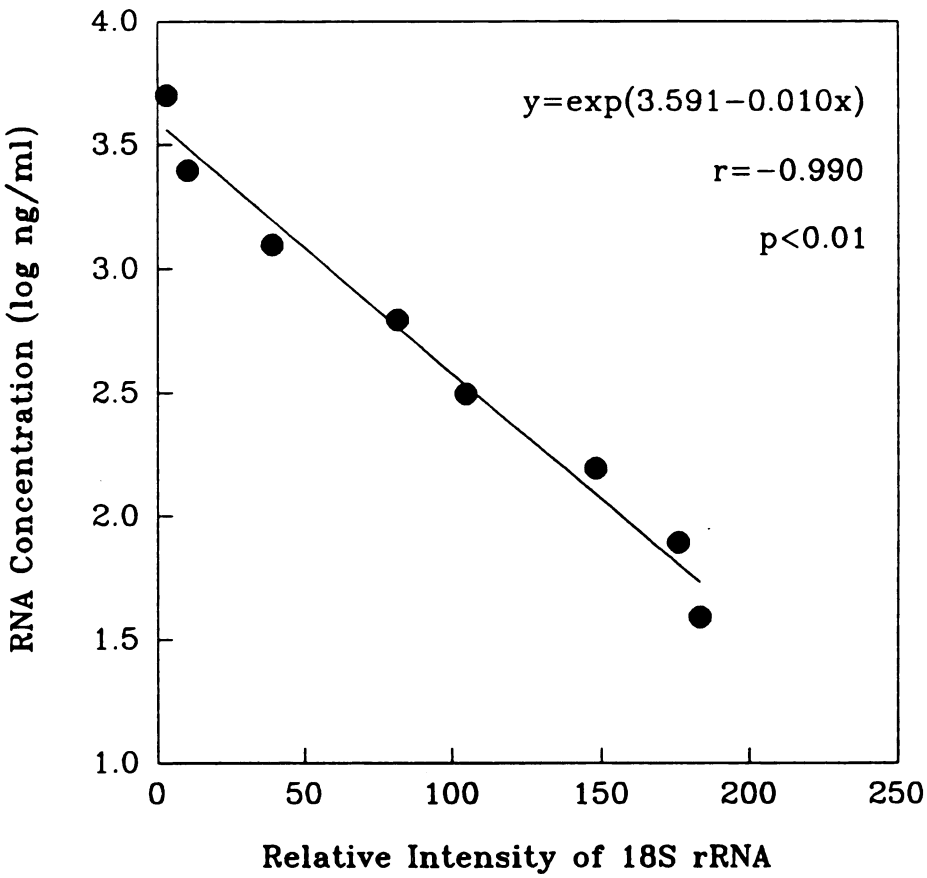

Figure 19. Standard curve for quantitative analysis of band intensity measured by computer-aided image analysis device in Northern analysis. A 10 μg/ml RNA sample was diluted in 2 fold sequence and hybridized with a [^{32}P] labeled PCR product. The intensities of 18S bands from non-specific binding were measured quantitatively by a computer-aid image analysis device which reads from the most darkness to the most brightness on a 0-255 scale basis. A linear correlation between relative intensity of 18S RNA and log transformed RNA concentration was found.

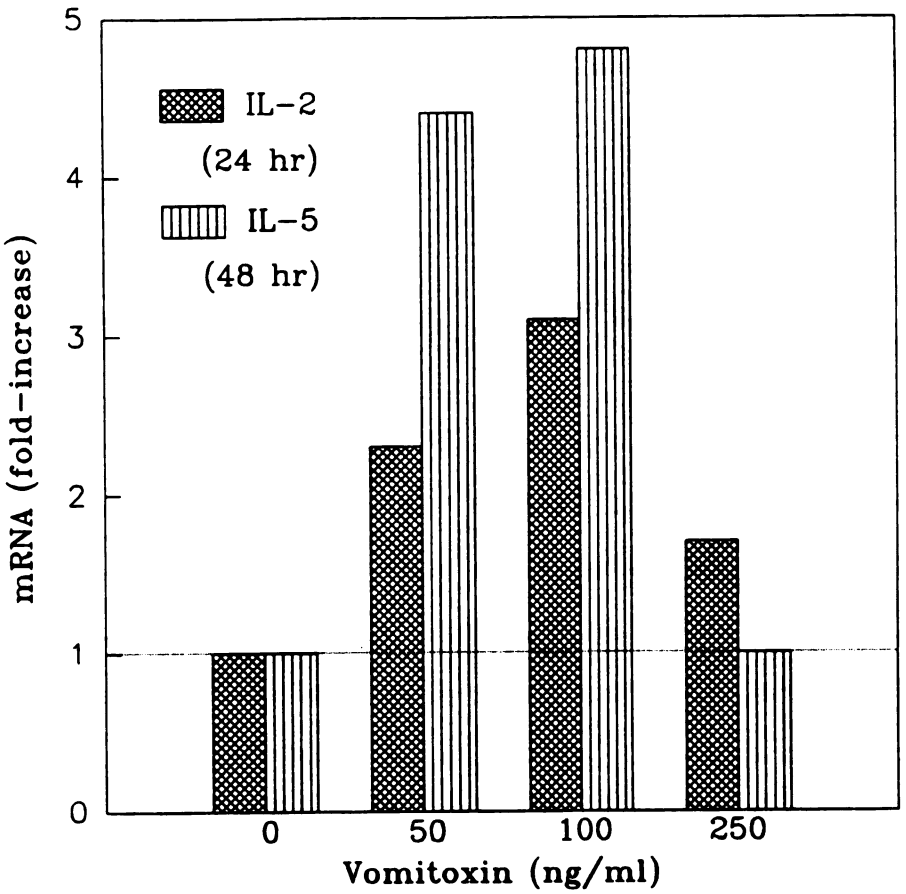

Figure 20. Effect of vomitoxin on IL-2 and IL-5 mRNA superinduction. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 24 or 48 hr. 10 μg of cellular RNA extracted were hybridized subsequently in Northern analysis by murine IL-2, IL-5, and rat GAPDH cDNA probes labeled with [^{32}P]. Band intensities of IL-2 and IL-5 were measured by a computer-aid image analysis device which reads from the most darkness to the most brightness on a 0-255 scale basis and normalized against corresponding band intensities of rat GAPDH mRNA. Corresponding concentrations were found from a standard curve (Fig. 19). Fold-increase was calculated on the basis of control groups (0 ng/ml vomitoxin). Results are representative of two separate experiments.

mRNA, a 2-3 fold increase in IL-2 mRNA after 24 hr of incubation and 4-5 fold increase in IL-5 mRNA after 48 hr of incubation were found after induction by 50-100 ng/ml vomitoxin treatment in a dose-response analysis (Fig. 20). Both IL-2 and IL-5 mRNA levels were similar at 0 and 250 ng/ml vomitoxin.

3.2 Effect of Vomitoxin on IL-2 and IL-5 Production by EL4-IL2 Cells

To assess the effects of *in vitro* vomitoxin treatment on cytokine secretion of cloned T cell line, IL-2 and IL-5 in EL4-IL2 culture supernatants were detected by MTT bioassay and ELISA, respectively. IL-2 and IL-5 levels were determined in culture supernatant from the same cultures that IL-2 and IL-5 mRNA were tested. IL-2 production in the presence of 50 ng/ml vomitoxin was increased early at 12 hr of incubation (Fig. 21). IL-5 production in the presence of 50 ng/ml vomitoxin was only increased slightly at 12 hr of incubation (Fig. 22), but was significantly stimulated by both 50 and 100 ng/ml of vomitoxin after 24 and 48 hr of incubation.

To confirm the above results, 6 independent EL4-IL2 cell populations were treated with 0, 50, 100, and 250 ng/ml vomitoxin and the production of IL-2 and IL-5 in culture supernatant was detected after 2 and 8 days of incubation. IL-2 production was significantly increased early in 2-day culture supernatant in the 50 and 100 ng/ml vomitoxin groups

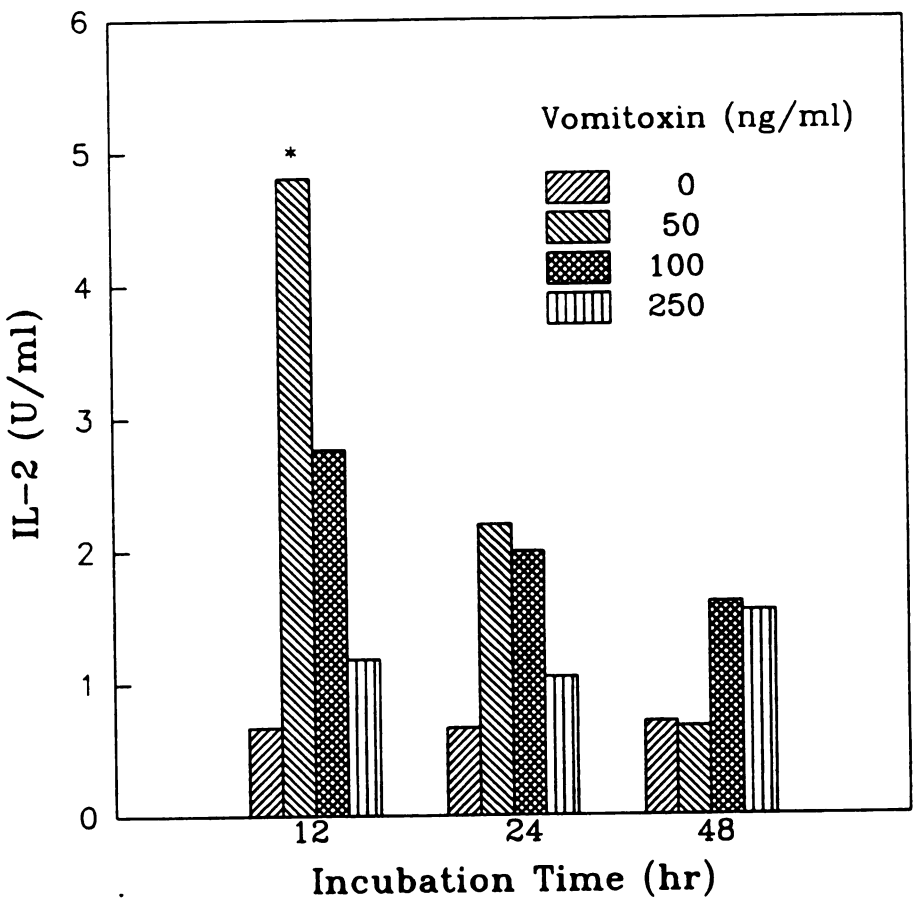

Figure 21. Effect of vomitoxin on IL-2 production in EL4-IL2 cells. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 12, 24, or 48 hr. IL-2 production in culture supernatant was determined by MTT bioassay using IL-2 dependent CTLL-2 cell line. Results are averages of two separate experiments. Random blocked ANOVA and lsd tests were applied. * indicate significant difference at 0.05 level.

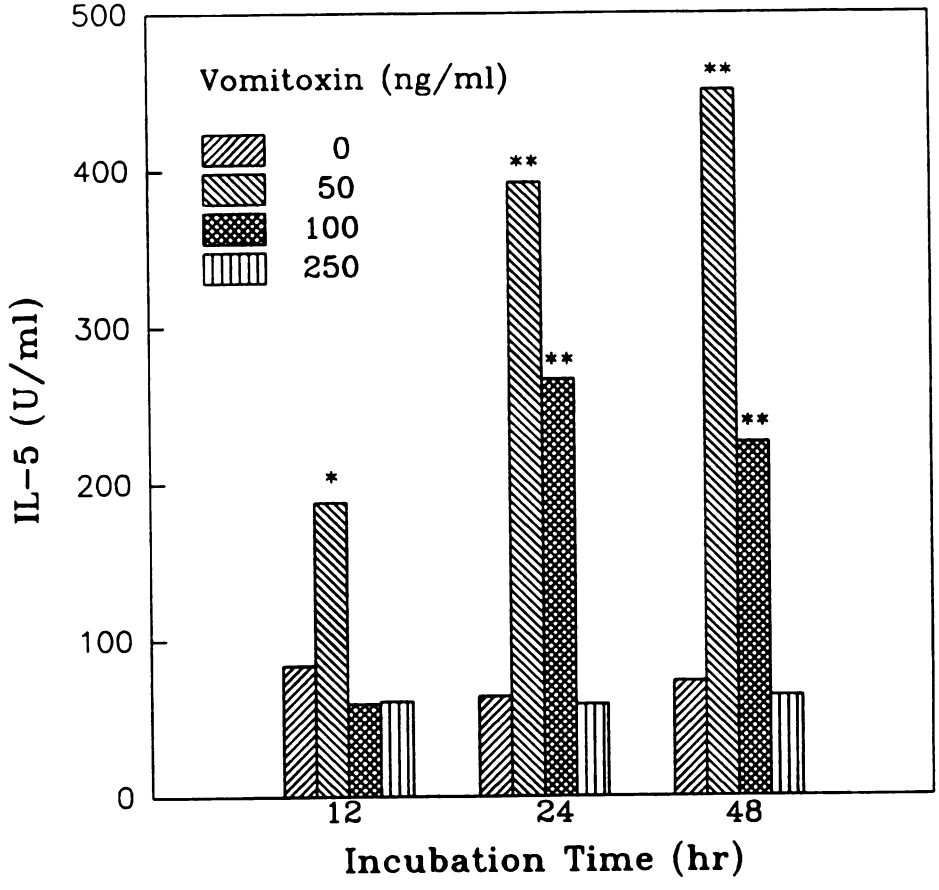

Figure 22. IL-2 production induced by vomitoxin in 2-day culture. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 12, 24, or 48 hr. IL-5 production in culture supernatant was detected by sandwich ELISA using capture and biotinylated goat anti-mouse IL-5 antibodies. Results are averages of two separate experiments. Random blocked ANOVA and lsd tests were applied. * indicate significant difference at 0.05 level. ** indicate significant difference at 0.01 level.

(Fig. 23). IL-5 production was slightly increased in the 50 ng/ml vomitoxin treatment group, but appeared to be slightly inhibited in 100 and 250 ng/ml vomitoxin treatment in 2-day culture supernatant (Fig. 24). In contrast, IL-5 production was significantly increased in 50 ng/ml and sightly increased in 100 ng/ml vomitoxin treatment groups later in 8-day culture supernatants (Fig. 25).

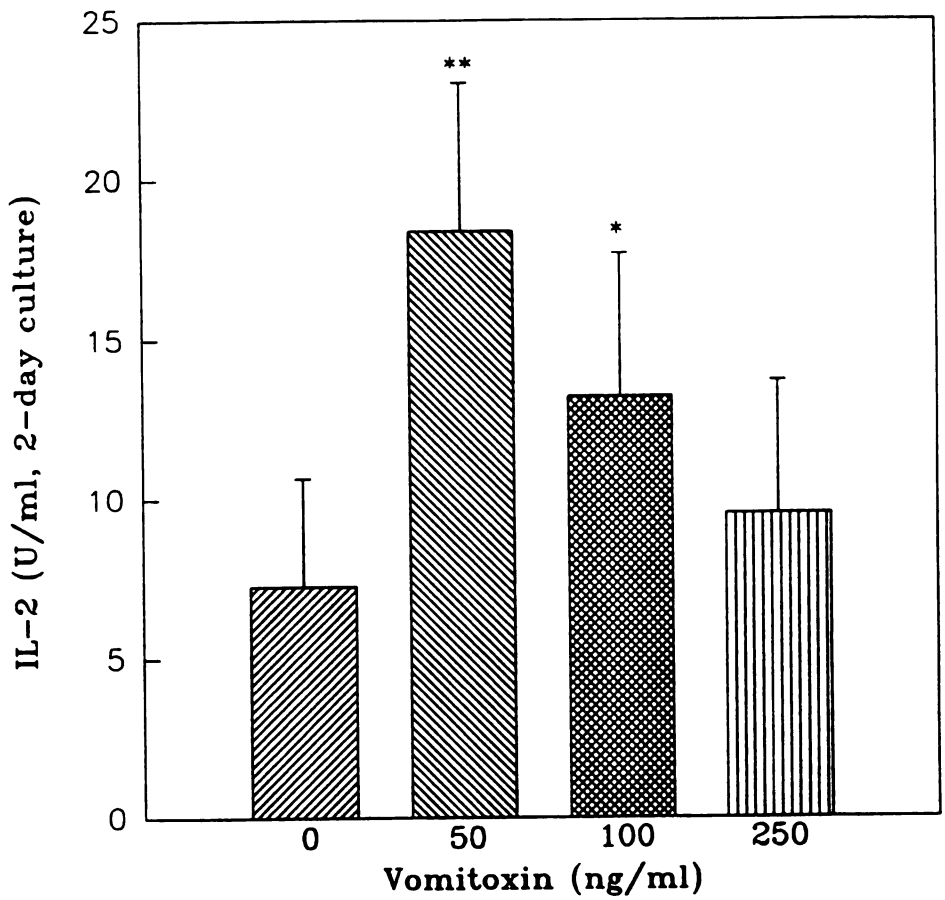

Figure 23. Effect of vomitoxin on PMA-induced IL-2 production in 2-day EL4-IL2 culture. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 2 days. IL-2 production in culture supernatant was determined by MTT bioassay using IL-2 dependent CTLL-2 cell line. Results are averages of six separate experiments. Data are means ± SE (n=6). Random blocked ANOVA and lsd tests were applied. * indicate significant difference at 0.05 level. ** indicate significant difference at 0.01 level.

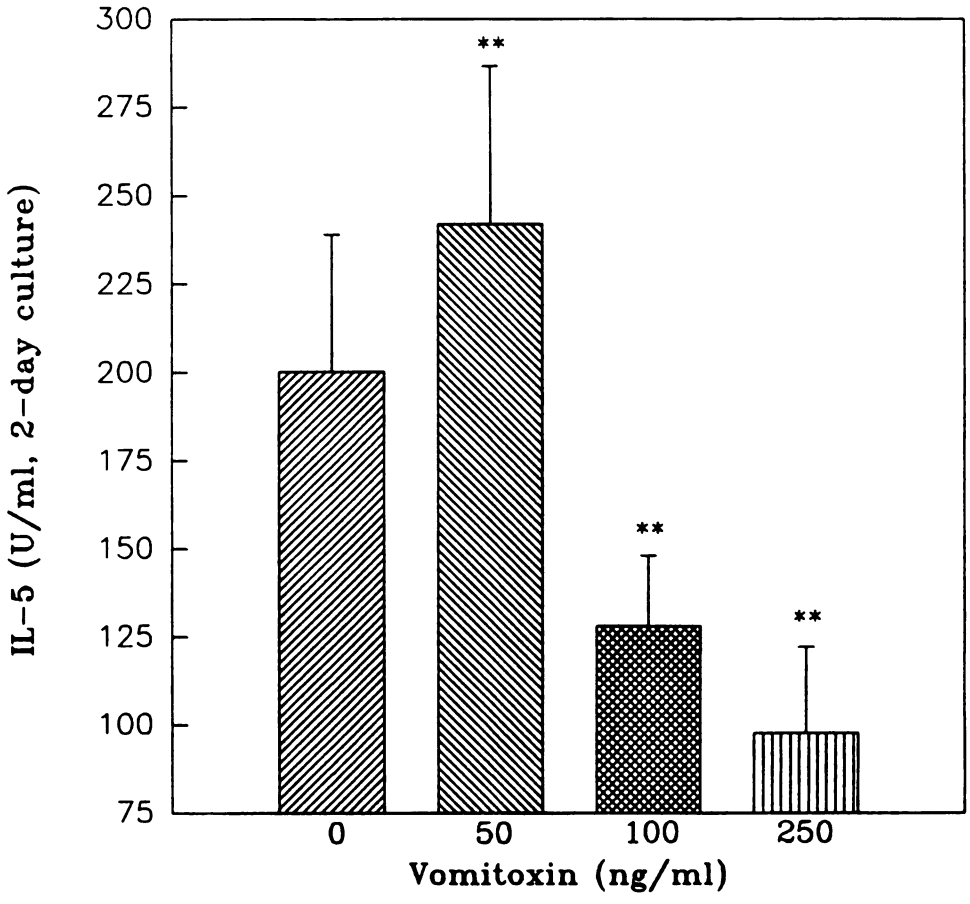

Figure 24. IL-5 production induced by vomitoxin in 2-day culture. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 2 days. IL-5 production in culture supernatant was detected by sandwich ELISA using capture and biotinylated goat anti mouse IL-5 antibodies. Results are averages of six separate experiments. Data are means ± SE (n=6). Random blocked ANOVA and lsd tests were applied. ** indicate significant difference at 0.01 level.

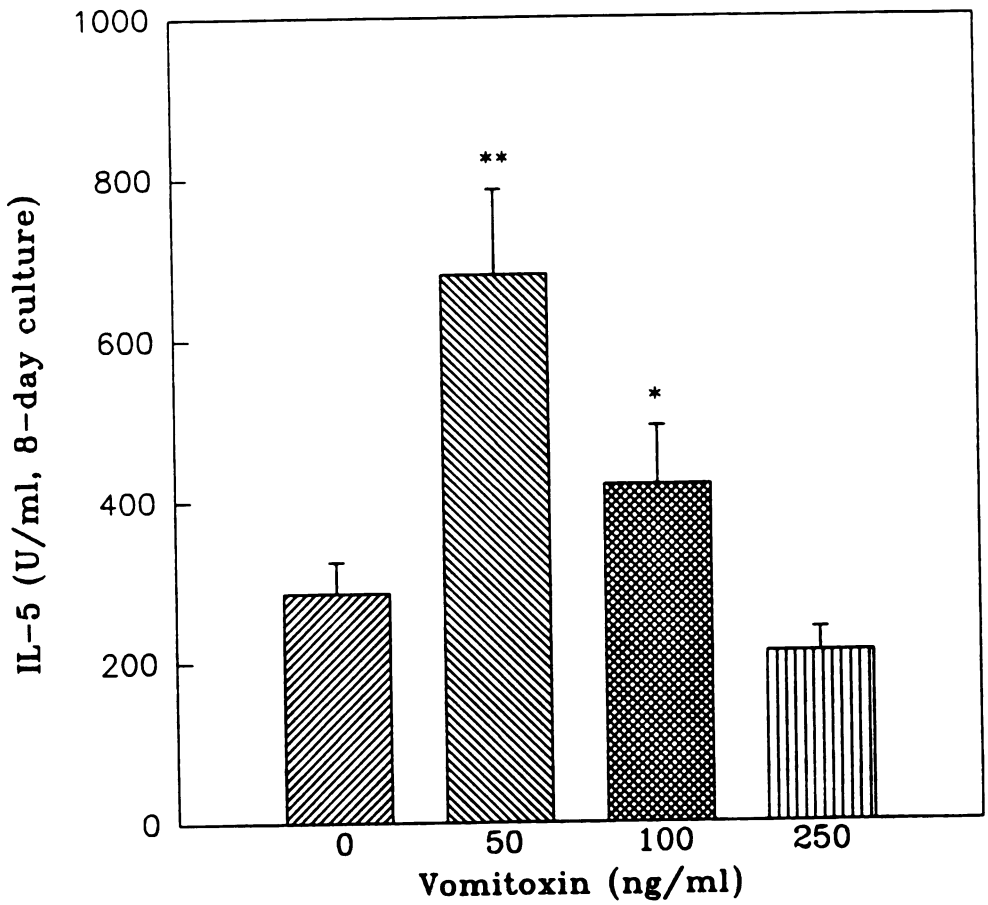

Figure 25. IL-5 production induced by vomitoxin in 8-day culture. EL4-IL2 cells were treated with 10 ng/ml PMA and 0, 50, 100, or 250 ng/ml vomitoxin for 8 days. IL-5 production in culture supernatant was detected by sandwich ELISA using capture and biotinylated goat anti mouse IL-5 antibodies. Results are averages of six separate experiments. Data are means ± SE (n=6). Random blocked ANOVA and lsd tests were applied. * indicate significant difference at 0.05 level. ** indicate significant difference at 0.01 level.

4.0 DISCUSSION

In this project, an *in vitro* model of vomitoxin-induced cytokine production and regulation in EL4-IL2 cells was developed to serve as a tool for understanding of vomitoxin-induced IgA dysregulation and cytokine gene dysregulation. This model can also be used to study immunomodulation caused by other potential toxins.

EL-4 ($H\text{-}2^b$) is a mouse thymoma T cell line. The wild-type EL-4 cell line produced very high levels of IL-2 (Farrar *et al.*, 1980; Lasek *et al.*, 1989). Many mutated variant type EL-4 cell lines have been obtained with characteristics of producing IL-2/IL-4 (Dornand *et al.*, 1992), IL-2/IL-6 (Espevik *et al.*, 1990), IL-2/Colony stimulating factor (CSF; Hilfiker *et al.*, 1981), and IL-5 (Tominaga *et al.*, 1988) upon the stimulation of T cell mitogen such as PMA. The EL4-IL2 cell line was employed in this study because of its capability of producing IL-2, IL-4, IL-5, and IL-6.

Consistent with many other studies, cytokine mRNA superinduction or increased cytokine production was found only in mitogen-stimulated EL4-IL2 cells. No detectable cytokine mRNA and little cytokine production were observed in non-mitogen-stimulated culture (Lasek *et al.*, 1989). Two

nuclear factors and proto-oncogene products, *c-jun* and *c-fos* proteins, are involved in regulation of cytokine gene expression (Abbas *et al.*, 1991; Altman *et al.*, 1990). PMA may activate cytosol protein kinase C (PKC) which can phosphorylate these nuclear factors. Nuclear factors then synergistically act on transcriptional regulatory sequences on cytokine gene.

IL-2 mRNA superinduction and IL-2 supernatant production increased early at 12-24 hr, while IL-5 increased at 2-8 days in EL4-IL2 cells after vomitoxin exposure. Although there has been no evidence to date that suggests IL-2 can switch B cells to IgA-producing cells, IL-5 is a well known cytokine that is capable of inducing proliferation and terminal differentiation of B cells committed to IgA secretion (Harriman *et al.*, 1988). It was reported that TGF-β and IL-5 act additively in stimulating IgA production only when TGF-β is added to the culture simultaneously with IL-5 or before IL-5 (Sonoda *et al.*, 1989). Further *in vivo* studies are needed in order to understand roles of cytokines in vomitoxin-induced IgA dysregulation.

It is notable in this study that 50-100 ng/ml are the optimal doses for vomitoxin to stimulate cytokine gene transcription and cytokine production in cell culture supernatant. Vomitoxin is a protein synthesis inhibitor and is possible it may share the same shutoff mechanism for IL-2 production with cycloheximide, another protein synthesis inhibitor. Although they are two completely different cell

culture system, vomitoxin appears to be more effective in EL4-IL2 cells than cycloheximide in human lymphocytes in induction of IL-2 mRNA, since 20 μg/ml cycloheximide were used (Efrat and Kaempfer, 1984) compared with 50-100 ng/ml vomitoxin used in EL4-IL2 cells. It is possible that vomitoxin selectively inhibits the protein repressor that shuts off active IL-2 formation than cycloheximide, thus it requires less amount of vomitoxin. The ID_{50} of vomitoxin on protein synthesis in CH12LX B cells is about 120 ng/ml (Minervini *et al.*, 1993). However, 250 ng/ml vomitoxin did not completely inhibit cytokine production and cytokine gene transcription in this study, the cytokine production and cytokine gene expression rather returned to the unstimulated level. This suggests that the inhibition of cytokine production in EL4-IL2 cell line require higher concentration of vomitoxin than inhibition of Ig production in CH12LX B cell line.

Most cytokine gene expression is believed to be regulated at the transcriptional level (Taniguchi, 1988). There are several possible mechanisms by which vomitoxin can superinduce IL-2 and IL-5 mRNA at this level. First, it is possible that vomitoxin binds directly to T cell receptor and an antigen binding signal may be conducted by the CD3 complex to trigger release of second messengers for regulating cytokine gene transcription. Second, vomitoxin may directly open membrane calcium channel to dramatically increase intercellular calcium concentration which activates

protein kinase C (PKC). PKC is essential to the synthesis of nuclear factors which regulate cytokine gene expression. Third, since vomitoxin might diffuse across cell membrane and selectively inhibit the synthesis of a protein which negatively regulates nuclear factor synthesis and cytokine gene expression. None of the above potential mechanisms has been tested so far.

Regulation of cytokine genes at the posttranscriptional level can also be very important (Taniguchi, 1988). Some possible mechanisms for posttranscriptional cytokine gene dysregulation can be hypothesized. Cytokine mRNAs have relatively short half-life. It is possible for vomitoxin to inhibit *de novo* synthesis of a short-lived protein(s) that plays important roles in control of cytokine mRNA level. Alternatively, there are highly conserved AU-rich sequence motifs within the 3' nontranslated regions of cytokine mRNA, which account for instability of cytokine mRNA. Vomitoxin may inhibit the synthesis of an unstable protein that binds to AU-rich sequences and triggers degradation of cytokine mRNA.

Numerous studies have shown that Th2 clones are involved in the regulation of IgA synthesis (Murray *et al.*, 1987; Coffman *et al.*, 1987), while only very a few reports agree that Th1 clone support IgA production (Lebman *et al.*, 1990). EL4-IL2 cells secrete cytokines produced by both Th1 and Th2 subsets. IL-2 and IL-5 are representative cytokines produced by Th1 and Th2, respectively. Nevertheless, other

unknown bioactive factors may also be involved since IL-2 production was determined by bioassay in which cells may be responding to yet-to-be-discovered cytokines.

5.0 SUMMARY AND FUTURE RESEARCH NEEDS

Vomitoxin (50-100 ng/ml) superinduced IL-2 and IL-5 mRNA transcripts and increased IL-2 and IL-5 levels in culture supernatant of PMA-stimulated EL4-IL2 cells. IL-2 was superinduced after 12-24 hr of incubation, while IL-5 was superinduced after 2-8 days of incubation. Approximately 2.5 fold increase in IL-2 and 4.5 fold increase in IL-5 mRNA transcripts were observed. Cytokine transcription and translation returned to the unstimulated level in the presence of 250 ng/ml vomitoxin.

Additional studies are needed to understand the level of this vomitoxin-induced cytokine dysregulation. (1) The possibility of other cytokine involvement such as TGF-β, IL-1, IL-4, IL-7, and IL-10 remains to be studied. ELISA, bioassay, and Northern analysis can be used to determine the level of cytokine production and cytokine gene expression. (2) Cycloheximide treatment of EL4-IL2 may be beneficial for understanding mechanisms of vomitoxin-induced cytokine dysregulation, since both of them are known protein synthesis inhibitors. (3) A runoff transcriptional analysis can be employed to determine whether cytokine superinduction by vomitoxin is regulated at the transcriptional level by monitoring the completion of nascent cytokine transcripts

from nuclei isolated at intervals from cells exposed to inducer in the presence or in the absence of vomitoxin. (4) Increased cytokine transcription rate may be accompanied by decreased cytokine degradation rate. Cytokine half-life can be detected by monitoring cytokine transcripts in cells incubated with inducer in the presence or in the absence of vomitoxin and then added a transcriptional inhibitor such as actinomycin. (5) The effects of vomitoxin on membrane signal transduction and intracellular second messages can be studied by monitoring calcium influx through plasma membrane calcium channel, protein kinase C and tyrosine kinase activities, and phosphorylation of various proteins such as nuclear factors.

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