# INFLUENCE OF SEX HORMONES ON IMMUNOGLOBULIN-A DYSREGULATION FOLLOWING *IN VIVO* AND *IN VITRO* EXPOSURE TO VOMITOXIN

By

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## ABSTRACT

INFLUENCE OF SEX HORMONES ON IMMUNOGLOBULIN-A DYSREGULATION FOLLOWING *IN VIVO* AND *IN VITRO* EXPOSURE TO VOMITOXIN

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Prolonged dietary exposure of female B6C3F1 mice to the trichothecene vomitoxin (VT) results in hyperproduction of immunoglobulin A (IgA), manifested as a marked elevation of total and autoreactive IgA, IgA immune complexes, and mesangial IgA deposition concurrent immunopathology that mimics human IgA nephropathy (IgAN). The purpose of this research was to (1) determine the susceptibility of various strains of mice to VT toxicity, (2) evaluate increased male sensitivity to the toxin, (3) assess the role of sex hormones in the modulation of VT toxicity. To assess the role of gender and strain in the mouse model, a diet containing 25 ppm VT was fed to B6C3F1 male mice and to B6C3F1, Balb/C, C3H/HeN, C3H/HeJ, and C57BL/6 female mice for 8 weeks and immunopathologic indicators of IgAN were compared with the same parameters in mice fed clean diet. The results suggested that male B6C3F1 mice and the five strains of female mice exhibited many of the immunopathologic effects characteristic of IgAN and that IgA

elevation was most marked in male B6C3F1 mice. In a follow-up study, immunopathologic markers indicative of IgAN were compared in male and female B6C3F1 mice fed diets containing 0, 2, 10 or 25 ppm VT for 12 wks. Based on the immunological parameters analyzed, males appeared more susceptible than female mice to VT-induced IgA dysregulation and IgA nephropathy in terms of latency, threshold dose, and severity. Since previous research indicated that male mice were more susceptible to the effects of VT toxicity, a subsequent study investigating the effects of castration of male and female mice and sex hormone supplementation on the development of IgAN was conducted. The results of this research suggested that the sex hormones modulate VT toxicity and that enhanced male susceptibility to VT-induced IgA nephropathy may be related to the regulation by the biologically active androgen, dihydrotestosterone.

This is dedicated to my family who has taught me the science of perseverance and endurance and instilled in me the thirst for knowledge. Their love and support have kept me motivated during times when self-motivation was not forthcoming. Most of all, to Duron, my nephew, whose birth was my greatest motivation!

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| | |
|---|---|
| ELISA | enzyme linked immunosorbent assay |
| IC/ICs | immune complex(es) |
| Ig | immunoglobulin |
| IgA | immunoglobulin A |
| IgAN | IgA nephropathy |
| IgE | immunoglobulin E |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| PP | Peyer's patch |
| RBC | red blood cells |
| VT | vomitoxin |

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# Chapter 1. Literature Review

## Mycotoxins

### General Aspects

Mycotoxins are fungal secondary metabolites of public concern because they have been implicated in a variety of human and animal diseases (Norred, 1993). These chemically diverse compounds are often strain-specific although they are not requisite for the successful growth and survival of the producing organisms by which they are produced (Pestka and Casale, 1990). Conditions favoring growth and production of mycotoxins by fungi include high substrate moisture levels (>14%), structural damage to crops by insects or water and high temperature stress, high crop densities, and weed competition (CAST, 1989). The invasion of grain and cereal crops can occur either in the field or post-harvest during transport, storage or processing (Marassas and Nelson, 1987; Pestka, 1988; Wood and Carter, 1989). The precise mechanism by which mycotoxin formation is initiated is not well defined (Marassas and Nelson, 1987).

Mycotoxins are produced by various fungal genera of which the most important are considered to be *Aspergillus sp.*, which are the best known for production of highly carcinogenic aflatoxins and the ochratoxins (Norred, 1993) and *Fusarium*, known for trichothecene, fumonisin, and zearalenone elaboration

(CAST,1989; Samson, 1992). Mycotoxins elicit a wide range of toxic effects in food-producing animals and consumption of contaminated feed has resulted in severe economic losses to livestock producers and farmers. In addition, and more importantly, mycotoxins pose a hazard to humans exposed to contaminated food products (Pestka, 1988). Consumption of low levels of some mycotoxins will not produce overt clinical signs, but might result in immunosuppression that could decrease host resistance to infectious agents (Corrier, 1991).

Of the mycotoxins found in foods and feeds, the most common are aflatoxins, zearalenone, vomitoxin (VT or deoxynivalenol), and the fumonisins (Richard *et al.*, 1993). Four general types of toxicity have been described for the mycotoxins: acute, chronic, mutagenic/carcinogenic, and teratogenic (Samson, 1992). Acute exposure is defined as a severe illness resulting from exposure occurring over a short time course, usually 12 to 24 hours after contact with the causative agent (Blood and Studdert, 1988). Acute mycotoxicosis are most often characterized by deterioration of liver, kidney, or intestinal function, which in severe instances of exposure can be fatal. Other mycotoxins elicit toxicity through protein synthesis inhibition producing effects as extreme as necrosis and immunodeficiency or as mild as skin sensitivity (Samson, 1992). A chronic illness is described as one that persists for a long periods of time with the disease showing little change or very slow progression during the period. Often the duration is undefined and varies with circumstances (Blood and Studdert, 1988). Chronic mycotoxicosis is characterized by continuous exposure to low concentrations (<2 ppm) of the mycotoxin resulting in persistent illness with nonspecific and subtle effects identified with poor performance (Prelusky *et al.*,

1994). One chronic effect of aflatoxins is induction of hepatocarcinomas (Samson, 1992). Other mycotoxins capable of affecting DNA replication have the potential to be mutagenic (causing genetic mutation) or teratogenic (causing physical defects in the developing embryo), with a variety of effects ranging from low birth weight to physical deformities (Blood and Studdert, 1988; Samson, 1992).

There are few measures that can be taken to control the climatic and biological determinants that encourage mycotoxin production and since these compounds are recalcitrant to ordinary chemical and physical means of degradation and detoxification, early detection is paramount. In the past, detection of mycotoxins was restricted to more conventional methods such as thin layer, liquid, or gas chromatography and mass spectroscopy (Pestka, 1988). However, in more recent years, immunochemical assays based on polyclonal and monoclonal antibodies to the different mycotoxins have been successfully employed in identifying these compounds (Abouzied *et al.*, 1991; Pestka, 1988, 1994).

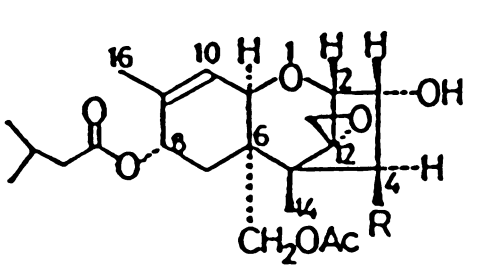
## Trichothecenes

### General Aspects

Trichothecenes perhaps represent the greatest health risk to animals and humans, with the exception of aflatoxin (Samson, 1992). Trichothecenes are naturally occurring, structurally related esters of sesquiterpenoid alcohols (contain 15 carbons) (Ishii, 1983). At least six genera of fungi are responsible for their production: *Fusarium, Myrothecium, Trichoderma, Trichothecium, Cephalosporium,* and *Stachybotrys* (Wyllie and Morehouse, 1978). Trichothecenes are composed of

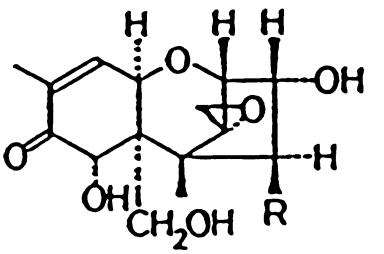
hydrogen, oxygen, and carbon, possess a tricyclic epoxide ring system, and have molecular weights ranging from 200-400 (Vidal, 1990). Most compounds that are categorized as trichothecenes include a six-membered, oxygen-containing ring, a spiro epoxy ($CH_2$--O) at the 12, 13 position of the structure and a double bond at the 9, 10 carbons (Figure 1.1)(Bamburg, 1983). Therefore, they are referred to as 12, 13-epoxytrichothecenes (Tamm, 1977; Wyllie and Morehouse, 1978). Elimination or replacement of the cyclic epoxide at the 12 and 13 carbons renders these compounds nontoxic while addition of acetyl groups (which increases lipid solubility) increases their toxicity (Bamburg, 1983; Vidal, 1990).

More than 148 structurally related trichothecenes have been identified, of which the ones usually encountered by humans and animals are T-2 toxin and VT (CAST, 1987). The trichothecenes have been divided into four groups based upon their chemical structures and characteristics, producing fungi, and power of biological activity (Figure 1.1) (Ueno, 1983; Wyllie and Morehouse, 1978). Group A consists of the hydroxy and acyloxy substituted trichothecenes such as T-2 and HT-2. T-2 toxin is the most thoroughly studied trichothecene to date because of its severe toxicity, broad range of immunosuppressive effects and its potential as a chemical and biological warfare agent (Corrier, 1991). Group B contains the 8 keto derivatives and includes VT and nivalenol. The macrocyclic derivatives are classified as Group C which consists of compounds such as verrucarin A and roridin A and the 7, 8 epoxy derivatives such as crotocin and the epoxyroridins constitute Group D (Ueno, 1977). Substitutions at the 3, 4, 7, 8 and 15 carbons give the trichothecenes their different names and toxic properties (Vidal, 1990).



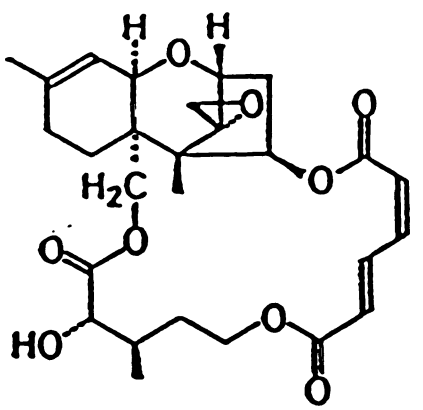
T-2 toxin R=OAc
HT-2 toxin R=OH

GROUP A



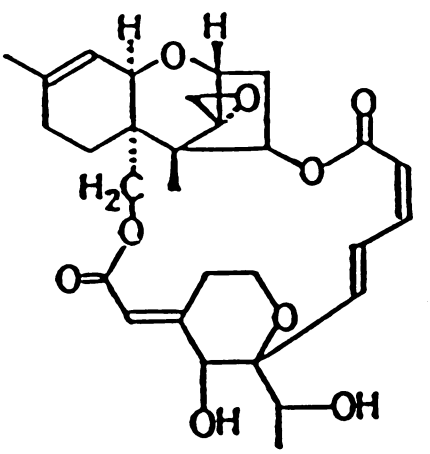
Nivalenol R=OH
Vomitoxin R=H

GROUP B



Verrucarin A

GROUP C



Satratoxin H

GROUP D

Figure 1.1. Chemical structures of the four types of trichothecenes (from Ueno, 1988)

The role of trichothecenes in the life cycle of their producing fungi has yet to be identified. However, in studies conducted by Bamburg and Strong (1971), several plant species were damaged following exposure to trichothecenes. Additionally, it has been proposed that mycotoxins are produced to enhance the severity of diseases caused by *Fusarium* in host plants (Salch and Beremand, 1993; Desjardins *et al.*, 1993). Most trichothecenes possess fungicidal and insecticidal properties and are therefore thought to be involved in the invasion of plant tissues that compete with their producing fungi (Bamburg and Strong, 1971).

Toxicity of Trichothecenes

Within the group of trichothecenes are some of the most potent protein and DNA syntheses inhibitors known (Ueno, 1977, 1983; McLaughlin, 1977). These compounds restrict protein and ultimately DNA synthesis by inhibiting chain initiation, elongation and/or termination. Additionally, those compounds that impair chain initiation alter the function of intact ribosomes or prevent the formation of the 80S initiation complex through selective binding of the trichothecene to the 60S subunit of eukaryotic ribosomes (Bamburg, 1983; Ueno, 1988). The basic mechanism by which trichothecenes affect protein synthesis is through inhibition of peptidyl transferase activity (Corrier, 1991; McLaughlin *et al.*, 1977).

Furthermore, trichothecenes are acutely toxic to actively dividing cell populations in tissues such as lymph nodes, thymus, spleen and bone marrow as well as intestinal mucosa (Ueno, 1977). Consequently, these compounds have the capacity to alter immune function by depressing humoral and cellular immunity and are

therefore considered to have immunosuppressive properties (Miller and Atkinson, 1987; Pestka and Forsell, 1988; Pestka *et al.*, 1987; LaFarge-Frayssinet, *et al.*, 1979). Immunotoxicity can be divided into two categories: immunosuppresion and immunostimulation. In the case of the former, the trichothecene restrains operations of the immune system often resulting in increased susceptibility to viral or bacterial infections and neoplasia. In the latter, the trichothecene stimulates various components of the immune system eliciting hypersensitivity or invoking an autoimmune-like dysfunction (Pestka and Bondy, 1994).

In many cases of fatal animal and human toxicoses, trichothecenes have been identified as the causative agent (Cote *et al.*, 1984; Corrier, 1991). Ingestion, which is the usual route of exposure to trichothecenes, causes a voluntary feed refusal, a decreased feed consumption with subsequent reductions in body weight gain, diarrhea, intestinal irritation and inflammation, emesis, decreased immune response, skin irritation, hemorrhaging of muscular tissues, degeneration of nerve cells, and hemolytic alteration (Trenholm, *et al.*, 1984; Bauer, *et al.*, 1989; Samson, 1992). Trichothecenes also have the capacity to decrease immunoglobulin synthesis and antibody responses as well as impair macrophage activity in domestic and lab animals (Corrier, 1991).

## Vomitoxin

### Occurrence

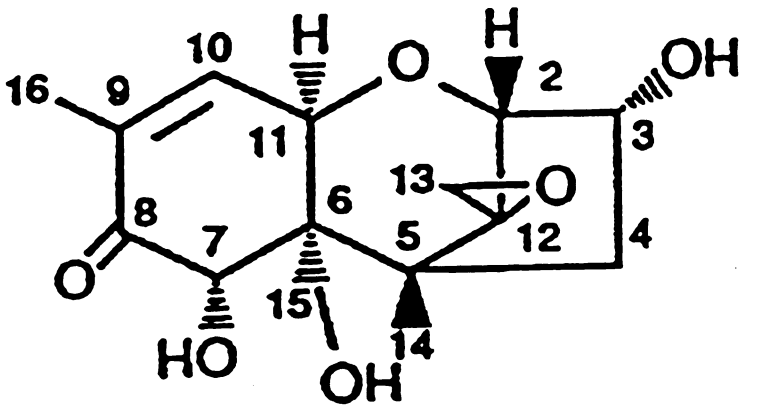
Vesonder and colleagues isolated VT from *Fusarium*-infected corn in 1973 (Vesonder *et al.*, 1973). During that same year, Yoshizawa and collaborators isolated

the same compound in culture (Yoshizawa *et al.*, 1973). The trichothecene deoxynivalenol, more commonly known as VT due to its emetic effects in swine, is a primary mycotoxin responsible for feed refusal and emetic syndromes (Marassas and Nelson, 1987). VT, which is mainly produced by *Fusarium graminearum*, is an 8-keto derivative (group B) that possesses hydrogen at $R_1$, hydroxyls at $R_2$, $R_3$ and $R_4$ and has a molecular weight of 296 (Figure 1.2)(Talmage, 1983; Ueno, 1983; Marassas and Nelson, 1987).

VT is a major contaminant of crops grown in temperate climate zones (Ueno, 1973; Miller and Atkinson, 1987) and has been detected in wheat, barley, corn, rice and other grains worldwide (Hunder *et al.*, 1991). In a 1991 study conducted by Abouzied and colleagues, VT was found in retail grain-based food products at levels high enough ( $>15$ ppm) to cause toxicity in animal models (mouse, swine, rats). However, it is important to note that the calculated $LD_{50}$ of VT for man is approximately six times greater than that for mice. In the United States, the Food and Drug Administration (FDA) has set an "advisory tolerance limit" of 2000 $\mu$g/kg in wheat and 1000 $\mu$g/kg in finished wheat products intended for human consumption (House, 1991; Hietaniemi and Kumpulainen, 1991). These limits are currently undergoing revision which will provide better safety margins and regulation.

## Toxicity and Immunomodulation of Vomitoxin

The $LD_{50}$ for VT is 70 mg/kg body weight if administered to mice intraperitoneally (ip) and 78 mg/kg body weight *per os* (po) (Forsell *et al.*, 1987), thus, VT is considered to be less toxic than trichothecenes such as T-2 toxin,




$C_{15}H_{20}O_6$

M.W. 296.1260

Species:

*F. graminearum*
*F. culmorum*

Figure 1.2. Chemical structure of vomitoxin (from Savard and Blackwell, 1994)

nivalenol and fusarenon-X (Scott *et al.*, 1980). VT toxicity is most often a result ofprotein synthesis inhibition that prevents the elongation-termination process by suppressing the formation of peptide bonds through interaction with the peptidyl transferase center located on the 60S ribosomal component (Kiessling, 1986). VT is also capable of suppressing DNA and RNA synthesis, but to a much lesser degree than protein synthesis, which seems to indicate that the former occurs as a secondary result of the latter (Ueno, 1985).

In an investigation by Ueno (1983), protein synthesis inhibition caused by VT occurred at concentrations as low as 2 $\mu$g/ml in rabbit reticulocytes. Furthermore, VT was demonstrated to be cytoxic at 0.25-1 $\mu$g/ml in HE and HeLa cells (Ueno, 1983). Interestingly, according to the authors, DNA damage in cells exposed to VT is probably not a result of unscheduled DNA synthesis. This was concluded because VT is still cytotoxic in cells that possess defective DNA repair systems (Robbana-Barnat *et al.*, 1988; Bradlaw *et al.*, 1985). Instead, the injury is generated by restricted protein synthesis which ultimately leads to DNA damage.

Chronic and subchronic exposure to VT results in reduced weight gain, voluntary feed refusal, and a reduced feed efficiency. Such clinical signs are also observed with acute exposure to VT and indicate that the gastrointestinal tract is one of the target organs of this compound (Hunder *et al.*, 1991). Notably, VT is immunosuppressive and immunostimulatory (Pestka *et al.*, 1987; Pestka and Bondy, 1990). Webster mice fed sublethal doses of VT experienced a significant decline in serum $\alpha_1$ and $\alpha_2$-globulins, serum IgM to sheep red blood cells (SRBC), plaque forming cell numbers and an increase in total serum albumin and the albumin:globulin

ratio (Tryphonas *et al.*, 1984, 1986). In addition, mice demonstrated a dose-related decrease in survival time following exposure to *Listeria*. In a study utilizing B6C3F1 mice exposed to VT and *Listeria*, splenic clearance of the organism indicated a reduced resistance to the pathogen (Pestka *et al.*, 1987). However, increased counts of *Listeria* in the spleen may be a result of VT-induced feed refusal as opposed to alteration in the immune functioning of the host animal (Pestka and Bondy, 1990). In a similar study using Balb/c mice, the immunosuppressive properties of VT were confirmed by a decrease in the stimulation of B and T cells by mitogens, and an inhibition of cellular proliferation in populations exposed to VT *in vitro* (Robbana-Barnat *et al.*, 1988). Furthermore, VT has been shown to elicit dose-reliant decreases in lymphocyte, monocyte and white blood cell numbers while neutrophil levels increase (Robbana-Barnat *et al.*,, 1988; Forsell *et al.*, 1986).

Most notably, VT immunotoxicity is manifested by a dramatic elevation (>3 fold increase) in serum immunoglobulin A (IgA), an increase in the polymeric to monomeric IgA ratio in serum, and an accumulation of mesangial IgA in the kidney glomerulus (Bondy and Pestka, 1991), all which are indicative of dysregulation of IgA production and an aberrant immune response. The immunostimulatory effects of VT on $IgA^+$ cells were investigated by Pestka and colleagues (Pestka *et al.*, 1990b). Mice fed VT exhibited an increase in the size and frequency of PP. In addition, mitogen-stimulated and unstimulated PP lymphocytes from these mice produced significantly larger amounts of IgA and demonstrated an increase in the number of IgA secreting cells in the PP as well as spleen, while IgG secreting cell numbers decreased (Pestka *et al.*, 1990a). The percentage of membrane IgA-bearing cells, in

addition to T helper cells ($CD4^+$), was increased in PP and spleen of the VT-exposed mice. There was also an increase in the ratio of $CD4^+$:$CD8^+$ T cells (Pestka *et al.*, 1990a).

Pestka and coworkers proposed that following VT exposure, B-cells located in the PP undergo terminal differentiation into IgA-secreting plasma cells. In a follow-up study (Bondy and Pestka, 1991), the number of IgA-secreting cells was greatly increased after only one day of *in vitro* culture which seemed to suggest a role for premature differentiation of the IgA secreting cells in the PP. In addition, terminal differentiation of B-cells in the PP as a result of VT stimulation may be T cell mediated. This was indicated by the increase in $CD4^+$ T cells, T cell help, and the altered ratio of $CD4^+$:$CD8^+$ in the spleen and PP (Bondy and Pestka, 1991). It has also been suggested that VT incidentally alters B cell differentiation by direct action on T cells (Warner *et al.*,1994).

In addition, $CD4^+$/$CD8^+$ cells from VT-treated mice (25ppm) co-cultured with control B cells from untreated mice produced 5 times more IgA than those T cells taken from control mice (Bondy and Pestka, 1991). Similarly, $CD4^+$ and $CD8^+$ T cells exposed to VT for a 24 hour period and then co-cultured with splenic B cells for 7 days induced 3- to 5- fold increases in IgA production when compared to control cultures. However, no increases in IgG and IgM were observed. This indicated that VT exposed $CD4^+$ lymphocytes are not only capable of increasing IgA production when cultured with B cells, but that they may also be the cellular population responsible for inducing production of IgA by control B cells *in vitro* and possibly *in vivo* subsequent to VT exposure (Warner *et al.*, 1994).

Bendtzen (1983) reported that compounds capable of inhibiting protein synthesis also have the capacity to superinduce the production of interleukins which are responsible for regulating immune responses. Based on this information, Miller and Atkinson (1987) investigated the effects of low concentrations of VT on interleukin production. They demonstrated that peripheral blood lymphocytes (PBL) exposed to phytohemagglutinin (PHA) and VT experienced enhanced stimulation of PHA-induced blastogenesis. They also observed an increase in IL-1 production by macrophages. More recently, Warner, *et al.* (1994) found increases in the levels of interleukin-4 (IL-4), IL-5, and IL-6 by cultured $CD4^+$ cells pulsed with VT. Thus, this supports the potential for VT to superinduce cytokine production. In a recent study by Dong and coworkers (1994), the elevation of gene expression and interleukin production by clonal T cells exposed to VT was investigated. Using the EL4.IL-2 (EL4) thymoma cell line this group demonstrated that VT administered in levels between 50 and 100 ng/ml mRNAs of IL-2, 4, 5 and 6 were superinduced in phorbol 12-myristate 13-acetate (PMA)-stimulated EL4 cells (Dong *et al.*, 1994). The authors suggested that ribosomal-bound VT causes the inhibition of protein synthesis, however, mRNA of the interleukins continues to accumulate until the toxin is withdrawn. Once the cell recovers and begins to function normally, there is a barrage of translation resulting in the production of massive quantities of the newly synthesized cytokines (Warner *et al.*, 1994). Ultimately, the increased levels of interleukins prompt polyclonal expansion and differentiation of the-IgA secreting cells (Warner *et al.*, 1994).

## Immunoglobulin A

### Characteristics

For a long time, IgA was believed to be an insignificant class of immunoglobulin. However, it is now known that the body manufactures more IgA than all the other isotypes combined (Mestecky and McGhee, 1987; Mazanec *et al.*, 1993). Although IgA is not researched as often as the other isotypes (IgG and IgM), it is of major concern not only because it accounts for approximately 66% of daily antibody synthesis in humans, but because it is the crucial immunoglobulin involved in mucosal immunity (Abbas *et al.*, 1991). Of the immunoglobulins, IgA is the only one that can be ferried across mucosal barriers into the lumens of mucosa-lined organs where it operates to inactivate deleterious agents in mucosal secretions (Abbas *et al.*, 1991). Quantitation of Ig-producing cells in tissues throughout the body showed that 1-2 m of human intestine contain a larger amount of Ig-synthesizing cells than any other tissue in the body, even if combined (Andre *et al.*, 1977; Beagley *et al.*, 1989).

Originally, IgA was considered to provide an immune barrier by obstructing the absorption and/or adherence of foreign antigens to epithelial cells of the mucosal lining. More recently, two new roles for mucosal IgA have been suggested. It has been proposed that mucosal IgA's defensive properties are not restricted to external secretions, but that IgA may possibly play a useful role through (1) antigen interaction in the lamina propria and (2) through the lining of epithelium (Mazanec *et al.*, 1993).

Structure of IgA

In humans, two IgA subclasses exist, $IgA_1$ ($\alpha 1$) and $IgA_2$ ($\alpha 2$), of which the former is present in serum at concentrations about 6 times greater than the latter (Abbas *et al.*, 1991). In mice, IgA has no subclasses, which makes class switching less complicated in this species (McGhee *et al.*, 1989). IgA occurs on the membrane of B cells in its monomeric form (Mestecky and McGhee, 1987). The alpha chain consists of 472 amino acid residues and blunt constant regions that allow for the formation of four Ig domains. This isotype contains extensions referred to as tail pieces (Fc) that enable intermolecular interactions to occur resulting in formation of multimeric molecules (Abbas *et al.*, 1991). The secretory form of IgA (Abbas *et al.*, 1991; McGhee *et al.*, 1989), exists as dimers or trimers with 4 heavy chains and 4 light chains or 6 heavy chains and 6 light chains, respectively (Abbas, *et al.*, 1991) (Figure 1.3). Polymeric IgA also accommodates an additional 15 kD polypeptide, known as the J chain, or joining chain that is attached to the tail piece by a disulfide bond. The J chain is requisite to connect the two monomeric subunits of the polymeric form and also functions to stabilize the multimer (Abbas *et al.*, 1991) (Figure 1.4). Polymeric IgA binds the secretory component (approximately 70,000 kDa) which facilitates the transport of the molecule into secretions and protects it from proteolytic attack (Abbas *et al.*, 1991).

Synthesis of IgA

The mechanisms and processes responsible for the regulation of differentiation of lymphoid cells into IgA producers are only partially understood and the

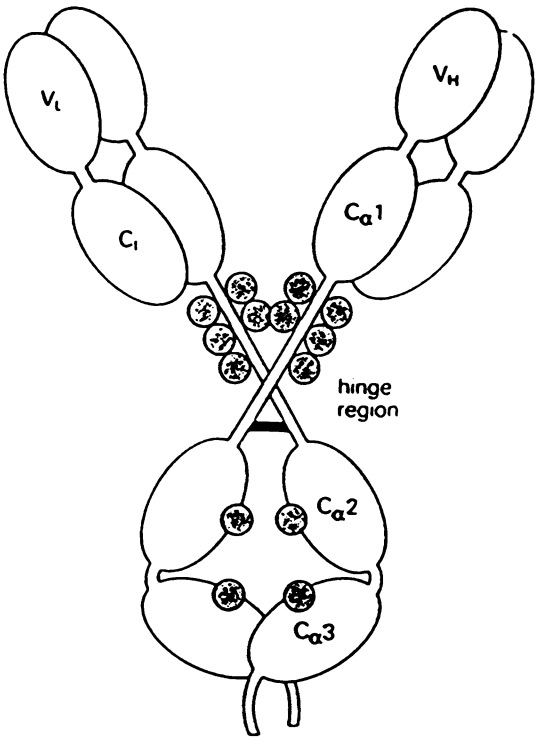



Figure 1.3. Structure of monomeric IgA (from Roitt, *et al.*, 1989)

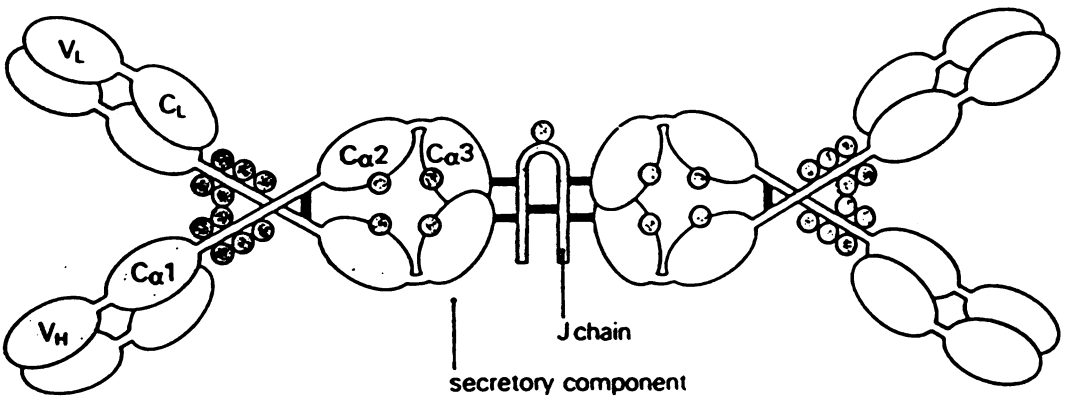



Figure 1.4. Structure of dimeric IgA with secretory component and J chain (from Roitt, *et al.*, 1989)

mechanisms that govern the distribution of IgA in mucosal tissues are also incompletely defined. Serum IgA is mostly monomeric and derived primarily from the bone marrow (Julian *et al.*, 1988). IgA for external secretions exists as $IgA_1$ and $IgA_2$ with the polymeric form predominating and is produced locally in mucosa-associated tissues and glands (Conley and Delacroix, 1987; McGhee *et al.*, 1989; Julian *et al.*, 1988). IgA enters mucosal secretions through epithelial transcytosis and occurs with the binding of the molecule to the transmembrane, polymeric Ig receptor, or secretory component which is situated on the basolateral surface of mucosal epithelial cells (Mazanec *et al.*, 1993; Tomasi, 1992). Subsequently, the complex undergoes endocytosis and is shuttled to the apical surface where proteolytic cleavage of the extracellular domains of secretory component causes release of secretory IgA (polymeric IgA + soluble extracellular domain of secretory component) into the lumen (Mazanec *et al.*, 1993).

The majority of studies focusing on the mechanisms of IgA production and secretions have demonstrated that IgA responses are greatly diminished with decreased numbers of T cells or in the complete absence of T cells (Elson, *et al.*, 1979; McGhee *et al.*, 1989). The predominant research done to determine the role of T cell populations in differentiation of B cells into IgA plasma cells has been performed in animal models (rat, rabbit, and primarily, mouse). In these species, gut-associated lymphoid tissue (GALT) consists of the appendix and lymphoid nodules (McGhee *et al.*, 1989). Additionally, situated throughout the small intestine are lymphoid aggregates, referred to as Peyer's Patches (PP), which are primary IgA inductive sites (Tomasi, 1992). The Peyer's patch dome region is enriched for T and

B cells and macrophages, while the follicles are comprised of germinal centers, that possess elevated numbers of $mIgA^+$ B cells (McGhee *et al.*, 1989). Within the germinal centers active division of B cells that are precursors of IgA plasma cells takes place (Mestecky and McGhee, 1987). Also located in the PP are $T_{helper}$ (Th) cells that uniquely support IgA responses. Ultimately, the germinal centers serve to populate distant tissues which include respiratory tract, mammary, lacrimal, uterine and salivary glands (McGhee *et al.*, 1989).

A study conducted by Elson and colleagues (1979) provided the first direct proof that T cells from the PP manage IgA synthesis. During this investigation, Con-A stimulated T cells from PP induced IgA production by lipopolysaccharide (LPS)-stimulated PP or spleen B-cell cultures, while T cells from spleen that were treated in the same fashion suppressed IgA, IgM and IgG synthesis in these cultures. This study also implied that IgA-specific T cells are present in elevated numbers in the PP and provided an explanation as to why IgA responses are explicitly induced in mucosal tissues (McGhee *et al.*, 1993).

IgA synthesis and response are modulated by the production of cytokines by other immune cells (McGhee *et al.*, 1989). These substances influence B-cell activation, proliferation, and terminal differentiation as well as class-switching from other isotypes. More specifically, interleukin 5 (IL-5) produced by activated T-cells, amplifies IgA synthesis by B-cells, an effect that is enhanced by IL-4 (Beagley *et al.*, 1988). It has been proposed that the steps involved in terminal B-cell differentiation are primarily regulated by IL-4, IL-5 and IL-6 (Beagley *et al.*, 1988).

## IgA Nephropathy

### General Aspects of the Disease

Human IgA nephropathy (IgAN), also known as Berger's Disease (Berger and Hinglais, 1968), is considered to be the most common type of glomerulonephritis in the world (D'Amico, 1983). This idiopathic disease was originally characterized in 1968 by Berger and Hinglais and was described as a chronic mesangiopathic glomerulonephritis identified by prevalent granular and electron-dense accumulations of IgA often accompanied by C3 in the mesangial region of the kidney (Berger and Hinglais, 1968). In more than half the cases, codeposition of IgM and/or IgG have been reported (Montinaro *et al.*, 1992).

IgAN usually develops in the second or third decade of life (D'Amico, 1983; 1987; Schena, 1990) and occurs more often in males than in females with a ratio ranging from 2:1 to 6:1, respectively, depending on the country (D'Amico, 1987). There is also conflicting evidence concerning the frequency of the disease (in the overall population) which varies across different geographical regions (Schena, 1990; D'Amico, 1987). IgAN is one of the most significant glomerular diseases in that it occurs more frequently than other forms of glomerulonephritis and it involves the entire immune system (Bene and Faure, 1987; Julian *et al.*, 1988). Additionally, this disease has great capacity to progress to end-stage renal failure. Nevertheless, the etiology of IgAN remains unknown (Clarkson *et al.*, 1987).

### Occurrence of IgA Nephropathy in Humans

Of the patients who undergo renal biopsy in Asia, Australia, and Western

Europe, 20-40% are diagnosed with IgAN as the form of primary glomerulonephritis (Julian *et al.*, 1988). In contrast, in the United States, a much lower incidence rate of about 2-10% of glomerulonephritis patients are reported as having IgAN, excluding a 38% incidence rate in Navajo Indians (New Mexico) (Julian *et al.*, 1988). There is also a lower incidence of the disease in the black community as compared to the white population (D'Amico, 1987). The overall frequency of the disease as determined via renal biopsy ranges from 5-10% in northern Europe, the United Kingdom and North America while a frequency of 25-35% was determined for southern Europe and Australia (Emancipator and Lamm, 1989). An IgAN frequency of 50% or greater has been reported in Asia (Clarkson *et al.*, 1987; Emancipator and Lamm, 1989).

One explanation for the great discrepancies in the frequency of IgAN is the differences in health screening standards and biopsy policies in various countries. Countries with an active screening program and a more aggressive biopsy policy inevitably will have a greater frequency of the disease as opposed to a country that has a health plan with no active screening program and a conservative biopsy routine (Emancipator and Lamm, 1989; D'Amico, 1987). This also explains the higher incidence of IgAN in Asia, where many of the countries have an aggressive urinalysis screening program (Julian *et al.*, 1988; Emancipator and Lamm, 1989).

Clinical Signs and Causes of Disease

At the onset of the disease, glomeruli appear normal with little to moderate mesangial expansion. With disease progression, segmental lesions, mesangial hypertrophy, focal sclerosis and tubular atrophy are often observed (Bene and Faure,

1987). Often times, the onset and recurrence of IgAN is partnered with an upper respiratory tract infection, or less often, with vaccine administration or an infection of the gastrointestinal or urinary tracts (Rostoker *et al.*, 1993; Julian *et al.*, 1988). More recently, it has been suggested that extrarenal cytokines manufactured during these episodes of infection may contribute to the pathogenesis of IgAN (Montinaro *et al.*, 1992). In a 1992 study by Montinaro and colleagues, it was shown that proinflammatory IL-1 could induce important histopathologic alterations related to IgA deposition and that IL-6 combined with IL-1 has the capacity to mediate mesangial cell proliferation leading to diffuse proliferative glomerulonephritis. This investigation also supports that glomerular injury may be a result of circulating extrarenal cytokines influencing a local reaction to nephritogenic antigens in the IgA immune complex that is deposited. Moreover, the investigators speculate that the glomeruli are continually exposed to these circulating cytokines and thus the latter can be considered a signal modulator controlling the extent of glomerular injury (Montinaro *et al.*, 1992)

The majority of patients diagnosed with IgAN present macroscopic hematuria or microscopic hematuria accompanied by mild proteinuria (< 2g/day) (D'Amico, 1987). A number of patients do not present with macroscopic hematuria, and other patients considered to have a very mild form of the disease do not present with proteinuria (Julian *et al.*, 1988). These patients often experience a remission of the disease as indicated by a normal urinalysis. However, subsequent renal biopsies from these individuals continue to show immunohistologic features of IgAN (Julian, *et al.*, 1988).

As iterated earlier, the cause of IgAN remains unknown. It has been proposed that IgAN is a result of an immune response to continuous exposure to certain food antigens resulting from a repetitious diet, an abnormal lining of the intestine or aberrations of the GALT (Nagy *et al.*, 1988; Pestka, 1993; Rostoker *et al.*, 1993). However, based on the knowledge that IgAN patients experience relapses with mucosal infection, it has been suggested that an increase in IgA production following antigenic stimulation at the mucosal surface may contribute to the formation of IgA immune complexes during bacterial and viral challenge, thus exacerbating the nephropathy (Rostoker *et al.*, 1993). It has also been theorized that increased intestinal permeability plays a major role in the onset and progression of IgAN in two ways. The first is based on increased permeability permitting exorbitant presentation of antigens to the mucosal immune system which leads to increased release of antibodies or complexed antigens into the general circulation. This in turn leads to the generation of nephritogenic immune structures complexed to IgA (Rostoker *et al.*, 1993). The second manner in which increased intestinal permeability may influence IgAN is based on excessive antigen entry into circulation, leading to prompting of the mucosal compartment to form nephritogenic IgA immune complexes or to bind mesangial antigens to specific IgA antibodies (Rostoker *et al.*, 1993).

Irrespective of the etiology, some clinical and histological features that seem to have prognostic value for IgAN include the age of the patient, the severity of proteinuria ($>$ 2g/day), the sex of the individual, hypertension, and the presence of renal impairment (Julian *et al.*, 1988). Still, the only definite means of diagnosis is by renal biopsy to determine deposition and necrosis (Bene and Faure, 1987).

Patients with Berger's disease display mesangial deposits of IgA in the kidney (Hisano *et al.*, 1991; Emancipator *et al.*, 1987). The deposited IgA is most often dimeric possessing the J chain and having the capability to bind secretory component (Hisano *et al.*, 1991; Bene and Faure, 1987). Some investigators believe that the J chains present do not belong to the IgA at all, but actually are from segmental subendothelial deposits of IgM that are sometimes co-deposited, but seems to be unrelated to the disease (Bene and Faure, 1987; Clarkson *et al.*, 1987). Furthermore, some investigators have identified the deposited IgA as $IgA_2$, however, the identity of which subclass is actually represented is still under debate (Bene and Faure, 1987).

Additionally, glomerular deposits have been suggested to be circulating IgA immune complexes (IgA-IC) and/or macromolecular IgA that may be antibodies against an exogenous or self-antigen. The latter implicates a possible autoimmune mechanism that may initiate the accumulation of IgA in the mesangium (Hiki *et al.*, 1991). Patients also exhibit changes in the mucosal surfaces associated with hyperproduction of IgA or impaired removal of this isotype, although the exact mechanism is not clearly defined.

It has been postulated that inhibition, saturation, or dysfunction of the monocyte-macrophage system is responsible for IgAN. If this is the case, the decreased rate of clearance of immune complexes would result in their accumulation in the kidney (Bene and Faure, 1987). It has also been shown that a dysfunction in phagocytosis may contribute to the progression of the disease and that a dramatic decrease in glomerular passenger macrophages may also influence the accumulation of the IgA in the mesangium (Bene and Faure, 1987). In addition, this disease also

involves increased number of IgA-bearing lymphocytes, levels of serum IgA, and ratio of polymeric to monomeric IgA (D'Amico, 1987; Emancipator and Lamm, 1989).

Experimental Animal Models

In an attempt to identify and understand the etiology and mechanism of human IgA nephropathy, many investigators have turned to experimental animal models. Several ivestigations have concluded that increased IgA production alone can not be held accountable for the pathogenesis of the disease. Two mechanisms for IgAN induction in experimental animals are known. The first is a passive model that involves administration of large quantities of preconstructed immune complexes. This method is influenced not only by the size of the immune complex, but also the dose of the IC that is administered, mononuclear phagocyte function, and vascular permeability (Chen *et al.*, 1988). The second mechanism involves binding of free antibody to an antigen that has been implanted in the kidney. This is *in situ* immune complex formation (Chen *et al.*, 1988).

One of the first groups to develop an experimental animal model for IgAN was Rifai and co-workers in 1979. The model they produced incorporated a protein from an IgA myeloma that was reactive with dinitrophenol-bovine serum albumin (DNP-BSA). This was used as the antibody component of an immune complex that was prepared *in vitro* and then injected. This group reported only IgA-IC that were composed of polymeric IgA deposited in the kidney mesangium as a result of the DNP treatment. Although this model is useful in that it may provide answers in

reference to the physicochemical exploitation of immune complexes, it has been criticized for its passive nature (Issacs *et al.*, 1981). Generally, the passivity of the approach renders it unable to indicate the initial circumstances that bring about the early disease in a patient.

Another experimental model was proposed by Issacs and colleagues in 1981 in an effort to contribute an active model that would be more applicable to the mechanisms and processes of the human disease. In development of this model, male Swiss-Webster mice were injected intraperitoneally with dextran derived antigens (neutral dextran or dextran sulfate) to induce IC-glomerulonephritis. As a result, investigators observed increased IgA and IgM deposits in the mesangium as well as hypercellularity and filling of Bowman's space. The disease process varied in severity in treatment animals but it was basically focal in nature involving mild hypercellularity and mesangial accumulation of IgA (Issacs *et al.*, 1981)

Interestingly, these investigators also noticed an increase in the periodic acid-Schiff positive (PAS-+) matter in the mesangial area in addition to an increase in the number of mesangial cells. Therefore, they concluded that dextrans were a credible antigen capable of inducing IgA-IC disease. This brought about questions concerning the possible role of carbohydrates as antigenic components in IC diseases. Frequently, the onset of IC-glomerulonephritis is exacerbated by or occurs concomitantly with respiratory or gastrointestinal viral infection syndromes (Jessen *et al.*, 1986; Issacs *et al.*, 1981). Based upon this, the developers of the dextran model speculated about whether the potential for exposure to viral and/or bacterial carbohydrates if chaperoned by a suitable antibody reply could represent an essential

factor in the pathogenesis of various types of glomerulonephritis (Issacs *et al.*, 1981).

In 1983, a group from Case Western Reserve University (Cleveland, OH) also began to develop a valuable experimental model for IgAN. The group sought to elucidate the role of mucosal immunity in the pathogenesis of different forms of human IC-glomerulonephritis (Emancipator *et al.*, 1983). They too acknowledged the clinical association of IgA-IC glomerulonephritis with respiratory and gastrointestinal viral-like syndromes and suggested that increased IgA invoked by a response to oral immunization may cause or contribute to the development of the disease. Female Balb/c mice were given an antigen, either ovalbumin, bovine gamma globulin or ferritin, in water for 14 wks (Emancipator *et al.*, 1983). As a result of continued oral exposure, an increase in IgA-producing cells in the bronchi and intestines was observed. Specific immunogen was demonstrated in the glomeruli of treated animals. There was also codeposition of specific IgA and J-chain. The researchers concluded that antigen deposition and antibody specificity were dependent upon the antigen that was utilized. The association of specific IgA in the serum with immune complexes in the glomeruli as well as expansion of IgA-producing plasma cells indicated an inherent role for the secretory immune system in the pathological mechanisms of this model (Emancipator *et al.*, 1983). The group also considered that antigen deposition occurred early in the immune response and that immune complex formation may occur in the lumen of the gut, *in situ* within the mesangial matrix, or within a compartment of the extracellular fluid (Emancipator *et al.*, 1983).

While the findings of the Emancipator *et al.* (1983) study were mostly consistent with those of other models and the human disease, the immunized animals

lacked increased mesangial matrix, mesangial proliferation, hematuria/proteinuria, and complement ($C_3$) deposition which usually accompanies IgA deposits. To account for these differences, the authors submitted that possibly stimulation of an immune response by low doses of an exogenous antigen as opposed to acute, intense exposure may be responsible. It was suggested that in this model, periodic antigen exposure resulted in slow deposition of the antibody which somehow limited the feedback mechanics of the alternative pathway thereby limiting $C_3$ deposition. In addition, IgG and IgM together can fully activate $C_3$ while the pure IgA in this study could only trigger $C_3$ partially (Emancipator *et al.*, 1983).

Several endeavors to produce a satisfactory experimental model for IgAN have followed. One such investigation showed that disabling the biliary transport of IgA contributed to mesangial IgA deposition (Gormly *et al.*, 1983; Melvin *et al.*, 1983) and several others demonstrated that decreased phagocytosis may also play an important role in the effectiveness of IgA and IgA-immune complex clearance (Rifai and Mannik, 1984). A research group in Japan formed an experimental model that scrutinized the role of a dysfunctioning reticuloendothelial system (RES) in the progression of the disease (Sato *et al.*, 1986). In this effort, female ddY mice which develop spontaneous IgAN (Imai *et al.*, 1985) were fed lactalbumin (Lalb), Lalb plus colloidal carbon injection to block RES, or colloidal carbon injection alone. All treated animals exhibited an increase, not only in serum IgA, but also in IgA deposition in the mesangium. However, the group fed Lalb and injected with colloidal carbon had the highest IgA and increased proteinuria with no significant deposition of IgG and IgM. The authors theorized that a portion of the Lalb antigen

enters the blood through the gastrointestinal mucosa. It is at this location that antibodies against Lalb are produced. Thus, with long-term, continuous oral exposure to the antigen, antibody is constantly manufactured (Sato *et al.*, 1986). As a result, a portion of the excessive IgA is transferred into the circulation and eventually to the liver in the form of IgA-IC or IgA aggregates. Ultimately, the body is unable to phagocytize these complexes which are then free to accumulate in the glomerular mesangium (Sato *et al.*, 1986). The authors made a direct association between the decreased phagocytosis and a dysfunctioning or blocked RES which contributed to IgAN.

Jessen *et al.* (1986) developed a mouse model for IgAN induced by Sendai virus. The purpose of this study was to develop an animal model which would use a naturally infectious pathogen to initiate the disease based again on the association between concomitant onset of IgAN with respiratory or viral infection. Male, 129/J and B6D2 mice from Sendai virus-free colonies were immunized with inactivated virus intranasally followed by live virus (Group 1), with purified viral envelope protein (Group 2), or were naturally infected (Group 3). Animals from groups 1 and 2 had slightly elevated glomerular deposits of IgM while IgG deposits were low or absent. In mice from all three groups, IgA was the predominant immunoglobulin deposited in the mesangium in addition to significant amounts $C_3$. Only animals from group 1 presented hematuria. Therefore, the investigators concluded that immunization and infection with a respiratory virus such as Sendai followed by viremia produces some of the clinical and histopathological features of human IgAN. Mice infected naturally also had significant IgA deposition, but to a lesser degree than

those infected through injections. Furthermore, naturally infected mice did not exhibit the major clinical features of the disease (Jessen *et al.*, 1986).

The group from Japan did a subsequent study using their previously developed model based on a dysfunctioned reticuloendothelial system (Sato *et al.*, 1987). In the 1987 study, female ddy mice were fed Lalb and then given colloidal carbon for three weeks. The animals were then divided into two groups, one that was administered the anti-allergic agent sodium cromoglycate (SCG) and a second group that was untreated. Since a strong relationship between IgAN and food hypersensitivity had been previously established by several groups, the investigators proposed that SCG would limit the gastrointestinal immune response to the Lalb (Sato *et al.*, 1987). The $SCG^-$ mice demonstrated an increase in IgA deposition, fluorescence intensity and frequency of electron dense deposits in the mesangium as a result of Lalb antigen. Deposits increased in number and in size, mesangial expansion was observed to a greater degree, and serum IgA was significantly elevated in the $SCG^-$ mice. In comparison, $SCG^+$ mice experienced none of these pathological changes and serum IgA was not different when compared to control animals. The researchers concluded that SCG markedly decreased mesangial depositions, expansions, and other pathological changes concurrent with IgAN. The results also supported that SCG prevented the effects of orally-induced IgAN and a dysfunctioning RES most probably by reacting to the local immunity of the gastrointestinal tract to eliminate an excessive, prolonged immune response to Lalb (Sato *et al.*, 1987).

In 1988, a group from China joined the search for an applicable experimental model for IgAN. Chen and colleagues utilized the MOPC-315 plasmacytoma as an

IgA source in an effort to determine *in vivo* the nature of the IgA-antigen interaction in the IgAN disease process and also to understand the relationship between the molecular form of IgA and that of the glomerular deposit. Female Balb/C mice were given iodinated ficoll conjugated to DNP (I-ficoll-DNP) intravenously (iv) in addition to iodinated polymeric IgA (I-pIgA) intraperitoneally (ip) (group 1). A second group of animals was given I-ficoll-DNP iv and I-biotinylated-monomeric IgA (I-biotinylated-mIgA). The third group was administered I-ficoll-DNP and unlabelled pIgA plus I-biotinylated-mIgA. Three sets of controls were also utilized and they received I-pIgA, I-ficoll-DNP or I-mIgA.

Of the treated animals, group 1 had IgA deposits limited to the mesangial area and the capillary loops at 4 and 6 hours following treatment administration, while group 2 had no deposits. Group 3 mice had IgA deposits that included significant quantities of mIgA, but this was observed only at 6 hours following the treatment. Of those animals with glomerular deposition of IgA, group 1 mice had the largest sized IgA-IC that were formed continually between 2 and 6 hours after treatment. The deposits formed in mice from group 2 were small in size, with no intermediate or large sized complexes constructed (Chen *et al.*, 1988). Interestingly, the investigators observed a reduction in the circulating levels of pIgA and an increase in the mIgA. One explanation for the former may be that the majority of pIgA was depleted for the formation of very large IC that were insoluble, and thus they were unable to diffuse into the circulation and be quickly removed by the liver (Chen *et al.*, 1988). The authors speculated that the increase in mIgA was a result of a decreased rate of diffusion of the small IC formed between the intravascular and extravascular

compartments (Chen *et al.*, 1988)

In addition, the authors demonstrated a positive correlation between the size of IC and glomerular localization as well as the importance of molecular form of IgA in the mode of IC construction. It was established in earlier *in vitro* investigations that pIgA has the capacity to form large or intermediate IgA-IC, which is requisite for glomerular deposition. This study reconfirmed these findings. Furthermore, mIgA was unable to form the properly sized IC because the low avidity of this molecular form resulted in unstable complexes. It was also shown that IgA-IC were preformed in the circulation and eventually were the inducers of glomerular IgA deposition. Some quantities of mIgA were found in the mesangium because the polymeric complexes provide free antigenic determinants that bind to the monomeric form. Finally, the IgA complexes that are deposited most probably are composed of preformed pIgA-IC and *in situ* formed mIgA-IC (Chen *et al.*, 1988).

Dextran was again utilized in 1989 when a group from Spain continued work with this model (Gonzalez *et al.*, 1989). Here, the role of defective hepatic handling of IgA in the initiation and progression of IgAN was studied. Since IgA is primarily cleared by the liver, a reduction or suppression of this organ's ability to effectually eliminate IgA polymers or IgA-IC could expedite their persistence and eventual deposition in the glomerulus. Inbred male ICR swiss mice were treated with repeated injections (ip) of dextran sulfate while control animals received saline. No alterations in the normal serum profile of IgG and IgM were observed in the treated mice. However, these same animals experienced a significant increase in total serum IgA as compared to controls.

This study also confirmed a positive relationship between elevated serum IgA and increase glomerular deposition. These same results had been previously demonstrated in a 1986 study by Genin and associates who induced IgAN by oral immunization with ferritin and in an early 1983 study by Melvin and coworkers who ligated the bile duct of animals to induce glomerular deposition. Chen and colleagues (1989) additionally demonstrated that with dextran administration, the percentage of hepatocytes with receptors to pIgA decreased. This occurred concomitantly with delayed blood clearance of pIgA conglomerates and diminution of antibody uptake by the liver. In previous studies, it has been shown that hepatocytes in the liver are responsible for IgA clearance in rats, rabbits and chickens (Orlans *et al.*, 1983). Furthermore, pIgA is preferentially conveyed from the circulation via the liver into the bile. This is accomplished when the secretory component of IgA binds to high affinity receptors in these cells (Gonzalez *et al.*, 1989).

A follow-up study a year later (Gonzalez-Cabrero *et al.*, 1990) on the mechanisms responsible for the formation of mesangial IgA deposits. Again, dextran sulphate was administered to male ICR swiss mice over a period of time while control mice received saline injections. Upon termination of the mice, kidneys were analyzed and little or no dextran was detected in the mesangium of treated animals. The researchers hypothesized that antigen driven help can direct polyclonal B-cell activation resulting in the production of non-specific IgA and that it is this non-specific IgA (predominantly pIgA )that eventually is incorporated into the complexes that are ultimately sedimented in the glomerulus (Gonzalez-Cabrero *et al.*, 1990).

A more molecular approach to IgAN was taken in 1991 when another group

from Japan pursued the characterization of the pathogenic subpopulation of IgA in the initiation of IgAN (Muso *et al.*, 1991). In order to make these characterizations, they analyzed the molecular size and electric charge of glomeruli-bound IgA. Female ddy and Balb/c mice were utilized. It is important to note that this same group had demonstrated a pathogenic role of retroviral gp70 IC in spontaneous initiation of glomerulonephritis in the ddy mice (Takeuchi *et al.*, 1989). Serum IgA levels were elevated in both types of animals, however, it was 2 fold higher in the ddY mice than in the Balb/c mice. Isoelectric focusing was utilized to determine the isoelectric point (pI) of IgA in serum and kidney eluates in an attempt to characterize the population of immunoglobulin involved in the disease. In older mice with a more severe disease state, IgA was predominantly dimeric or polymeric in serum and mostly polymeric in kidney eluates. In the younger animals, serum IgA was predominantly monomeric, however, IgA in kidney eluate was still chiefly in the polymeric form. It was suggested that the increase in polymeric IgA detected in serum may be a result of polyclonal stimulation of IgA-specific B cell clones. Muso and coworkers (1991) intimated that with increasing age there is a proportional increase in pIgA and that this form may possess a significant quality of polyclonally expanded IgA which contributes to the development of IgAN (Muso *et al.*, 1991).

Interestingly, Muso, *et al.* (1991) also confirmed that dimeric and polymeric IgA are related to the development and extent of glomerular injury based on detection of these two forms exclusively in the kidney eluates. However, it is important to acknowledge that all forms of IgA increased in production. Thus, it appears as though there is an explicit mesangial trapping mechanism for pIgA. A possible

explanation is that mesangial cells bind to the Fc portion of pIgA which is then followed by phagocytosis. There is an upregulation of the activation of the cell surface IgA Fc receptor on mesangial cells that is brought about by the increase in serum IgA. Thus, it seems as though pIgA is the pathogenic form (Muso *et al.*, 1991).

Montinaro *et al.* (1991) tested the hypothesis that antigen in immune deposits is the actual mediator of glomerular injury in IgAN (Montinaro *et al.*, 1991). This group produced an experimental design for IgAN characterized by the deposition of passively formed IgA/IgA-IC that had the capacity to capture antigen *in situ*. Through the use of plasmacytoma-derived monoclonal IgAs (IgA-$\alpha$-DNP and DNP-IgA-$\alpha$-PC), they incorporated IgA that could serve not only as antigen, but also as the antibody. Female C57BL/6 mice were injected (iv) with both monoclonal IgAs thus ensuring that the IgA-IC were comprised only of IgA that was constructed *in vivo*. Two hours after antibody administration some animals were also given one of three antigens that share a coinciding antigenic determinant--one of two carbohydrate antigens, pneumococcal C polysaccharide (PnC), or phosphorylcholine conjugated to Ficoll (PC-Ficoll) or a protein antigen, bovine serum albumin conjugated with phosphorylcholine (BSA-PC). This protocol assured that all antigens had the same opportunity to associate with the binding site of the antibody already bound to the glomeruli (Montinaro *et al.*, 1991).

Those mice receiving both antibodies demonstrated diffuse mesangial deposition of IgA while mice receiving only one of the two did not. $C_3$ was also deposited in the same manner as that observed for IgA. Animals treated with both

antibodies experienced an increase in mesangial matrix and proliferation of cells in the mesangial area (Montinaro *et al.*, 1991). Animals treated with the antibody followed by antigen administration experienced an expansion in mesangial matrix and hypercellularity, however, the most critical lesions were observed in the group that was given PnC. Notably, the severity of the lesions was directly proportional to the amount of PnC that was given. The seriousness of histopathological and functional alterations accompanying IgAN were diverse and dependant upon the characteristics and quantities of the antigen captured by deposited IC. The pathophysiologic modifications often represented by hematuria and proteinuria were also reliant upon the nature and quantity of antigen dispensed. Montinaro and coworkers (1991) concluded that the antigenic element of the IgA-IC gives the deposit its nephritogenic capacity and that antigens of viral or bacterial derivation play an integral role in the induction of glomerular damage (Montinaro *et al.*, 1991).

In 1992, Gesualdo *et al.* (1992) developed an experimental model for IgAN using various strains of rats for the purpose of pathophysiologic investigations (Gesualdo *et al.*, 1992). Here, the investigators tried to induce IgAN in rats by generating a sustained mucosal immune response via a particular immunization protocol. Male Lewis, Wistar, Fischer and Sprague-Dawley rats were given clean water or water containing a 0.1% concentration of bovine gamma globulin (BGG). The animals were also injected (iv) for three successive days. A separate group of Lewis rats were given BGG by an oral route. Lewis and Wistar rats proved to be high IgA responders, as evidenced by their elevated levels of serum IgA anti BGG as compared to controls (39 fold and 9.5 fold increases, respectively). In addition, the

Lewis rats also had a significantly elevated IgG response. In contrast, the Fischer and Sprague-Dawley rats were poor IgA responders with increases in specific IgA levels of only 3.9 and 4.5 fold, respectively. In the glomeruli from Lewis and Wistar rats, deposits of IgA, BGG and $C_3$ were detected on a more frequent basis than their controls and the immunized Fischer and Sprague Dawley mice (Gesualdo *et al.*, 1992). In a previous study, this same group (Gesualdo *et al.*, 1990) demonstrated that lengthening the period of oral immunization beyond 6 weeks will result in an increase in the IgA:IgG ratio due to oral tolerance suggesting that protracted antigen exposure will also heighten glomerular immune deposits and proclivity of IgA.

More recently, Endo and coworkers (1993) developed an experimental model of IgAN utilizing various strains of gram (-) bacteria as the causative antigen in an attempt to investigate the nephritogenicity of these organisms and their cellular components. Female C3H/HeN mice were given bacterial fractions or cell wall components (i.e. lipopolysaccharide--LPS) *per os* or intraperitoneally. Those mice that received the antigen ip experienced mild to marked mesangial deposition of IgA and $C_3$, while their po treated counterparts experienced the same but to a much lesser degree. Interestingly, $C_3$ deposition did not appear until 20-30 weeks following treatment. This was in agreement with previous findings by Takeuchi *et al.*, (1989) and Imai *et al.*,(1985) who showed that 30 weeks of age seems to be crucial for the spontaneous development of IgAN, accompanied by $C_3$ deposition, in ddy mice. Additionally, those groups treated with bacterial cell wall components demonstrated much more intense deposition of IgA and $C_3$ than those receiving bacterial supernatant fractions. It was concluded that factors responsible for inducing IgA and $C_3$

accumulation in glomeruli of animals with IgAN are closely affiliated with cell wall components common among gram (-) bacteria. However, in this particular study, LPS did not appear to be involved in this induction.

Many researchers involved with experimental models of IgAN agree that the antigen partially responsible for inducing the disease may be of dietary origin. However, most of the models previously discussed did not utilize a naturally occurring dietary antigen to induce disease nor was the role of the mucosal imune system studied in depth. As described earlier in the introduction, our laboratory presented a model utilizing VT for IgAN inducement in 1989 (Pestka *et al.*, 1989) that has continuously undergone modifications and improvements to the present time.

In the initial investigation of VT immunotoxicity, female B6C3F1 mice were orally exposed to VT at various concentrations (0, 2, 10, 25 and 50 ppm) for 24 weeks (Pestka *et al.*, 1989). Mice fed 25 ppm VT had the greatest elevations in serum IgA as early as 4 weeks following initial exposure. By 24 weeks, the serum IgA concentration in this treatment group was more than 17-fold greater than the control value. In addition, concurrent decreases in serum IgG and IgM were observed indicating that the immunostimulatory effects of the toxin were specific for IgA (Pestka *et al.*, 1989; Forsell *et al.*, 1986). In addition, polyacrylamide gel electrophoresis (PAGE) revealed that the predominate form of IgA in serum from mice fed 25 ppm VT was pIgA, as compared to control serum (Pestka *et al.*, 1989).

In this same investigation, the capacity of the systemic compartment to contribute to IgA elevation was studied. Splenocytes from treated and control mice were cultured and Ig production quantitated. The IgA production was elevated in

cultures from treated mice while IgG and IgM levels were not affected. This was in contrast to cultures from controls in which IgA levels did not increase. Similarly, IgA accumulation in the mesangium of treated mice was greater than that of controls. However, IgG and $C_3$ deposition was not observed (Pestka *et al.*, 1989). The authors resolved that increased IgA as a result of VT exposure was most likely due to increased production. PPs situated throughout the small intestine house accessory, T helper, and T suppressor cells that regulate isotype switching, as well as activation and differentiation of IgA progenitors with antigen exposure (Pestka *et al.*, 1989). The authors suggested that B cells derived from the PP migrate through the systemic compartment to mucosal sites such as the lamina propria. This may possibly provide explanation for the involvement of the mucosal and systemic compartments as indicated by the cultured splenocyte results (Pestka *et al.*, 1989).

In a follow-up study, our group examined the effects of VT on the histological and lymphocytic profiles of immune organs in the mucosal lymphocyte migratory pathway in an effort to further explain the mechanisms of IgAN induced by this compound (Pestka *et al.*, 1990b). B6C3F1 females were fed a 25 ppm VT diet and killed at intervals. PP, mesenteric lymph nodes, spleen and thymus were removed and fixed. Additionally, cells from PP and spleen were distributed and stained for total population quantifications. Treated mice had elevated serum IgA levels and decreased or unaffected IgG quantities, while PP from these animals were 1.5- to 2-fold larger than PP from their control counterparts. This tissue from treated mice had prominent germinal centers representative of lymphocyte proliferation. This was absent in PP tissue removed from control mice. Consistent with the histological

findings, cell recoveries from PP of treated mice increased with length of VT exposure (Pestka *et al.*, 1990b). In addition, the percentage of B and T cells in PP from treatment animals were higher than that of controls. Similarly, the percentage of T cells in the spleens of treated animals was also greater when compared to those from control mice. Notably, there was also an increase in the number of $IgA^+$ and $CD4^+$ cells in PP and $IgA^+$ B cells in spleen from VT-exposed animals. Furthermore, the mesenteric lymph nodes from mice fed the toxin were 2 to 3 times larger with a greater number of germinal centers when compared to lymph nodes from controls (Pestka *et al.*, 1990b).

From this study, the authors determined that VT induced qualitative and transient modifications in PP, spleen and mesenteric lymph nodes that were eminently characteristic of elevated lymphocyte travel through the mucosal migratory pathway (Pestka *et al.*, 1990b). Moreover, dietary VT has the capacity to modulate regulation of the mucosal immune response at the PP level as illustrated by modified lymphocyte distribution in the mucosal and systemic compartments. To our knowledge, VT is the first naturally occurring environmental toxin to induce immunopathologic parameters consistent with IgAN, indicating a possible etiologic role. Of extreme relevance is the similarity between features of human IgAN and those of the VT model which include: (1) elevated $CD4^+$:$CD8^+$ ratio; (2) elevated serum IgA; (3) increased polymeric:monomeric IgA ratio; (4) increased synthesis of IgA by isolated lymphocytes (spontaneous or mitogen-stimulated); and (5) elevated IgA titres to dietary antigens (Pestka *et al.*, 1990b).

In summary, many models for IgAN have been developed within the past

decade which have provided enlightenment concerning this common disease. Glomerular IgA deposition has been induced by a variety of approaches, passive and active, including ip injection of apoferritin or dextran into mice, iv injection of IgA-DNP-IC, and oral exposure to lactalbumin and colloidal carbon to block the RES. These investigations have made general key findings. First, IgA deposits are naturally present in the kidney. Therefore, in order to cause glomerular damage/kidney dysfunction, the deposits must possess nephritogenic properties (Scivattaro *et al.*, 1993). Second, IgA and IgA-IC in and of themselves can not elicit glomerular dysfunction (Emancipator *et al.*, 1983). Third, oligomeric IgA containing J-chain is important in glomerular deposition (Emancipator *et al.*, 1983; Scivattaro *et al.*, 1993). Fourth, exposure to viral and/or bacterial carbohydrates (i.e. cell wall components) probably represents an essential factor in pathogenesis (Endo *et al.*, 1993; Issacs *et al.*, 1981). Fifth, antigen deposition and antibody specificity depend on the antigen utilized while codeposition of IgG and IgM (which have high $C_3$ fixation activity) is required for $C_3$ deposition, which is in turn required for hematuria (Scivattaro *et al.*, 1993; Emancipator *et al.*, 1983). Sixth, size and electrostatic charge of IC play an integral role in glomerular localization (Chen *et al.*, 1988). Finally, there is a direct relationship between elevated serum IgA and increased glomerular deposition (Chen *et al.*, 1988; Melvin *et al.*, 1983; Genin *et al.*, 1986). A key question that remains to be examined in animal models is related to the apparent higher male susceptibility to this disease. Endogenous sex hormones may be central to this question.

## Sex Hormones

### Testosterone

In males, 95% of the principal circulating androgen in humans, dogs, and rodents, testosterone, is produced by the testes (Neubauer *et al.*, 1993). The action of this steroid hormone in target cells depends not only on the quantity that is present, but also on the magnitude of metabolic transformations that occur within the cells, associations with the appropriate receptors, and the action of the androgenic receptor at the genomic level (Rommerts, 1990). Androgens, and specifically testosterone, are crucial in the development, as well as the subsistence, of reproductive tissues (i.e., prostate, epididymis, testis) and other masculine characteristics such as increased hair growth and muscle strength (Rommerts, 1990).

Androgens are initially produced during early embryonic development. This production continue at very low levels until puberty at which time there is a surge in testosterone. High levels are continually produced from that time until around the fourth decade of life when levels begin to gradually decline (Curtis and Barnes, 1989). Cholesterol is the source the body uses to produce steroid hormones. This substrate is made in the cytosol and usually exists in an esterified form (Rawn, 1989). Cholesterol can be produced *de novo* from an acetate precursor or may be taken up from the lipoproteins in plasma. The majority of synthesis occurs in the Leydig cells of the testes, however, the adrenal cortex also contributes to androgen manufacturing (Rommerts, 1990). In addition, brain cells are also capable of producing small quantities of steroids (Hu *et al.*, 1987) and in females, diminutive amounts of androgens are produced by the ovaries (Rawn, 1989).

The initial step in androgen synthesis occurs in the mitochondria and involves cleavage of the cholesterol side chain to remove a six-carbon fragment from the hydrocarbon tail which is attached at $C_{17}$ in the D ring of the compound (Figure 1.5). Enzymes from the endoplasmic reticulum subsequently convert pregnenolone to a series of $C_{19}$-steroids. Thus, the bioproduction of biologically active androgen is a serial degradation of inactive pregnenolone (Rommerts, 1990). More specifically, pregnenolone is converted to progesterone in a two-step process. Initially, the $C_3$ hydroxy group of pregnenolone is oxidized to a keto group by 3-$\beta$-dehydrogenase. This is followed by rearrangement and migration of the double bond to form progesterone (Rawn, 1989)(Figure 1.6).

Progesterone is in turn converted to the androgens, testosterone and androstenedione. The $C_{17}$ of progesterone is hydroxylated which is followed by oxidative cleavage of the bond between $C_{17}$ and $C_{20}$ to remove acetaldehyde. This is succeeded by oxidation of the 17-hydroxyl group to form a keto group resulting in androstenedione. Further reduction of the $C_{17}$ keto group generates testosterone (Voet and Voet, 1990)(Figure 1.7). The entire biosynthetic process is under the influence of oxidative enzymes, a number of which utilize cytochrome P450 (Rommerts, 1990).

The active androgen in many target tissues is 5$\alpha$-dihydrotestosterone (DHT), and the androgenic activities in a variety of tissues are mediated essentially through the reduction of testosterone to DHT (Neubauer *et al.*, 1993). DHT is considered a second class steroid in that its activity depends upon peripheral mobilization (on-site metabolism within the target cell) (Araneo *et al.*, 1991). This active compound is produced through the conversion of testosterone by the enzyme 5$\alpha$-reductase

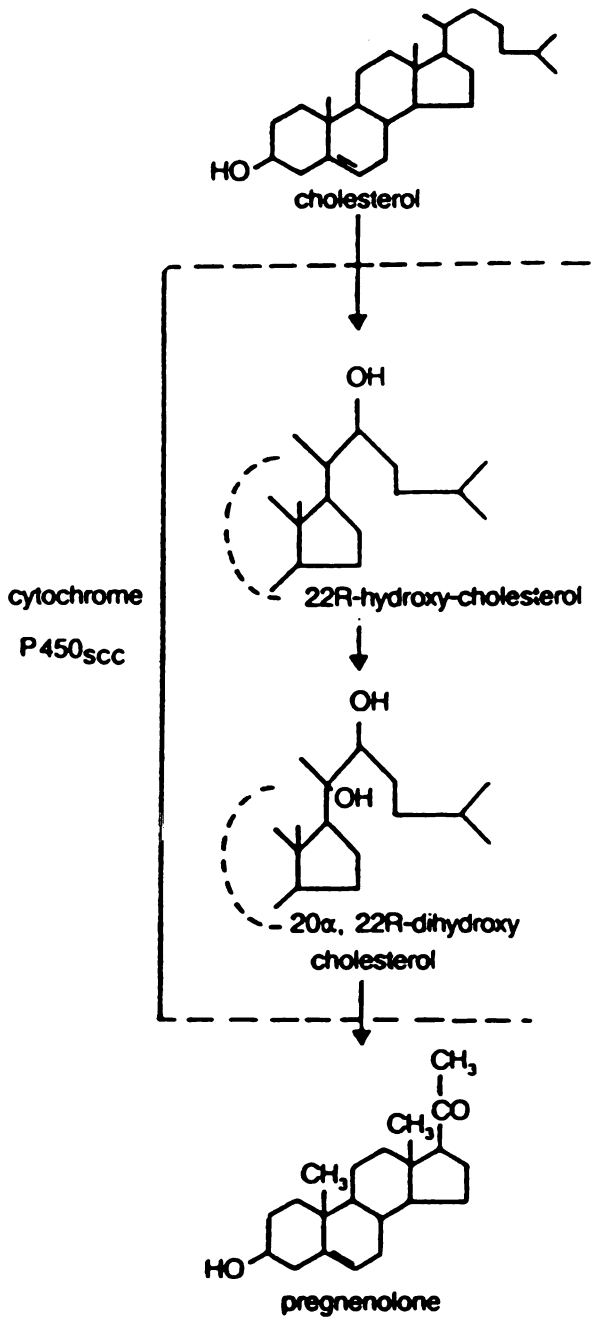



Figure 1.5. Initial step in androgen production--cleavage of the cholesterol side-chain to form pregnenolone (from Rommerts, 1990)

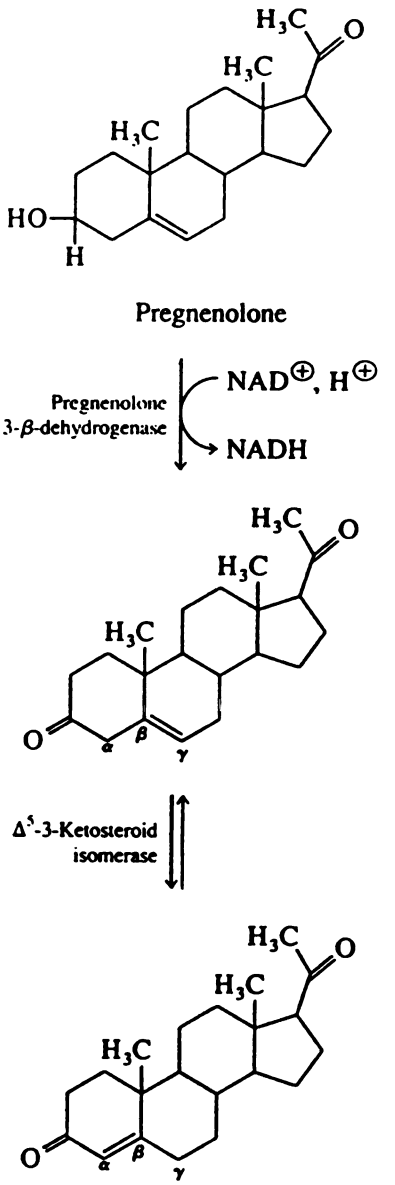



Figure 1.6. Two-step conversion of pregnenolone to form progesterone. (1) the $C_3$ hydroxy is oxidized to a keto group (3-β-dehydrogenase) (2) rearrangement and migration of the double bond by ketosteroid isomerase forms progesterone (from Rawn, 1989)

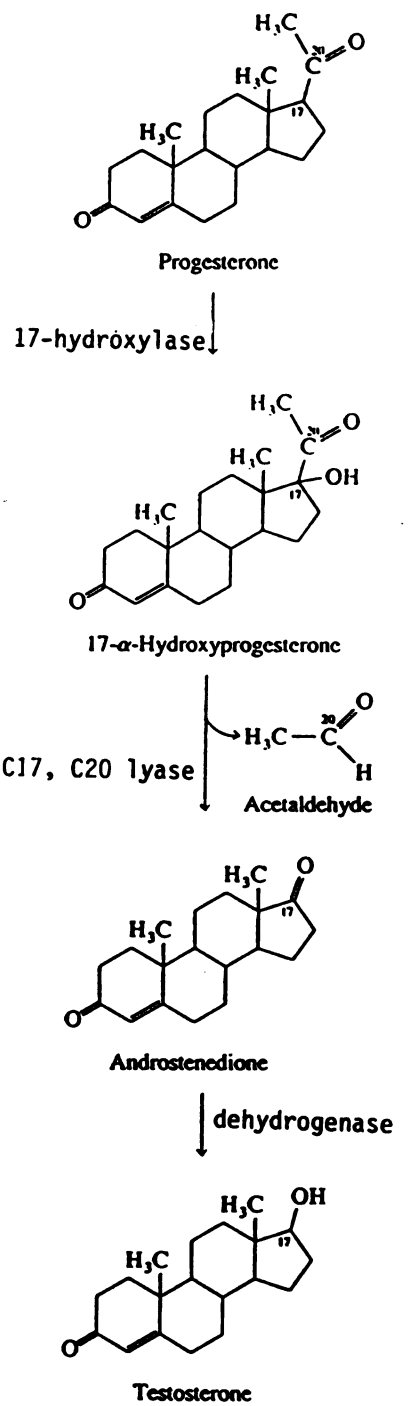



Figure 1.7. Conversion of progesterone to androstenedione and testosterone (from Voet and Voet, 1990)

(Schweikert and Romalo, 1990; Place *et al.*, 1990). DHT is the third fetal hormone and is requisite for normal male somatic differentiation. In addition, binding of DHT to its receptor has generated responses in target tissues such as hyperplasia, hypertrophy, and secretion (Neubauer *et al.*, 1993).

Estrogen

Estrogens are the hormones responsible for the promotion of estrus, and the development and maintenance of female sex organs (Cheesman, 1982). These compounds were first isolated from follicular fluid taken from sow ovaries (Goodman, 1988). Estrogens are found in the blood loosely bound to sex hormone binding globulin (SHBG) and are in the plasma in concentrations much lower than those of other gonadal hormones. However, during the menstrual cycle the concentration of estrogens varies over an approximated 20-fold range (Goodman, 1988).

The $C_{19}$ androgens serve as biosynthetic precursors to the estrogens. The transformation of androgens to estrogens occurs principally in the ovaries, and to a much lesser extent, in the adrenal cortex (Goodman, 1988; Voet and Voet, 1990). Some estrogens are produced in the testes of males (Rawn, 1989). The estrogenic steroids are characterized as the $C_{18}$ series and usually possess an A ring. Aromatization of the A ring of the androgen, androstenedione, results in withdrawal of methyl carbon at $C_{19}$ leading to formation of estrone (Goodman, 1988)(Figure 1.8). The major estrogen is 17$\beta$-estradiol, which possesses the greatest biological activity and is considered to be the most potent of the steroids (Cheesman, 1982). Estradiol is produced from testosterone through successive hydroxylations to convert

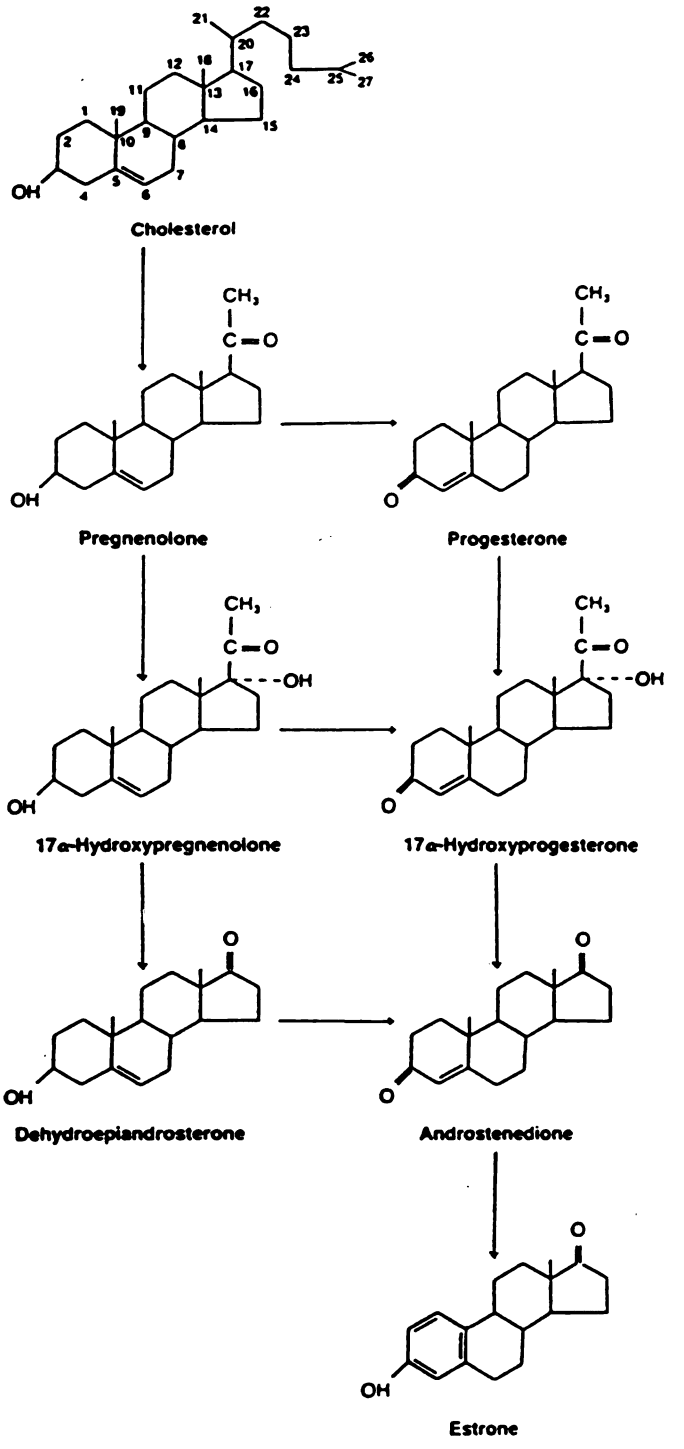



Figure 1.8. Conversion of the $C_{19}$ androgen, androstenedione to the estrogen, estrone (from Goodman, 1988)

the $C_{19}$ methyl to a gem diol. This is followed by a third hydroxylation at the $C_2$ position resulting in the formation of the compound (Rawn, 1989) (Figure 1.9).

Steroid Hormones and Immunity

Steroid hormones have been found to have profound effects on lymphoid cells (Daynes and Araneo, 1991). Extensive research efforts have been focused on the influence of gender and sex hormones on the immune response, yet the complete range of their effects remain poorly understood (Bhalla, 1989; Cohn, 1979). However, it is known that there are estrogen receptors on all tissues in the body, including lymphocytes. Estrogen elicits direct effects on these cells by binding to its receptor on the cell. Once inside the cell, it binds to an estrogen responsive element on the DNA (Denton *et al.*, 1992). In contrast, while there are also testosterone receptors on cells of many tissues in the body, including lymphocytes, they were not characterized as well. Some of the well documented effects of gender on immunity include (1) higher Ig levels in females as to compared to males, (2) more intense antibody responses of females to some microbial challenges, and (3) increased effectiveness of cell-mediated immunity in males (Schuurs and Verheul, 1990).

Studies focusing on estrogens have established a positive correlation between elevated levels of these female sex hormones and the incidence of occurrence of autoimmune diseases (Homo-DeLarche *et al.*, 1991; Siiteri, 1979; Carlsten *et al.*,1991). It has also been demonstrated that estradiol has the capacity to suppress natural killer cell activity, variably alter the functioning of several subsets of T-lymphocytes, facilitate the production of systemic antibody, and enhance B-cell

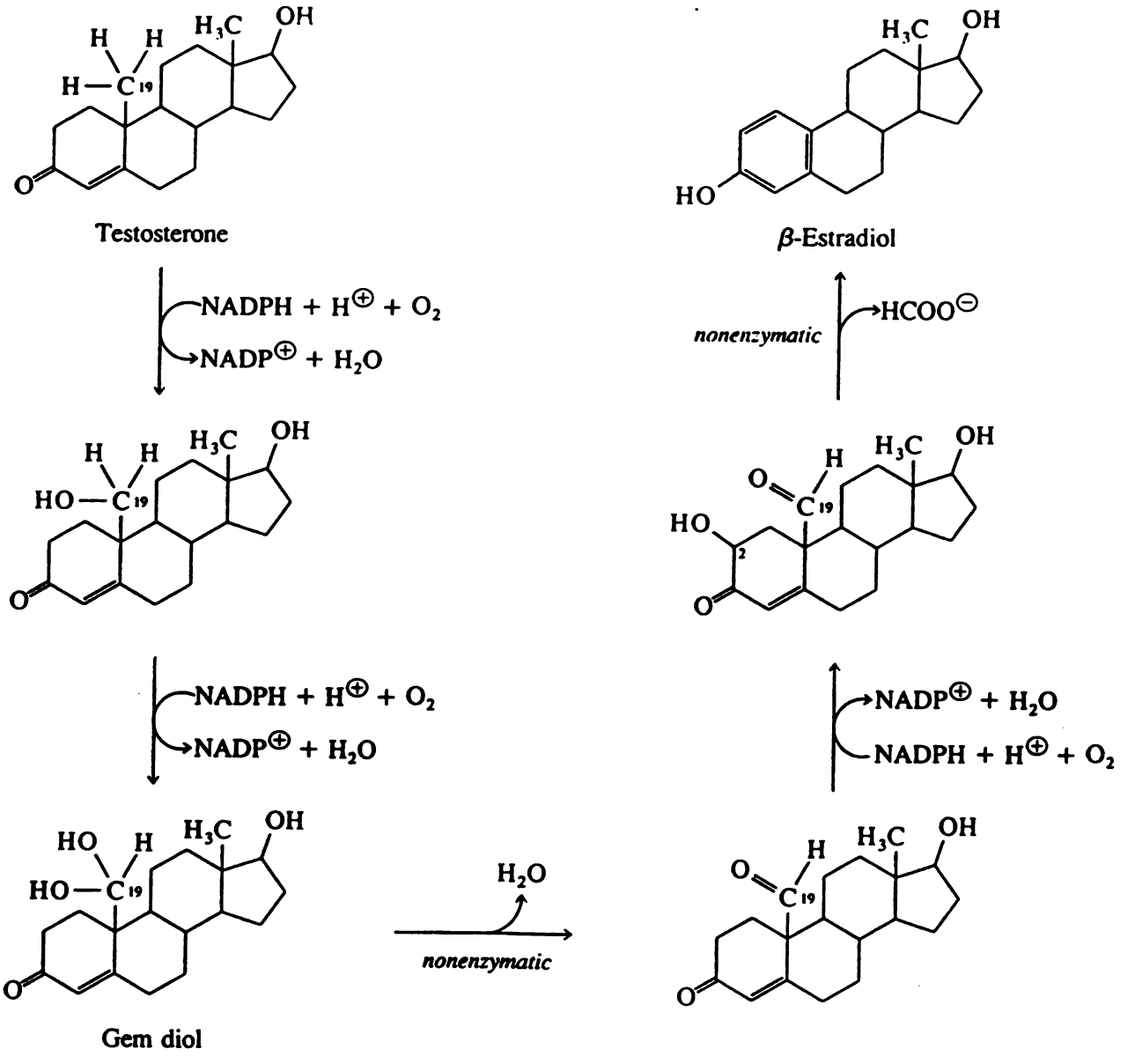



**Figure 1.9. Synthesis of the major estrogen, 17β-estradiol by successive hydroxylations of testosterone (from Rawn, 1989)**

response (Styrt and Sugarman, 1991). Several studies have also indicated thatestrogens suppress the normal functioning of phagocytes and depress macrophage activation by lymphokines (Pfeifer and Patterson, 1985).

The male sex hormones, androgens, have also been investigated to determine whether they affect the immune system and again a positive relationship was established. Araneo and colleagues (1991) illustrated that the active form of testosterone, dihydrotestosterone (DHT), exerts a suppressive effect on the production and elaboration of cytokines IL-4, IL-5, and $\gamma$-interferon by T-lymphocytes without altering their capacity to manufacture IL-2. Additionally, it has been shown that following castration of male mice there is an increase in the mass of the thymus, the spleen, and peripheral lymph nodes (Grossman, 1985). Furthermore, testosterone has been shown to inhibit the antibody response in mammals depending upon the antigen utilized and to interfere with lymphocyte transformation (Slater and Schreck, 1993). It has been proposed that males have a weaker immune response due to their degree of sensitivity to androgen (Cohn, 1979). Researchers also believe testosterone to be immunosuppressive in its ability to increase the activity of suppressor T cells (Cohn, 1979). Since no testosterone receptors have been identified in human peripheral T cells, it has been proposed that testosterone indirectly acts on peripheral T cells through a second messenger (Slater and Schreck, 1993).

**Thesis Rationale**

In previous studies, our laboratory has established that IgA production and dysregulation are induced by dietary VT exposure of B6C3F1 and Balb/C female mice in a manner highly analogous to human IgAN. Originally, the effects of strain and genetic background on VT toxicity were studied. In conjunction, the effect of gender on VT toxicity was investigated. Based on these results, an increase in male susceptibility to VT and VT-induced IgAN was of particular interest since IgAN occurs more frequently in males than females. In previous studies, the optimal dose of VT required to elicit a maximal IgA response had been determined for female B6C3F1 mice. A dose-response study was conducted to further assess gender differences and to determine the optimal VT dose for B6C3F1 male mice. Since it has also been established that VT exposure, *in vivo* or *in vitro*, alters cytokine-mediated T-cell regulation of B-cell activation, and subsequently IgA production, a castration and hormone supplementation experiment was proposed to further investigate the role of the sex hormones, 17$\beta$-estradiol and 5$\alpha$-dihydrotestosterone, in VT toxicity. Because of the effects of sex hormones on cytokine production, and the differences observed between male and female mice, there is the potential for the involvement of these hormones in the mechanism that causes IgA dysregulation.

# Chapter 2. Role of Gender and Strain in Vomitoxin-Induced Dysregulation of IgA Production and IgA Nephropathy in the Mouse

(Accepted in *Journal of Toxicology and Environmental Health*)


## ABSTRACT

Prolonged dietary exposure of female B6C3F1 mice to the trichothecene VT results in hyperproduction of immunoglobulin A (IgA) with a concurrent immunopathology that mimics human IgA nephropathy. To assess the role of gender and strain in the mouse model, semipurified AIN-76A diet containing 25 ppm VT was fed to B6C3F1 male mice and to B6C3F1, Balb/C, C3H/HeN, C3H/HeJ, and C57BL/6 female mice for 8 weeks and immunopathologic indicators of IgA nephropathy were compared to the same parameters in mice fed clean diet. At the cessation of the experiment, all treatment groups weighed less than respective controls. Serum IgA was increased in male and female B6C3F1 mice as well as in C3H/HeJ, C57BL/6, and Balb/c female mice compared to corresponding controls. Serum IgA levels were 2 to 6-fold higher in B6C3F1 male treatment animals compared to female treatment groups from all strains. In contrast, at wk 8 serum IgG levels were unaffected or decreased, and serum IgM was decreased in all groups at wk 8. There was a trend toward increased IgA production by Peyer's patch (PP) lymphocytes isolated from treatment mice as compared to controls in all groups except the C3H/HeJ mice. Notably, IgA levels were 18-fold higher in B6C3F1 male treatment PP cultures than in B6C3F1 female treatment cultures. Hematuria was

significantly greater in treatment mice than respective controls at both wks 4 and 8. Increased mesangial IgA deposition was also detectable in all treatment groups except the C57BL/6 mouse. The results suggested that the male B6C3F1 mouse and the five strains of female mice exhibited many of the immunopathologic effects found in IgA nephropathy and that IgA elevation was more marked in male B6C3F1 than female B6C3F1 mice.

# INTRODUCTION

VT (deoxynivalenol) is a trichothecene mycotoxin that occurs naturally in cereal grains and grain-based food products worldwide (Abouzied *et al.*, 1991; Tanaka *et al.*, 1988). This compound is a potent protein synthesis inhibitor and has been implicated in both animal and human toxicoses (Ueno, 1983). Chronic and subchronic dietary exposure to VT and other trichothecenes results in reduced weight gain, feed refusal, and a reduced feed efficiency. Of particular interest is the ability of VT to be simultaneously immunosuppressive and immunostimulatory (Pestka *et al.*, 1987; Pestka and Bondy, 1990) in a mouse model. Immune stimulation is manifested by a dramatic elevation in serum immunoglobulin A (IgA) production in female B6C3F1 mice that is indicative of an aberrant mucosal immune response (Forsell *et al.*, 1986; Pestka *et al.*, 1989, Dong and Pestka, 1993; Dong *et al.*, 1991; Bondy and Pestka, 1991). This and other IgA-related effects of VT closely parallel human IgA nephropathy, the most common glomerulonephritis in the world (D'Amico, 1987; Emancipator and Lamm, 1989; Julian *et al.*, 1988; Pestka *et al.*, 1989). The latter includes an increase in the ratio of polymeric to monomeric IgA (Pestka *et al.*, 1989), an increased number of IgA-bearing lymphocytes (Pestka *et al.*,1990a), and deposits of IgA in the kidney mesangium accompanied by hematuria (Dong *et al.*, 1991; Dong and Pestka, 1993).

The observation that dietary VT induces murine IgA nephropathy is significant because the toxin may be an etiological factor in the human disease and because it serves as a model for study of the immunopathologic sequelae associated with this

important glomerulonephritis. In order to assess the effects of gender and strain in this experimental model, a study was undertaken in male B6C3F1 mice and several different female mouse strains to compare both the susceptibility to and severity of VT-induced IgA nephropathy. The results suggested that (1) all five strains of female mice exhibited immunopathologic effects consistent with IgA nephropathy and (2) male B6C3F1 mice were more prone to IgA hyperproduction than female B6C3F1 mice.

## MATERIALS AND METHODS

Experimental Design. B6C3F1 male and female mice and female C57BL/6, C3H/HeJ, C3H/HeN, and Balb/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) and the Jackson Laboratory (Bar Harbor, ME). They were randomized and housed (6-8 mice per group; 3 females/cage and 2 males/cage) in environmentally protected cages (Nalgene, Rochester, NY) as formerly described (Pestka, *et al.* 1987). Animals were fed a powdered, semipurified AIN76A diet (ICN Nutritional Biochemical, Cleveland, Ohio) with distilled water provided *ad libitum*; food and water were changed every 3-4 days. Prior to feeding regimens, mice were acclimated for 7 days. Each mouse was weighed weekly and subjected to blood and urine collection at 4 wk intervals. The same mice were used for lymphocyte preparations at the termination of the study.

VT was produced in rice cultures inoculated with *Fusarium graminearum* and purified by water-saturated silica gel chromatography. VT purity was verified by finding a single band by TLC and a single peak using HPLC, as described by Witt, *et al.* (1985). Animals were fed clean diet or diet spiked with 25 ppm VT for 8 wks as reported by Forsell *et al.* (1986). In previous studies with the B6C3F1 female mouse model, this level has been shown to be optimal for inducing elevated serum IgA (Pestka, *et al.* 1989).

Immunoglobulin quantitation. At 4 wk intervals blood was collected from the retro-orbital plexus of ether-anesthetized animals. IgA, IgG, and IgM levels in sera were quantitated by ELISA (Bondy and Pestka, 1991) utilizing standard mouse reference serum (ICN Immunobiologicals, Lisle, IL) and heavy-chain specific goat

anti-mouse IgA, IgG, and IgM and corresponding peroxidase conjugates (Cappel, Malvern, PA).

Hematuria. Mice were placed in metabolism cages and urine samples were collected overnight at 0, 4, and 8 wks (2ml/mouse/12 hours) (Dong *et al.*, 1991). Samples were centrifuged at 500 x g for 10 minutes and sediment was microscopically examined for erythrocytes per microscopic field (x45).

Lymphocyte Cultures. After 8 wks mice were euthanized with $C0_2$, and Peyer's patches (PP) were excised, pooled (2-4 mice/pool) teased apart in Hanks balanced salts media (Hanks, Sigma Chemical Co., St. Louis, MO) with tissue forceps and then passed through a stainless steel screen. Cells were centrifuged at 450 x g for 10 min and resuspended in RPMI medium (pH 7.5) prepared as follows: RPMI-1640 (Sigma chemical Co., St. Louis, MO) supplemented with 5 x $10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 1 mM sodium pyruvate, 25 mM HEPES buffer, and 0.1 mM nonessential amino acids (RPMI). PP lymphocytes were counted with a hemacytometer (American Optical, Buffalo, NY) and viability ascertained by trypan blue dye exclusion. The cells were resuspended in RPMI-containing 10% (v/v) fetal calf serum (5 x $10^5$ cells/well)(Gibco, Laboratories, Chagrin Falls, IL) and cultured in 96 well tissue culture plates (10 replicates per pool) without mitogen. Following 6 d of incubation at 37°C in humidified 7% $C0_2$, supernatants were analyzed by ELISA for IgA production.

Mesangial IgA, IgG, and C3 deposition. At experiment cessation, kidneys were removed and immediately frozen in liquid nitrogen. They were sectioned to 7 $\mu$m on a cryostat (Riechert-Jung, Cambridge Instruments, Buffalo, NY) and stained

with FITC-labeled goat anti-mouse IgA, IgG, and C3 according to the procedure of Valenzuela and Deodhar (1981). Sections were coded, randomized, and viewed under a fluorescence microscope. Ten individual glomeruli per section were viewed and the mean fluorescence intensity was ranked on a scale of 1-6, with 6 indicating very intense fluorescence and 1 representing little or no fluorescence.

Statistical analysis. Data were analyzed using MStat (Michigan State University). Significant differences ($p < 0.05$) between control and VT-treated groups were analyzed by Mann Whitney for two independent groups and ANOVA and Student-Newman-Keul for multiple comparisons.

# RESULTS

To assess the role of gender and strain in the VT-induced IgA nephropathy mouse model, the effects of feeding 25 ppm VT to B6C3F1 male, and to B6C3F1, Balb/c, C3H/HeN, C3H/HeJ and C57BL/6 female mice for 8 wks were assessed. VT treated animals appeared ungroomed. Extensive mortality was observed in C57BL/6, C3H/HeJ, and C3H/HeN treatment groups following prolonged exposure (>4 wks) to toxin; 57, 33 and 25% survived in these groups, respectively, whereas, female B6C3F1, male B6C3F1 and Balb/c groups survived the 8 wk feeding trial with relatively low or no mortality. Significantly reduced body weight gains were observed in all treatment groups as compared to controls (Table 2.1). Only the C3H/HeJ treatment group had a significant weight loss compared to starting weight at wk 0. Although the B6C3F1 male controls gained an estimated 1.5 g more than the female controls, the treatment males exhibited a weight reduction of approximately 2 g more than the female.

As early as 4 wk, serum IgA was significantly increased in B6C3F1 male and female treatment groups, but not in the other strains (Figure 2.1). VT ingestion caused a significant elevation of serum IgA over controls at wk 8 in all treatment groups except C3H/HeN. Additionally, wk 8 serum IgA for B6C3F1 male treatment animals was significantly higher than that for B6C3F1 female animals exposed to the treatment diet.

Although there was a downward trend in serum IgG at 8 wk in treatment mice compared to controls, the effects on serum IgG were inconclusive (Figure 2.2). The B6C3F1, C3H/HeJ, HeN, and Balb/c female groups exposed to VT exhibited

Table 2.1. Effect of 8 wk dietary vomitoxin on body weight.[a]

| | Weight Change (g)[*] | | | |
|---|---|---|---|---|
| | Initial wt | Final wt | Initial wt | Final wt |
| | 0 wks ctrl. | 8 wk ctrl. | 0 wks trt. | 8 wks trt. |
| B6C3F1♀ | 22±9 | 26±1.3[b] | 21±0.3 | 20±0.2[c] |
| B6C3F1♂ | 26±0.9 | 32±1.4[b] | 27±1.2 | 23±1.7[c] |
| C57BL6 | 22±0.6 | 26±1.5[b] | 19±0.6 | 17±0.4[c] |
| C3H/HeJ | 22±1.1 | 27±1.0[b] | 21±0.7 | 15±0.9[b,c] |
| C3H/Hen | 17±0.3 | 29±1.5[b] | 19±0.9 | 19±0.7[c] |
| Balb C | 17±0.4 | 24±0.9[b] | 16±0.2 | 16±0.7[c] |

[*] n=5-8 mice, except C57BL6, C3H/HeJ, and C3H/Hen 8 wks treatment.
[a] Reported as mean weight at initiation and termination of study.
[b] Significantly different from original weight at beginning of study.
[c] Treatment value significantly different from corresponding control value.

Table 2.1. Effect of 8 wk dietary vomitoxin on body weight.[a]

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| C57BL6 | 22±0.6 | 26±1.5[b] | 19±0.6 | 17±0.4[c] |
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[*] n=5-8 mice, except C57BL6, C3H/HeJ, and C3H/Hen 8 wks treatment.
[a] Reported as mean weight at initiation and termination of study.
[b] Significantly different from original weight at beginning of study.
[c] Treatment value significantly different from corresponding control value.

Figure 2.1. Elevation of serum IgA of mice exposed to dietary vomitoxin. Data are means $\pm$ SE; (a) indicates treatment value is significantly different from respective control value ($p \leq 0.05$) and (b) indicates B6 male treatment value is significantly different from corresponding B6 female treatment value ($p \leq 0.05$). ** n=5-8 mice except C57BL6, C3H/HeJ, and C3H/Hen 8 wks treatment.

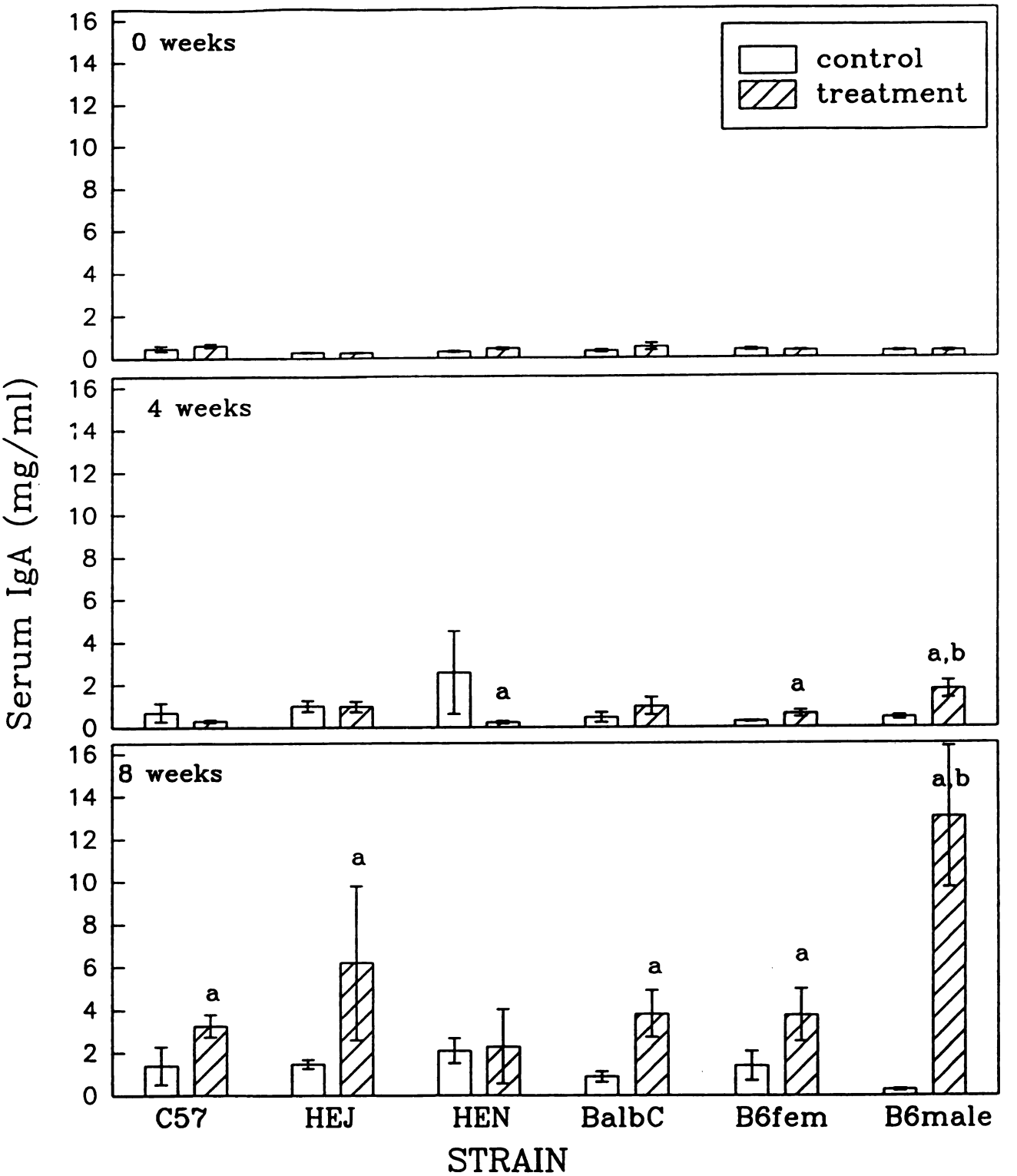

0 weeks
4 weeks
8 weeks
control
treatment
Serum IgA (mg/ml)
STRAIN
C57
HEJ
HEN
BalbC
B6fem
B6male
a
a,b

Figure 2.1

Figure 2.2. Effect of vomitoxin ingestion on serum IgG. Data are means $\pm$ SE; (a) indicates treatment value is significantly different from respective control value ($p \leq 0.05$), and (b) indicates B6 male treatment value is significantly different from corresponding B6 female treatment value ($p \leq 0.05$). ** n=5-8 mice except C57BL6, C3H/HeJ, and C3H/Hen 8 wks treatment.

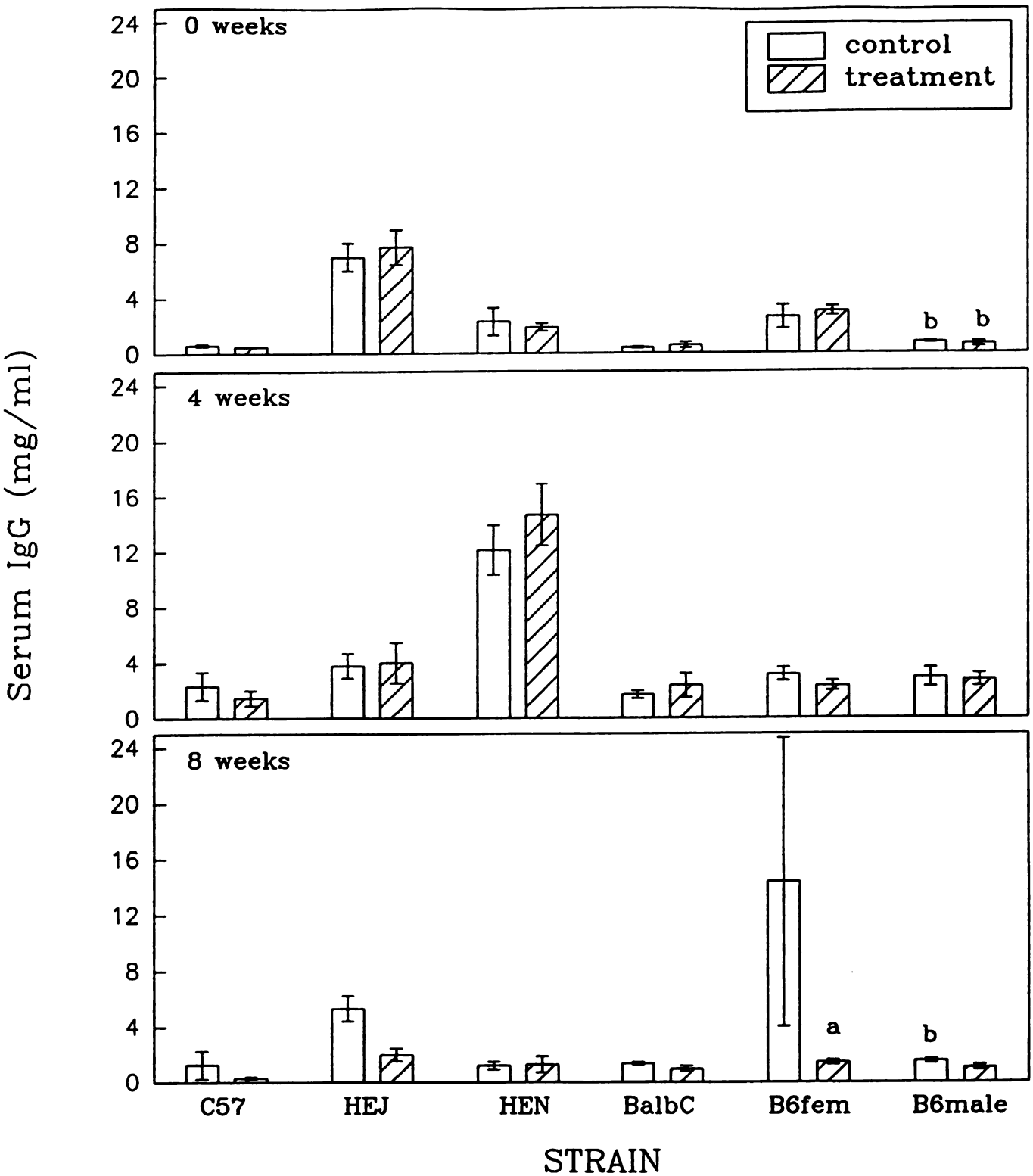

0 weeks
4 weeks
8 weeks
control
treatment
Serum IgG (mg/ml)
0
4
8
12
16
20
24
b
b
a
b
C57
HEJ
HEN
BalbC
B6fem
B6male
STRAIN

Figure 2.2

significantly depressed serum IgM levels at wk 4 whereas all groups had decreased IgM at wk 8 (Figure 2.3). Notably, the B6C3F1 treatment males had a significantly lower IgM level than the B6C3F1 treatment females at wk 8.

PP lymphocytes from all groups were cultured and IgA levels were quantitated to assess the effects on IgA production (Table 2.2). In cultures of treatment PP lymphocytes from Balb/c and C3H/HeN females and B6C3F1 males, IgA levels were approximately 100, 10 and 60-fold higher, respectively than controls. In B6C3F1 mice, IgA was 18-fold higher in treatment cultures from males compared to treatment cultures from females.

Splenic lymphocytes were also cultured in the presence of Con A or LPS or without mitogen stimulation, and IgA production was quantitated (Table 2.3). In unstimulated and stimulated cultures of lymphocytes from treatment B6C3F1 males and females, IgA levels were significantly elevated over the respective control, while the treatment B6C3F1 male values were also significantly elevated over the treatment B6C3F1 females. In addition, IgA levels from LPS stimulated and unstimulated cultured lymphocytes from treatment C3H/HeN and Balb/c, respectively, were significantly higher than their controls (Table 2.3).

When hematuria was used as an index of renal damage there were significantly increased numbers of red blood cells in urine as early as wk 4 in all groups except the B6C3F1 females (Fig. 2.4). At wk 8, all strains showed a significant increase over respective controls. Hematuria in males fed VT was significantly elevated over male control at both wks 4 and 8 and over the corresponding B6C3F1 female

Figure 2.3. Effect of vomitoxin ingestion on serum IgM. Data are means $\pm$ SE; (a) indicates treatment value is significantly different from respective control value ($p \leq 0.05$), and (b) indicates B6 male treatment value is significantly different from corresponding B6 female treatment value ($p \leq 0.05$). ** n=5-8 mice except C57BL6, C3H/HeJ, and C3H/Hen 8 wks treatment.

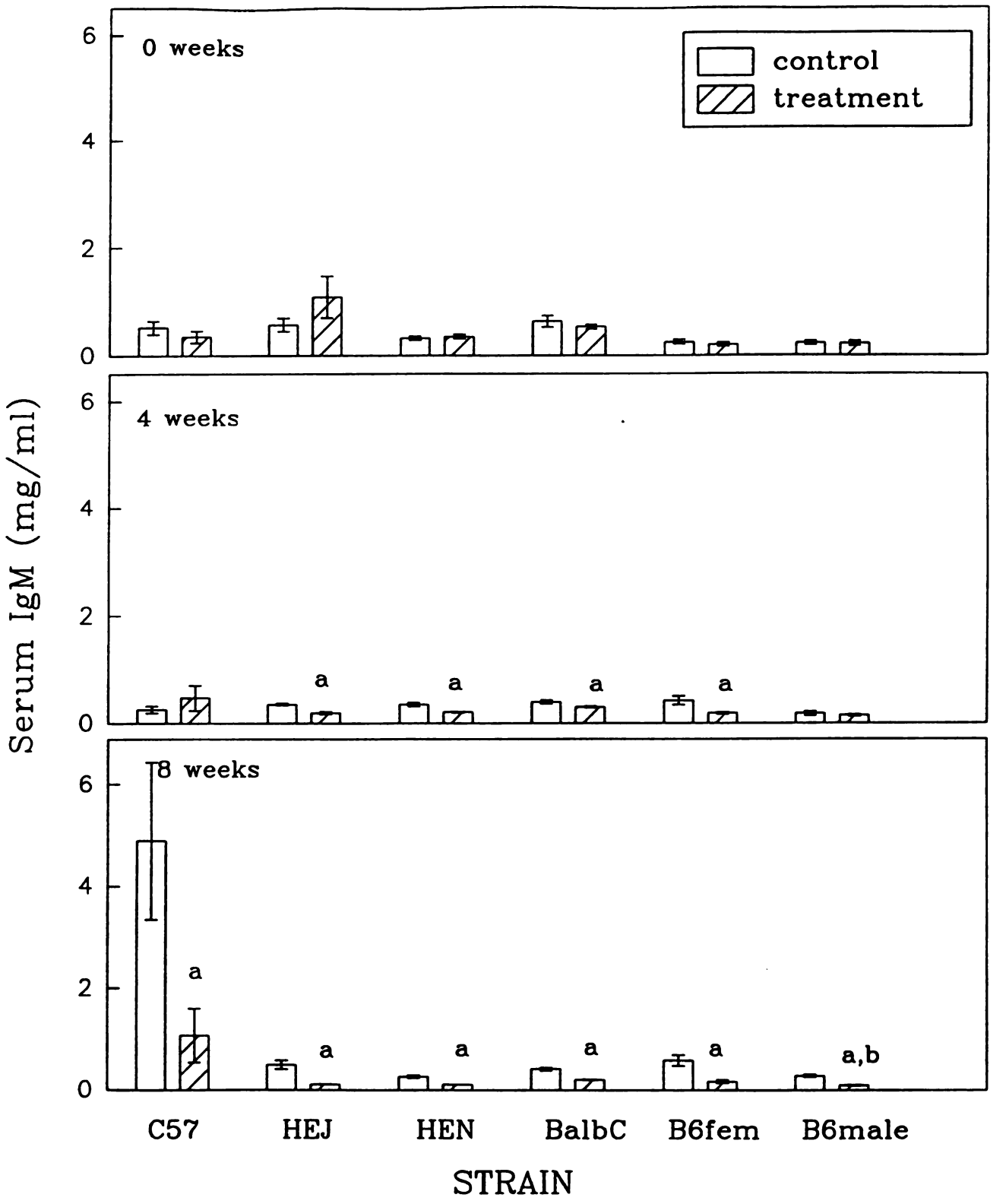

0 weeks
4 weeks
8 weeks
control
treatment
Serum IgM (mg/ml)
0
2
4
6
a
a,b
C57
HEJ
HEN
BalbC
B6fem
B6male
STRAIN

**Figure 2.3**

Table 2.2. Effect of dietary vomitoxin on IgA production in unstimulated Peyer's patch cultures.[a]

| | IgA (μg/ml) | |
|---|---|---|
| | Control | Treatment |
| B6♀ | 0.8±0.23 | 0.5±2.3 |
| B6♂ | 1.1±0.4 | 63.1±12.8[b] |
| C57BL6 | 1.4±0.1 | 3.6±0.7 |
| C3H/HeJ | 4.2±2.3 | 0.5±0.1 |
| C3H/HeN | 2.0±0.4 | 19.8±7.7 |
| Balb/c | 0.4±3.1 | 38.5 ± 16.2 |

[a] Data are means of two pooled groups ± SE
[b] Indicates treatment significantly different from control at $p \leq 0.05$

Table 2.3 Effect of dietary vomitoxin on IgA production in spleen cultures[a]

| | Control | Treatment |
|---|---|---|
| ConA | | |
| B6♀ | 51±12 | 102±15[b] |
| B6♂ | 167±21 | 1320±100[b,c] |
| C57BL6 | 700±160 | 780±79 |
| C3H/HeJ | 273±30 | 256±17 |
| C3H/HeN | 226±60 | 234±34 |
| BalbC | 9830±1382 | 15200±3600 |
| LPS | | |
| B6♀ | 1050±107 | 2230±209[b] |
| B6♂ | 975±100 | 4868±522[b,c] |
| C57BL6 | 2296±209 | 2583±300 |
| C3H/HeJ | 1425±348 | 979±138 |
| C3H/HeN | 1352±735 | 5440±1931[b] |
| Balb/c | 1007±65 | 1675±311 |
| Unstimulated | | |
| B6♀ | 299±49 | 497±60[b] |
| B6♂ | 776±241 | 2600±[b,c] |
| C57BL6 | 1066±483 | 1953±633 |
| C3H/HeJ | 11275±835 | 297±44 |
| C3H/HeN | 179±35 | 239±35 |
| Balb/c | 965±355 | 12500±6000[b] |

[a] Data reported as mean IgA level (ng/ml) ± SEM of two pooled groups
[b] Significantly different ($p<0.05$) from respective control
[c] Significantly different ($p<0.05$) from corresponding female counterpart

Figure 2.4. Effect of vomitoxin ingestion on microscopic hematuria. Data are means ± SE; (a) indicates treatment value is significantly different from respective control value ($p<0.05$) (b) indicates B6 male treatment value is significantly different from corresponding B6 female treatment value ($p<0.05$) . ** n=5-8 mice except C57BL6, C3H/HeJ, and C3H/HeN 8 wks treatment.

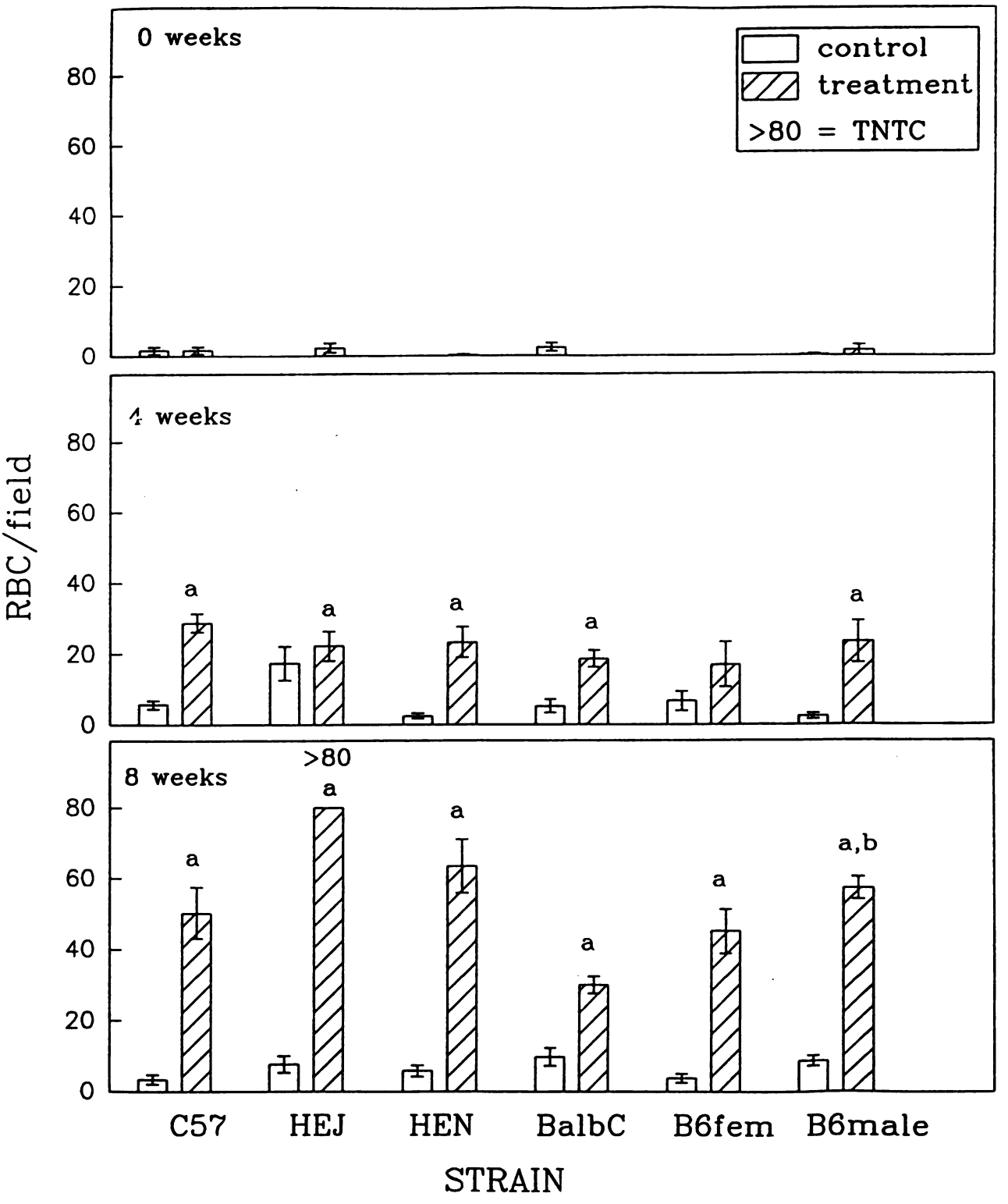

0 weeks
4 weeks
8 weeks
control
treatment
>80 = TNTC
RBC/field
0
20
40
60
80
a
>80
a,b
C57
HEJ
HEN
BalbC
B6fem
B6male
STRAIN

**Figure 2.4**

treatment group at wk 8. Glomerular IgA, IgG and C3 deposition were assessed with immunofluorescence microscopy (Table 2.4). Mesangial accumulation of IgA was evident in B6C3F1 males and all female treatment mice with the exception of the C57BL/6 (Figure 2.5). Mesangial deposition of IgG appeared to marginally decrease from control to treatment in B6C3F1 males and Balb/c mice, but similar patterns were not evident in other groups. Finally, increased mesangial C3 deposition was observed in B6C3F1 males and females and C3H/HeJ females (Figure 2.6).

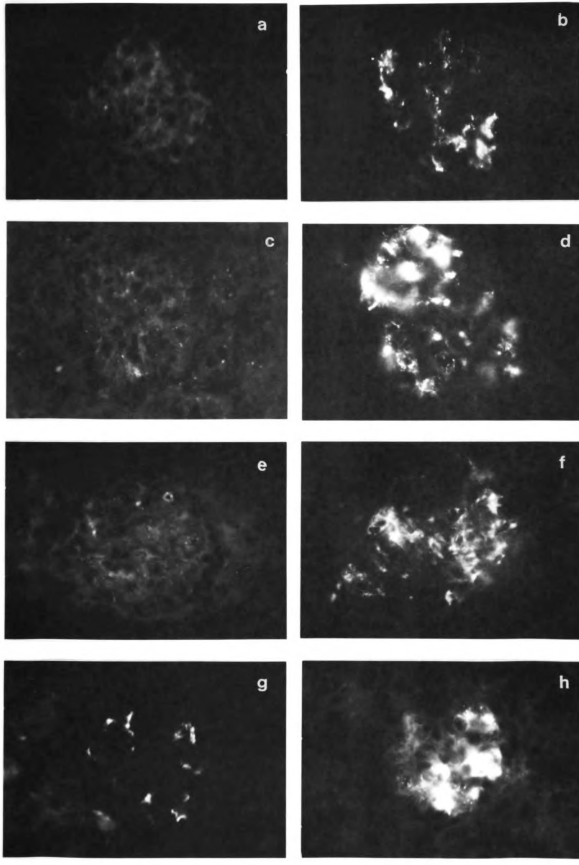
Table 2.4. Effect of dietary vomitoxin on mesangial deposition of IgA, IgG and $C_3$.[a]

| | Mean Fluorescence Intensity[*] | |
|---|---|---|
| Deposition | Control | Treatment |
| **IgA** | | |
| C57BL6 | 3.2 | 2.6 |
| C3H/HeJ | 2.8 | 3.7 |
| C3H/HeN | 2.7 | 2.9 |
| BalbC | 1.9 | 2.5 |
| B6♀ | 1.6 | 2.4 |
| B6♂ | 1.8 | 3.5 |
| **IgG** | | |
| C57BL6 | 1.7 | 1.9 |
| C3H/HeJ | 2.1 | 2.4 |
| C3H/HeN | 1.3 | 1.6 |
| BalbC | 1.9 | 1.0 |
| B6♀ | 1.1 | 1.4 |
| B6♂ | 1.8 | 1.5 |
| **$C_3$** | | |
| C57BL6 | 2.8 | 2.6 |
| C3H/HeJ | 1.4 | 2.9 |
| C3H/HeN | 2.0 | 2.0 |
| BalbC | 1.6 | 1.7 |
| B6♀ | 2.1 | 2.4 |
| B6♂ | 1.6 | 3.0 |

[*] n=40-80 except C3H/HeJ treatment, where n=20.

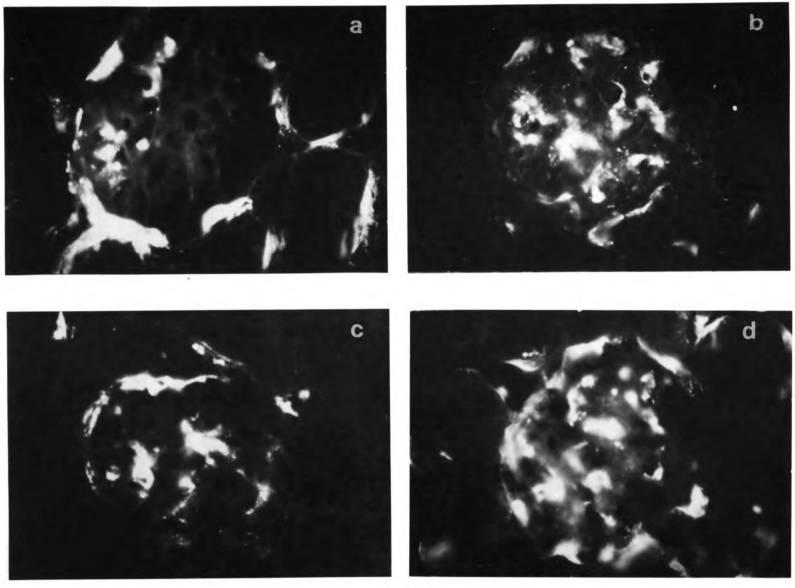
[a] Mice were fed 25 ppm vomitoxin for 8 wks. Results are mean rank on a scale of 1-6 as described in Materials and Methods.

Figure 2.5. Effect of vomitoxin ingestion on mesangial IgA deposition. Kidney sections prepared at wk 8 with FITC-labeled anti-mouse IgA. (a) C3H/HeJ control (b) C3H/HeJ treatment (c) Balb/c control (d) Balb/c treatment (e) B6C3F1 female control (f) B6C3F1 female treatment (g) B6C3F1 male control (h) B6C3F1 male treatment.


a
b
c
d
e
f
g
h

IgA S/C

Figure 2.6. Mesangial $C_3$ deposition in control and treatment groups. Kidney sections prepared at wk 8 with FITC-labeled anti-mouse $C_3$. (a) C3H/HeJ control (b) C3H/HeJ treatment (c) B6C3F1 male control (d) B6C3F1 male treatment.


a
b
c
d

# DISCUSSION

Exposure to dietary VT at the 25 ppm level was chosen for comparative purposes because it represents an optimal concentration for IgA elevation in the B6C3F1 female mouse (Pestka *et al.*, 1989). The concentration is equivalent to 1/20 of the LD50 mg/kg body weight/day for this mouse (Forsell *et al.*, 1986). The predominant toxic manifestations reported in B6C3F1 female mice fed VT at this level are reduced body weight gain, and organ weights, lymphopenia and disturbance of the typical serum Ig profile (Forsell *et al.*, 1986). Reduced body weight observed here in all treatment groups was consistent with earlier reported results in the B6C3F1 female mouse (Forsell *et al.*, 1986; Dong and Pestka, 1993). The decreases in weight gain can most likely be explained by a reduced feed conversion efficiency and/or feed refusal which was qualitatively observed. Rotter *et al.* (1992) reported reduced feed intake and efficiency of food conversion in outbred female mice fed VT. In comparing B6C3F1 mice fed VT, males had a greater net weight loss than females. It is possible that the variations in depressed body weight gain between male and female mice might be attributed to sex differences in the growth response to VT-spiked diet as has been previously reported in swine by Cote, *et al.* (1985).

Several strains of mice were chosen in this study to assess the potential contribution of genetic background to susceptibility to VT-induced dysregulation of IgA production. Chronic VT toxicity has been studied in the B6C3F1 mouse model in our laboratory because this hybrid strain is known for its hardiness and longevity and thus it has been typically used in carcinogenicity and immunotoxicity studies (Cameron *et al.*, 1985). The inbred C57BL/6 and C3H/HeN strains were selected to

monitor the effects of VT on the parental strains of the B6C3F1 mouse. The C3H/HeJ strain is genetically comparable to the C3H/HeN, but is LPS-nonresponsive and a high IgA responder (Babb and McGhee, 1980; Kiyono *et al.*, 1982). Finally, Balb/c mice were included because these are a common model in immunological studies. The relatively high mortality found in C3H/HeJ, C3H/HeN and C57BL/6 strains may have resulted form increased sensitivity to the toxin by these inbred strains and made it difficult to statistically compare immunopathologic effects among these groups.

Elevated serum IgA found for all female treatment animals was consistent with previous findings (Forsell *et al.*, 1986; Dong *et al.*, 1991; Pestka *et al.*, 1989). However, the largest increase over the respective control group was seen in the B6C3F1 male mice, which had serum IgA levels 47 fold higher than corresponding controls at wk 8, compared to a 2.7 fold increase in the B6C3F1 treatment females. Reduction in serum IgM might suggest that isotype switching contributed to the IgA increase or be the result of oral tolerance (Pestka *et al.*, 1990c; Rasooly and Pestka, 1992; Emancipator and Lamm, 1989). VT-induced IgA hyperproduction involves both the mucosal and systemic compartments of the immune system, although the exact mechanisms remain unclear (Pestka *et al.*, 1990b; Bondy and Pestka, 1991). PP are gut-associated lymphoid tissue that are situated along the small intestine and are the primary IgA inductive sites (Mestecky and McGhee, 1987; Mestecky, 1988). Our laboratory has previously shown that VT acts at the PP level by enhancing terminal differentiation to IgA-secreting cells (Pestka *et al.*, 1990a; 1990b; Bondy and Pestka, 1991). During VT exposure, quantitative changes are observed in the

lymphocyte profile that include increased IgA+ cells, CD4+ cells, T cells, and the CD4+: CD8+ ratio (Pestka *et al.*, 1990a), thus suggesting that T cells may play a role in this dysregulation. The results presented here suggested that isolated PP lymphocytes from various strains tested also produced larger amounts of IgA when obtained from VT-fed mice. Notably, as found for serum IgA, male mice produced larger amounts of IgA than female mice. Similarly, IgA levels of isolated splenocytes from treated males were also significantly elevated over their female counterpart which was consistent with serum IgA results.

Since hematuria is a marker of kidney damage, it was notable that the treatment animals exhibited this affect as early as wk 4. Male treatment mice had a higher number of red blood cells in their samples as compared to B6C3F1 female treatment mice, indicating a more severe hematuria and greater kidney damage. Increased accumulation of mesangial IgA in the glomeruli was observed in all treatment animals except the C57BL6, further suggesting that all strains experienced induction of glomerulonephritis. However, deposition of IgA in the kidney was most obvious in the B6C3F1 male mice. In human IgA nephropathy there is often codeposition of IgG and IgM with $C_3$ detectable in the Bowman's capsule (Emancipator *et al.*, 1987). In results reported here, IgG was detectable in sections from treatment and control animals, but was essentially the same. Mesangial $C_3$ appeared to increase marginally in the B6C3F1 females and to a much larger extent in the B6C3F1 males and C3H/HeJ females. This was inconsistent with observations reported previously by our laboratory (Dong *et al.*, 1991; Dong and Pestka, 1993) in which significant mesangial $C_3$ deposition was found in VT-fed B6C3F1 females, but

in agreement with several other experimental animal studies of IgA nephropathy (Emancipator and Lamm, 1989; Sategna-Guidetti, *et al.* 1992; Montinaro *et al.*, 1992). These latter investigations suggested that codeposits of IgG, IgM or $C_3$ may be responsible for the variations in glomerular function that occur in this glomerulonephritis (Emancipator *et al.*, 1987). A possible explanation for the discrepancy between results in our laboratory may be that Dong *et al.* (1993) employed an image analysis technique to measure fluorescence of glomeruli in polygonal areas that included Bowman's capsule. Since C3 is readily detectable in the Bowman's capsule of kidneys from control mice, subtle differences in mesangial accumulation may not have been not detectable. In contrast, this study employed visual ranking that focused on the mesangial matrix, since this is where the deposition occurs.

The reasons for increased sensitivity of male mice to VT and susceptibility to enhanced IgA production are problematic. It is possible that endogenous steroid hormones, specifically estrogens, enhance the cytochrome P-450-mediated detoxification of VT (Cote *et al.*, 1985). Estrogens also have the capability to inhibit lymphocyte proliferative responses (Forsberg, 1984), affect the quantity of lymphocytes and monocytes (Luster *et al.*, 1984), and exert a depressive influence on macrophage activation by lymphokines (Pfeifer and Patterson, 1985). Androgens, specifically dihydrotestosterone (DHT), have also been known to regulate the immune response. Males often exhibit an increased effectiveness of cell-mediated immunity (Schuurs and Verheul, 1990). Araneo *et al.* (1991) found that DHT suppressed production of cytokines IL-4, IL-5, and $\gamma$-interferon. It is thus possible that DHT

exacerbates the effects of VT by altering T cell-driven B cell expansion and differentiation to IgA secreting cells.

In summary, the results presented here suggest that all strains of mice studied were susceptible to VT-induced dysregulation of IgA production and IgA nephropathy. While consistent differences in the immunopathogenic parameters tested were not readily apparent among females of the various strains, male B6C3F1 mice exhibited more marked elevation of serum IgA, *in vitro* PP IgA production, impaired weight gain, hematuria, and IgA deposition in the kidney glomerulus than female B6C3F1 mice. Interestingly, these results mimic human IgA nephropathy in that this glomerulonephritis occurs more frequently in males than in females (Schena, 1990; Emancipator and Lamm, 1989; Bene and Faure, 1987). Future investigations will seek to clarify the role of gender and to investigate the contribution of sex hormones to VT-induced dysregulation of IgA production and IgA nephropathy.

# Chapter 3. Vomitoxin (Deoxynivalenol)-Induced IgA Nephropathy in the B6C3F1 Mouse: Dose Response and Male Predilection

(Accepted in *Toxicology*)


## ABSTRACT

Oral exposure to the trichothecene vomitoxin (VT or deoxynivalenol) in mice induces marked elevation of total and autoreactive IgA, IgA immune complexes, and mesangial IgA deposition in a manner that is highly analogous to human IgA nephropathy. In this study, immunopathologic markers indicative of IgA nephropathy were compared in male and female B6C3F1 mice fed semipurified AIN-76A diet containing 0, 2, 10 or 25 ppm VT for 12 wks. Males fed 10 and 25 ppm VT and females fed 25 ppm VT had increased serum IgA at 4 wks. At wk 8, male mice fed the minimal dose of 2 ppm VT and female mice fed 10 ppm also exhibited elevated serum IgA. IgA levels were consistently higher in treatment males than females with significant differences being observed in 10 ppm dose group at 4 and 12 wks. IgA coproantibodies (fecal antibodies) were marginally increased (maximum of 2-fold) in mice of both genders fed 10 and 25 VT. At 8 and 12 wks, serum IgM was depressed in male and female mice eating 10 and 25 ppm VT, whereas consistent effects on serum IgG or IgE were not observed. In similar fashion, male mice in the 2, 10 and 25 ppm VT groups exhibited microscopic hematuria as early as 4 wks, whereas this occurred in females fed 10 and 25 ppm VT only at wk 10 with urinary erythrocyte counts being lower than male

counterparts. Mesangial deposition of IgA and $C_3$ was significantly increased in males exposed to 2, 10 and 25 ppm VT and in females exposed to 10 and 25 ppm VT, with males exhibiting a greater deposition than corresponding females. Based on these immunological parameters, males appeared more susceptible than female mice to VT-induced IgA dysregulation and IgA nephropathy in terms of latency, threshold dose, and severity.

## INTRODUCTION

Vomitoxin (VT or deoxynivalenol) is a toxic, secondary metabolite of *Fusarium graminearum* that commonly occurs in grains and cereal-based food products (Abouzied *et al.*, 1991; Tanaka *et al.*, 1988). This compound is a potent protein synthesis inhibitor and has been associated with animal and human toxicoses (Ueno, 1983; CAST, 1989). Interestingly, VT and other trichothecenes can be simultaneously immunosuppressive and immunostimulatory (Pestka and Bondy, 1990). The latter is most prominently manifested by aberrant elevation of total and autoreactive serum IgA, increased polymeric to monomeric IgA ratio in serum and accumulation of IgA in the kidney glomerulus (Forsell *et al.*, 1986; Pestka *et al.*, 1989; Pestka *et al.*, 1990a,c; Bondy and Pestka, 1991; Rasooly and Pestka, 1992). All of these markers mimic human IgA nephropathy (Berger's Disease) (D'Amico, 1987). Although the etiology of this disease remains unknown, it is considered to be the most common glomerulonephritis in the world, affecting 2-5 times more males than females (Schena, 1990).

Recently, we have observed that dietary exposure to 25 ppm VT induces IgA nephropathy in several strains of mice and that male B6C3F1 mice appeared to exhibit greater increases in serum IgA and more mesangial IgA deposition than their female counterparts (Greene *et al.*, 1994b). Further investigation into possible differences in sensitivity between male and female animals to VT-induced IgA nephropathy is of considerable interest because foodborne VT may be one of several etiological factors in the human disease. Additionally, such a comparison can serve as a model for understanding immunopathologic mechanisms involved in male predilection for IgA nephropathy. The purpose of this study was to compare dose response effects

of dietary VT in male and female B6C3F1 mice. The results strongly indicate that males were more susceptible than female mice to VT-induced IgA dysregulation and IgA nephropathy in terms of exposure time, dose level, and severity.

## MATERIAL AND METHODS

Animals and Diet. B6C3F1 male and female mice (8 wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN), housed (2 males/cage and 3 females/cage) and acclimated for 1 wk in environmentally protected cages (Nalgene, Rochester, NY) as formerly described (Pestka *et al.*, 1987). Cages were kept in a temperature controlled room with a 12 hr light and dark cycle. Control animals (0ppm) were fed powdered, semipurified AIN-76A diet (ICN Biochemical, Cleveland, Ohio). Treatment groups were fed AIN-76A spiked with VT (2, 10 or 25 ppm) (Forsell *et al.*, 1986) that was purified from *Fusarium graminearum* cultures as described by Witt *et al.*, (1985). Feeding regimens were carried out for 12 wks with food and water being changed every 3-4 days and water provided *ad libitum*.

Immunoglobulin quantitation. Serum samples were collected from the orbital plexus of ether-anesthetized animals every 4 wks. Serum IgA, IgG, and IgM were quantitated by enzyme-linked immunosorbent assay (ELISA) (Pestka *et al.*, 1990a) utilizing standard mouse reference serum (ICN Immunobiologicals, Lisle, IL) heavy-chain specific goat, anti-mouse IgA, G or M as a capture antibody and corresponding goat, anti-mouse peroxidase conjugates (Cappel, Malvern, PA) detection antibodies. For IgE, a modified ELISA (Pestka and Dong, 1994) was used that employed mouse IgE, heavy chain specific, rat anti-mouse IgE and corresponding peroxidase conjugate from Pharmingen, San Diego, CA.

Fecal pellets were also collected for IgA measurement at 7 and 12 wks. IgA coproantibodies were detected by ELISA of fecal suspensions prepared as reported by deVos and Dick (1991).

Hematuria. Urine samples were collected overnight from individual mice in metabolic cages at 4 and 10 wks as described by Dong *et al.*, (1991). Samples were centrifuged at 500 x g for 10 min and sediment was microscopically examined for erythrocyte presence per microscopic field (x45).

Quantitation of Mesangial IgA, IgG, and C3. After 12 wks, animals were euthanized with $CO_2$ and kidneys were removed, separated into halves, mounted on corks in embedding media and immediately frozen in liquid nitrogen. These were sectioned to 7 $\mu$m on a cryostat (Riechert-Jung, Cambridge Instruments, Buffalo, NY) and stained for immunoglobulin or complement deposition with fluorescein-labeled goat anti-mouse IgA, IgG or $C_3$ according to the procedure of Valenzuela and Deodhar (1981). Sections from each animal were viewed under an epifluorescence microscope. Fluorescence intensities in ten glomeruli from each section were measured using an ITM densitometric video camera (Waltham, MA) and JAVA image analysis system (Jandel Scientific, San Rafael, CA). Representative photographs were taken on the same microscope using Kodak Tri-X Pan B & W film.

Statistical Analysis. Data were analyzed using MStat (Michigan State University). Differences between control and VT-treated groups were analyzed by Mann Whitney for two independent groups and by ANOVA and Student-Newman-Keul for multiple comparisons.

## RESULTS

Diet containing 0, 2, 10 and 25 ppm VT was fed to male and female B6C3F1 mice for 12 wks and various immunologic parameters indicative of IgA nephropathy were compared. All groups survived the feeding trial without significant mortality being observed. Ruffled fur without hair loss was noted in the 2 and 10 ppm male and female treatment groups, whereas alopecia (hair loss) in conjunction with a matted coat and an ungroomed appearance were observed in the males and females fed 25 ppm VT. Significantly reduced body weight gains were also observed in all male and female treatment groups when compared to controls (Table 3.1). Male and female control mice had mean weight gains of 7g and 9g, respectively, and in the 2 ppm treatment group, a 2 g difference was also observed between male and female mice. In the 10 ppm treatment groups, mean weight gain was approximately the same when comparing males with females. The only treatment group that experienced a significant weight loss was males fed 25 ppm VT.

Serum IgA was significantly elevated as early as wk 4 in the 10 and 25 ppm male treatment mice as well as in 25 ppm female treatment mice (Figure 3.1). At wk 8, males fed 2, 10 and 25 ppm VT had significantly elevated serum IgA (2, 8 and 10-fold increases, respectively) over controls whereas only females fed 10 and 25 ppm exhibited increased IgA (5 and 9 fold increases, respectively). At wk 12, only male and female mice fed 10 ppm VT had significantly increased serum IgA levels with males again exhibiting higher levels than females.

Coproantibodies were measured at 7 and 12 wks to assess the effects of VT on intestinal IgA (Table 3.2). There was a general trend towards increased IgA but the

Table 3.1. Effect of dietary vomitoxin on body weight changes in male and female mice.*†

| | Female | | Male | |
|---|---|---|---|---|
| DOSE | Initial wt. | Final wt. | Initial wt. | Final wt. |
| 0 ppm | 21±0.7 | 28±2 | 25±1 | 34±1 |
| 2 ppm | 20±0.2 | 24±0.6[a] | 25±0.5 | 31±1[a] |
| 10 ppm | 20±0.4 | 22±0.5[a] | 25±0.3 | 28±0.7[a,b] |
| 25 ppm | 20±0.3 | 20±0.4[a] | 25±0.3 | 24±0.8[a] |

* n = 7-9 mice per group.
† Reported mean weight at the initiation and termination of study ± SEM
[a] indicates significantly different ($p < 0.05$) from control (0 ppm).
[b] indicates significantly different ($p < 0.05$) from preceding lower dose.

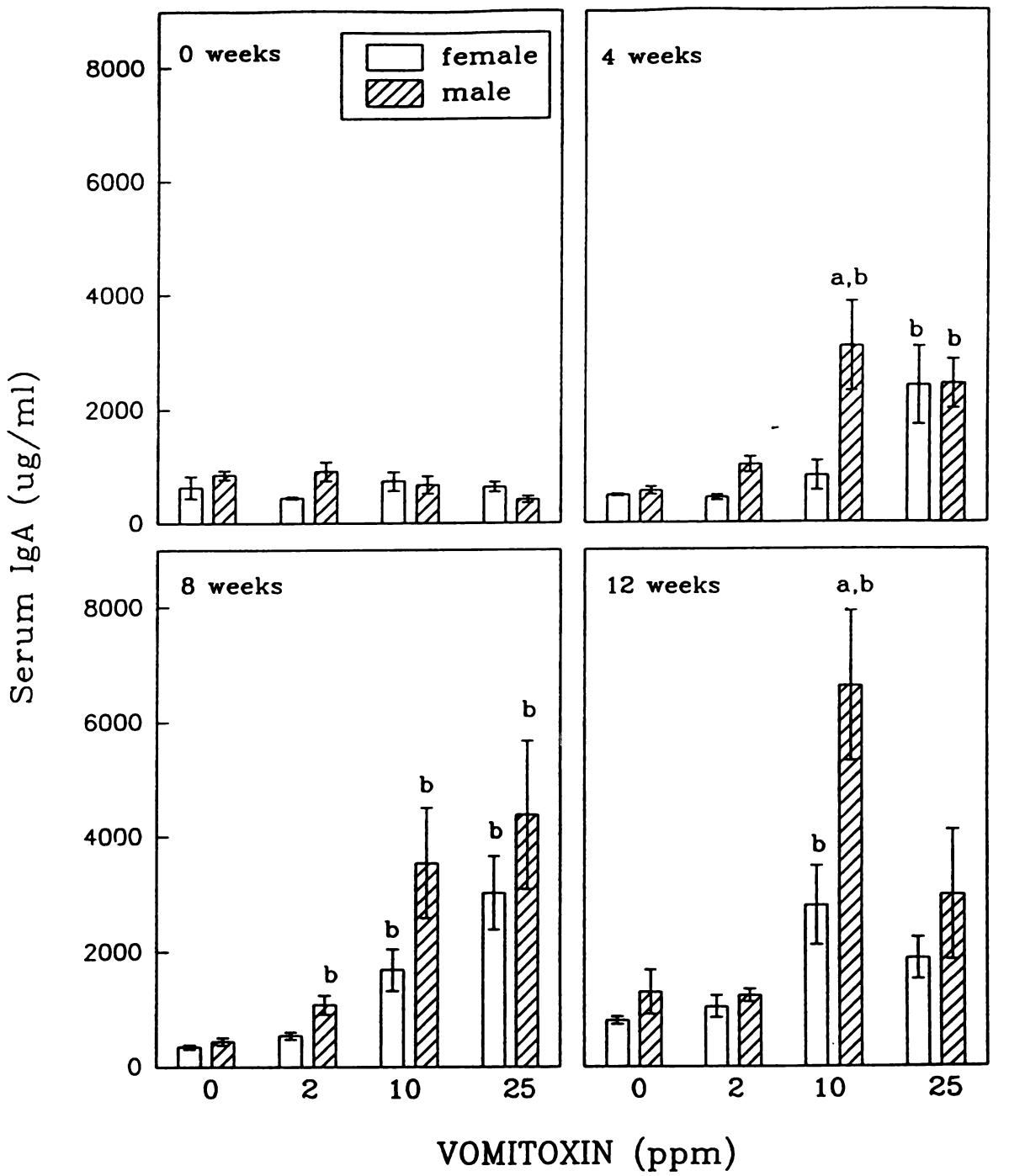



**Figure 3.1. Effect of dietary vomitoxin on serum IgA of male and female mice. Open and hatched bars indicate females and males, respectively. Data are means ± SEM (n = 7-9 mice per group). (a) indicates significantly different ($p < 0.05$) from corresponding female values and (b) indicates significantly different ($p < 0.05$) from respective control value.**

Table 3.2. Effect of dietary vomitoxin on IgA coproantibody in male and female mice.[a]

| | | Vomitoxin Dose[*] | | | |
|---|---|---|---|---|---|
| Wk | Group | 0 ppm | 2 ppm | 10 ppm | 25 ppm |
| 7 | female | 2.4±0.3 | 5.2±1.6 (2.2) | 2.4±0.3 (1.0) | 2.0±0.1 (0.8) |
| 7 | male | 1.3±0.2 | 1.6±0.2 (1.2) | 2.1±0.5[b](1.6) | 2.6±0.2[b](2.0) |
| 12 | female | 1.9±0.3 | 3.4±2.0[c](1.8) | 2.2±0.3 (1.2) | 3.9±0.7[c](2.1) |
| 12 | male | 1.8±0.3 | 1.8±0.2 (1.0) | 2.0±0.3 (1.1) | 3.8±0.6[b](2.1) |

[*] n = 7-9 mice per group
[a] Data are mean ± SEM, ( ) indicates fold difference from control
[b] indicates significantly different ($p<0.05$) from respective control (0ppm)
[c] indicates significantly different ($p<0.05$) from corresponding 7 wk value

magnitude of increase was not as dramatic as in serum IgA. Male mice fed the 10 ppmVT for 7 and 12 wks and 25 ppm for 7 wks exhibited significantly higher fecal IgA levels than their respective controls. Female mice exhibited similar trends but these were not significant.

Serum IgM appeared to increase over time in the 0 and 2 ppm treatment groups (Figure 3.2). A significant elevation was observed in the 2 ppm male mice at 4 wks. However, serum IgM was lower in 10 and 25 ppm VT-treated male and female animals than corresponding controls at 8 and 12 wks. In contrast to IgA and IgM, consistent patterns of elevation or depression were not observed for serum IgG and IgE over the 12 wk feeding period (Figures 3.3 and 3.4, respectively).

Potential for glomerulonephritis was monitored at 4 and 10 wks using microscopic hematuria as a marker (Figure 3.5). At wk 4, none of the female VT-treated groups exhibited significantly increased urinary erythrocytes when compared to controls. In contrast, all male treatment groups had significantly increased erythrocytes as early as 4 wks when compared both to their respective control, and to the corresponding female treatment group. By wk 10 the 2, 10, and 25 ppm male treatment groups and the 10 and 25 ppm female treatment groups exhibited marked hematuria. However, erythrocyte numbers for males fed 2 and 10 ppm VT at 12 wks were significantly elevated over corresponding female treatment groups.

Mesangial deposition of IgA, IgG and $C_3$ was detected by immunofluorescence microscopy using fluorescein-labelled antibodies and quantified by image analysis (Table 3.3). Males fed 2, 10 and 25 ppm VT and females fed 10 and 25 ppm VT exhibited significantly increased mesangial IgA deposition (Figure 3.6). Mean fluorescence

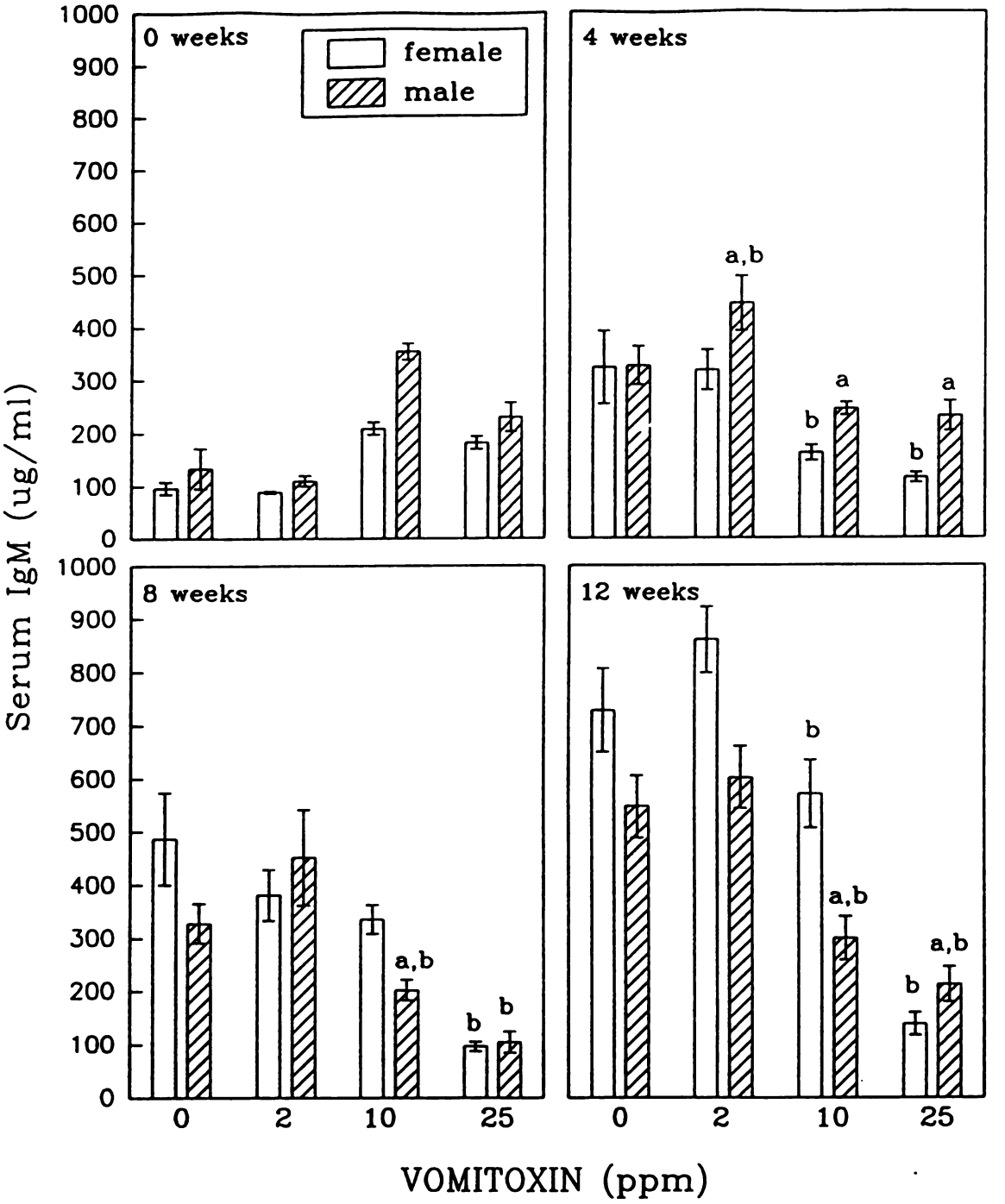



**Figure 3.2. Effect of dietary vomitoxin on serum IgM of male and female mice. Open and hatched bars indicate females and males, respectively. Data are mean ± SEM (n = 7-9 mice per group). (a) indicates significantly different ($p<0.05$) from corresponding female values and (b) indicates significantly different ($p<0.05$) from respective control value**

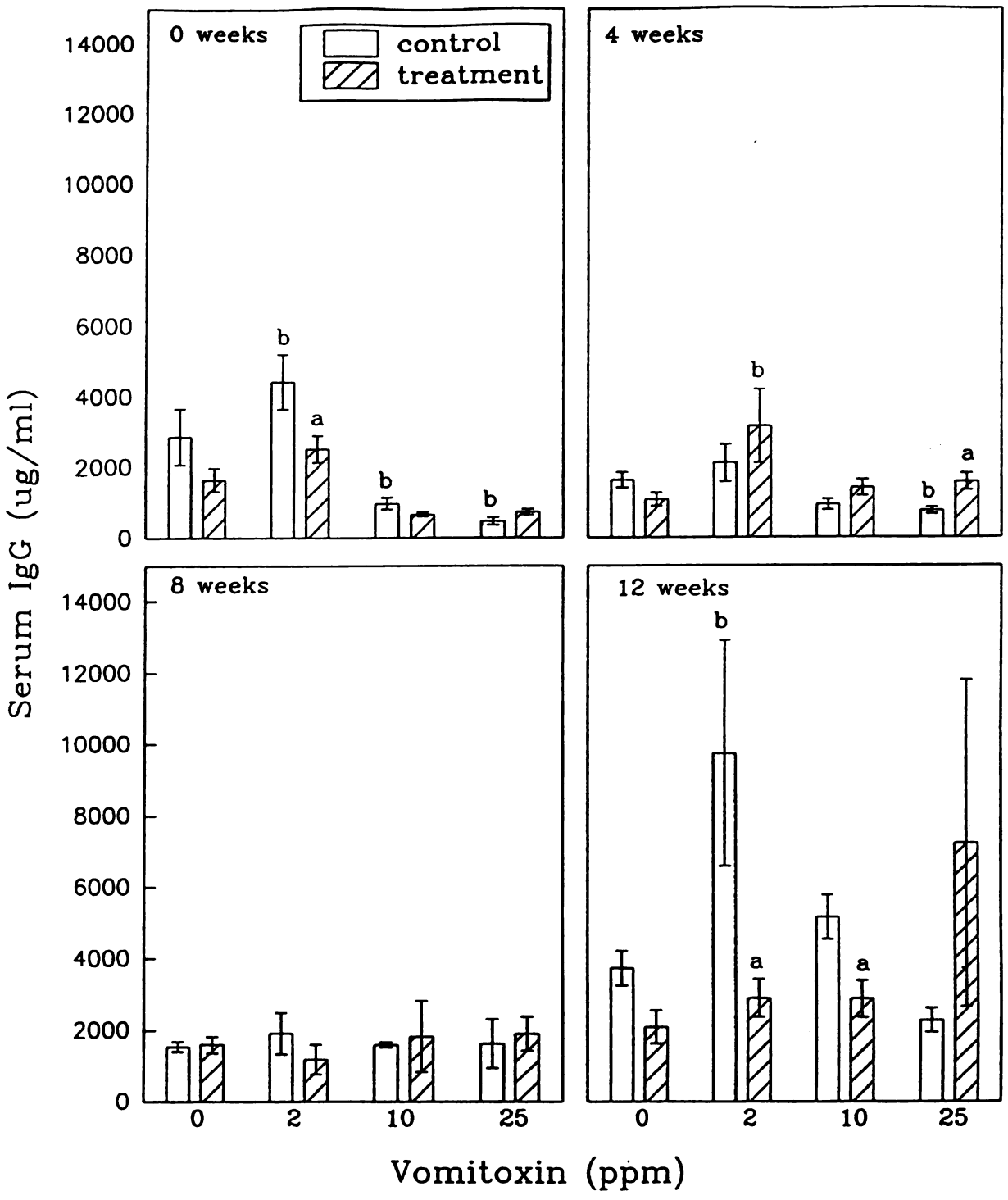



**Figure 3.3. Effect of dietary vomitoxin on serum IgG of male and female mice. Open and hatched bars indicate females and males, respectively. Data are means ± SEM (n = 7-9 mice per group). (a) indicates significantly different ($p<0.05$) from corresponding female values and (b) indicates significantly different ($p<0.05$) from respective control value.**

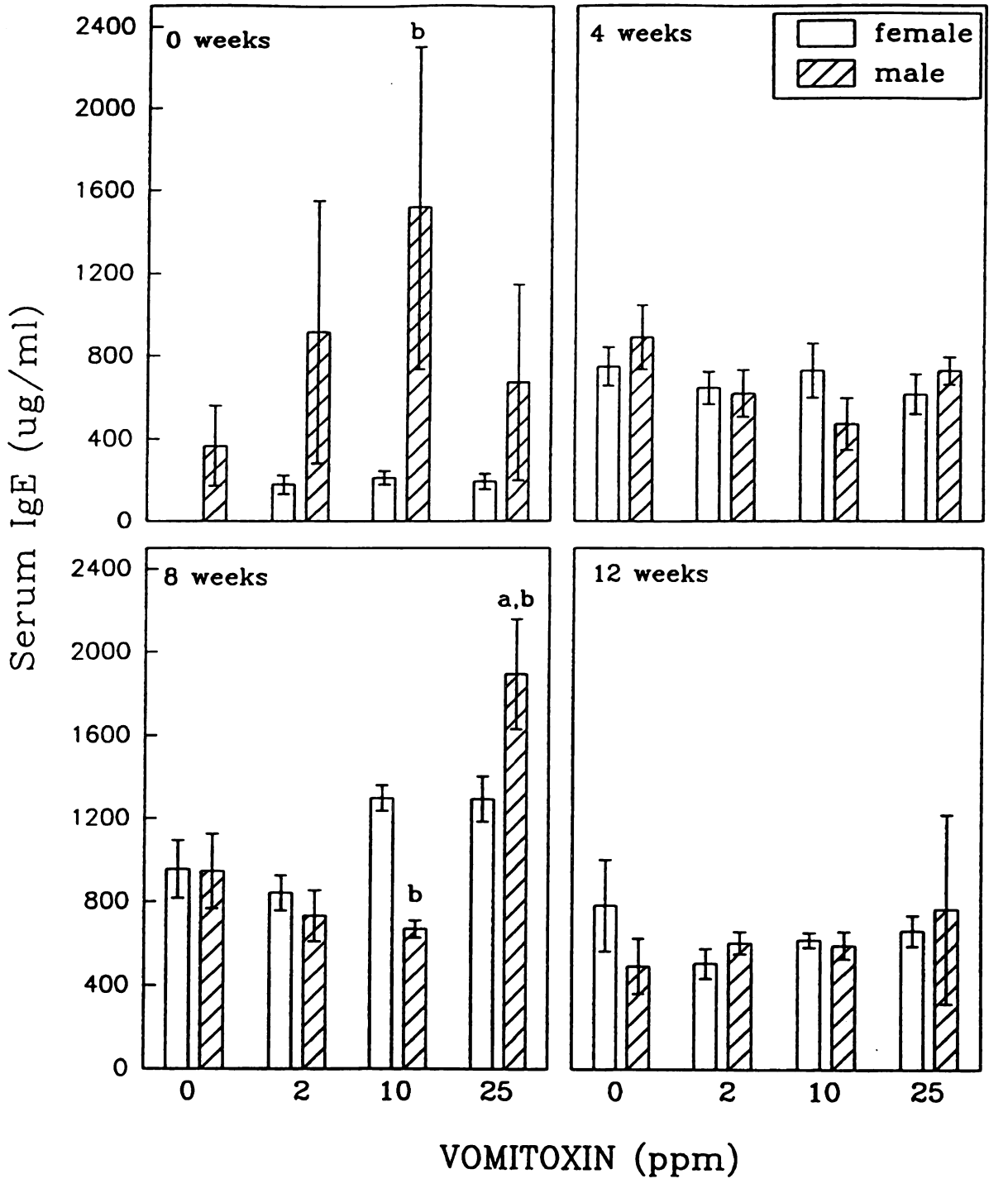



**Figure 3.4. Effect of dietary vomitoxin on serum IgE of male and female mice. Open and hatched bars indicate females and males, respectively. Data are means ± SEM (n = 7-9 mice per group). (a) indicates significantly different ($p<0.05$) from corresponding female values and (b) indicates significantly different ($p<0.05$) from respective control value.**

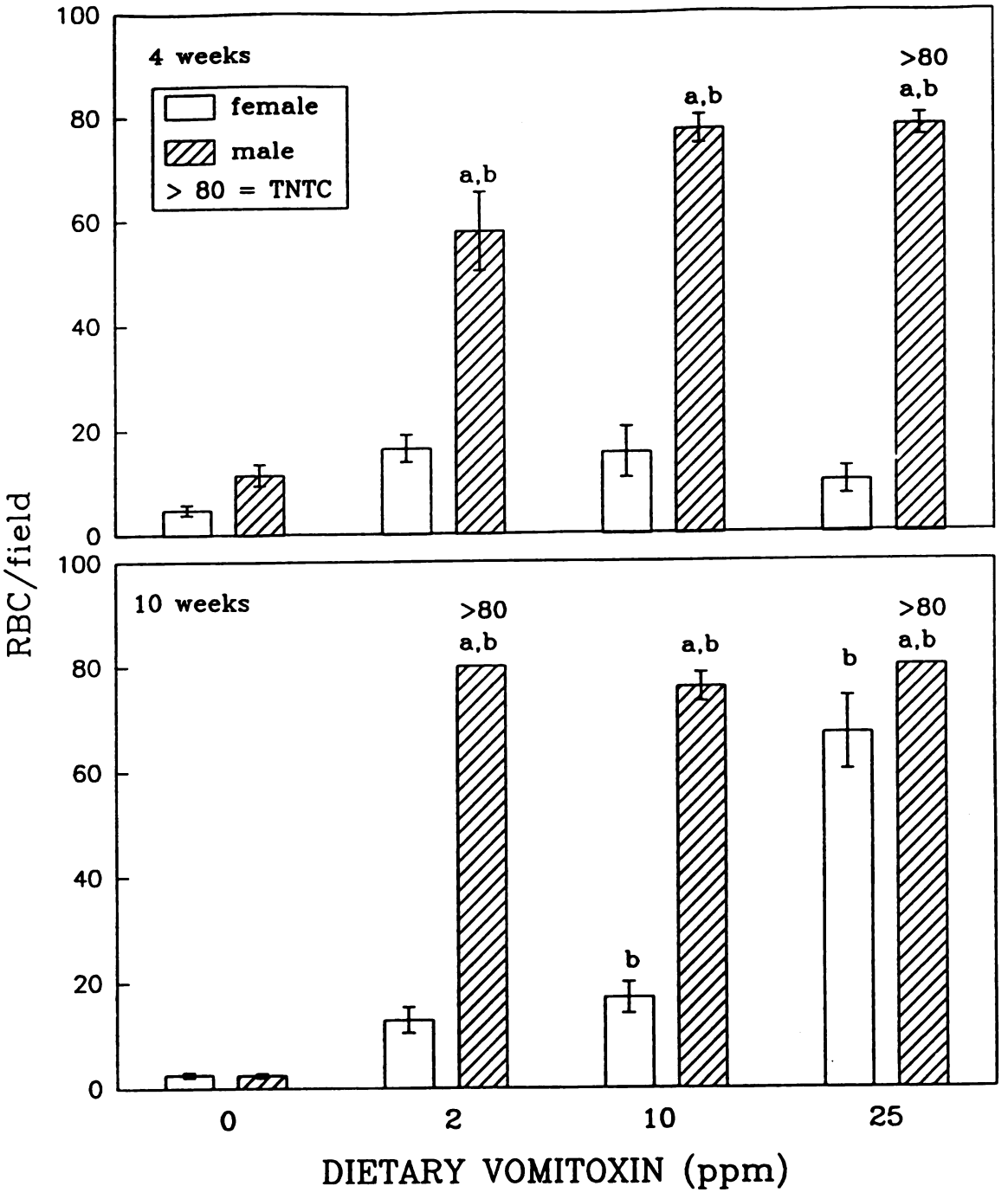


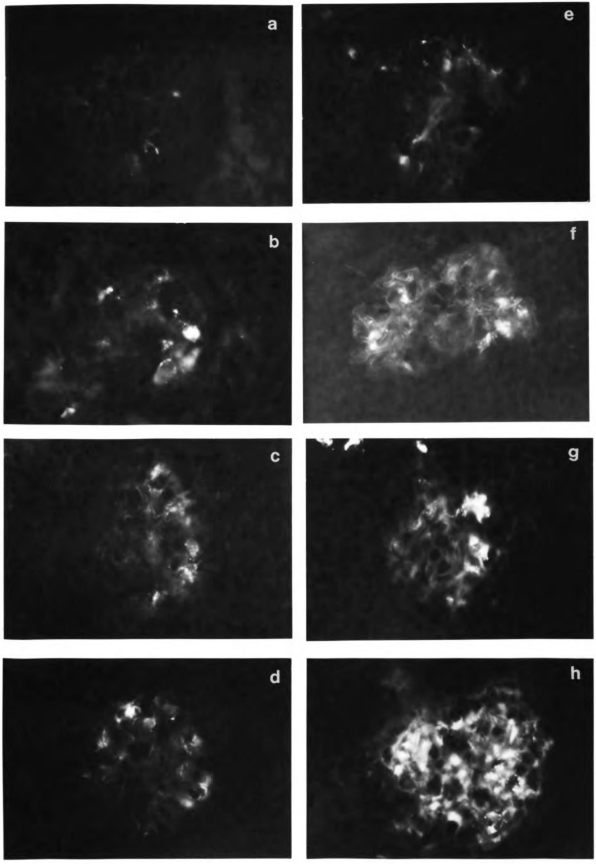

Figure 3.5. Effect of dietary vomitoxin on hematuria in male and female mice. Open and hatched bars indicate females and males, respectively. Data are means ± SEM (n = 7-9 mice per group). (a) indicates significantly different ($p<0.05$) from corresponding female values and (b) indicates significantly different ($p<0.05$) from respective control value.

Table 3.3. Effect of dietary vomitoxin on mesangial deposition of IgA, IgG and $C_3$.[a]

| | | Vomitoxin Dose[*] | | | |
|---|---|---|---|---|---|
| Type | Gender | 0ppm | 2ppm | 10ppm | 25ppm |
| IgA | female | 93±3 | 82±3[b] | 145±3[b] | 189±3[b] |
| | male | 74±2[c] | 102±2[b,c] | 154±3[b,c] | 182±3[b] |
| IgG | female | 103±2 | 78±2[b] | 106±5 | 74±3[b] |
| | male | 124±2[c] | 123±3[c] | 98±3[b] | 85±2[b,c] |
| $C_3$ | female | 175±3 | 156±4[b] | 187±3 | 200±3[b] |
| | male | 125±4[c] | 163±3[b] | 185±3[b] | 204±2[b] |

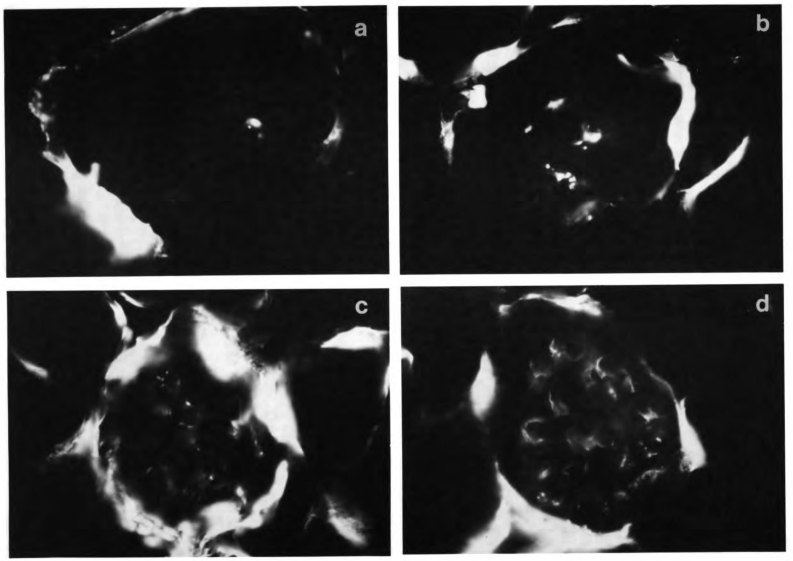
[*] n = 7-9 mice per group
[†] Reported as mean weight at initiation and termination of study
[a] indicates significantly different ($p<0.05$) from control (0ppm)
[b] indicates significantly different ($p<0.05$) from preceding lower dose

Figure 3.6. Effect of vomitoxin ingestion on mesangial IgA deposition in male and female mice. Kidney sections prepared at wk 12 with FITC-labeled anti-mouse IgA. (a) female control (b) female, 2 ppm (c) female, 10 ppm (d) female, 25 ppm (e) male, control (f) male, 2 ppm (g) male, 10 ppm (h) male, 25 ppm.


a
e
b
f
c
g
d
h

intensity was greater in males fed 2 and 10 ppm VT as compared to females exposed tothe same doses. In both genders, IgA deposition appeared to be dose dependent. Contrastingly, mesangial deposition of IgG decreased with increasing dose of the toxin in both genders (Table 3.3). Mean fluorescence intensity for $C_3$ was significantly increased in females fed 25 ppm VT and in males fed 2, 10 and 25 ppm VT. Mesangial C3 increased in males with increasing VT doses (Figure 3.7), whereas C3 deposition in females was not consistent across treatment doses and increases were marginal.

Figure 3.7. Effect of vomitoxin ingestion on mesangial $C_3$ deposition in male mice. Kidney sections prepared at wk 12 with FITC-labeled anti-mouse $C_3$. (a) male control, (b) male, 2 ppm (c) male, 10 ppm (d) male, 25 ppm.


a
b
c
d

# DISCUSSION

The results presented herein suggest that male B6C3F1 mice were more susceptible to the effects of VT than their female counterparts based on comparison of latency, threshold dose and/or severity of the following immunopathologic markers: (1) serum IgA, (2) hematuria, (3) mesangial IgA deposition, and (4) mesangial $C_3$ deposition. This research also helps clarify the minimal and optimal levels of VT required to elicit VT-induced IgA nephropathy. Decreased body weight gain with increasing VT dose as seen here was comparable to results described earlier in the B6C3F1 female mouse and other rodent models (Forsell *et al.*, 1986; Dong and Pestka, 1993; Rotter *et al.*, 1992). Reduction in weight was most probably a result of diminished feed conversion efficiency and/or feed refusal which was qualitatively observed in this study, and reported earlier (Morrissey and Norred, 1985; Forsell *et al*, 1986; Arnold *et al.*, 1986; Rotter *et al.*, 1992). It has been previously demonstrated that a minimum level of 2 ppm VT is required to elicit a reduced weight gain in mice, a level that is comparable to that seen in swine (Forsell *et al.*, 1986; Tryphonas *et al.*, 1986). In addition, the no observable effect level (NOEL) relative to the toxicological manifestations of VT is less than 0.25 mg/kg body wt in rats which is equivalent to 1.7-3.9 ppm (Arnold *et al.*, 1986). In contrast, in a study conducted by Hunder and colleagues (1991), a minimum level of 10 ppm VT was required for animals to exhibit a significantly reduced body weight gain.

Differences in body weight gain between males and females have been previously reported in swine (Cote *et al.*, 1985). At the beginning of the feeding trial, male mice

weighed approximately 4-6g more than the female mice (data not shown) and males eating clean diet weighed 2 g more than females at termination (Table 1). Males fed 25 ppm VT lost 0.5g while females gained 0.5g. Thus, it appeared that males experienced a greater net weight loss than females.

The increase in serum IgA level observed for male mice consuming the 2, 10 and 25 ppm VT diets and female treatment mice fed 10 and 25 ppm VT occurred in a dose-dependent manner and agree with previous findings that VT induces aberrant serum IgA elevation (Forsell *et al.*, 1986; Dong *et al.*, 1991; Pestka *et al.*, 1989). The possibility that males were more susceptible to VT-induced dysregulation of IgA production was supported by earlier appearance of this effect, lower dose threshold and the observation that the larger increases in serum IgA when compared to controls was observed in males consuming VT. Reductions in serum IgM levels in mice consuming higher doses of VT were not only consistent with earlier investigations but also appeared to be dose-related (Forsell *et al.*, 1986; Pestka *et al.*, 1989) and may indicate that isotype switching prompted the increase in serum IgA. The lack of an effect on serum IgG has also been observed previously (Pestka *et al.*, 1990a; Dong *et al.*, 1991; Dong and Pestka, 1993).

We have recently observed that long term VT feeding at 25 ppm (16 wks or longer) results in significant IgE increases in B6C3F1 female mice (Pestka and Dong, 1994). The isotype specific effects of VT have not been exclusively or collectively identified, therefore, the possibility that this toxin can alter IgE production has also been investigated to some extent by our laboratory in previous research efforts. Notably, in this study, serum IgE levels were significantly elevated when compared to control in only the male treatment animals consuming the 25 ppm dose of VT. This was consistent with

results reported by Pestka and Dong (1994) where serum IgE increased 2 to 5 fold with VT exposure, however, the level of increase in this study was much lower. The alterations in serum IgE profile suggest that foodborne mycotoxins such as VT may be an etiologic factor in diet-related hypersensitivities. Since our model mimics IgA nephropathy, of particular interest is a study by Yano and colleagues (1992) which found that patients suffering with IgA nephropathy present with elevated serum IgE levels that are significantly increased over control patients. In this study, no evidence for IgE elevation was noted for either gender at 12 wks suggesting that males did not show a predilection for this effect.

The results of this and a previous study (Pestka *et al.*, 1989) contrast somewhat with the report by Forsell *et al.*, (1986) from our lab in which female B6C3F1 mice exhibited significant IgA increases when fed 2 ppm VT and had maximum serum IgA increases at 10 ppm VT. There are several differences among these studies that might contribute to these observations. First, in the 1986 study, feed was replaced at 7 day intervals, whereas in subsequent studies it was replaced on a 3-4 day schedule. Second, the mice were housed one per cage in the original studies. Third, serum IgA was measured at a single time point (6 wks) in the early investigation. Finally, in the 1986 study, control animals had a mean serum IgA level of 1500 $\mu$g/ml, while control animals in the subsequent investigations had much lower mean levels, indicating that differences in mucosal immune status may be involved.

VT-induced IgA hyperelevation apparently involves both the mucosal and systemic components of the immune system (Pestka *et al.*, 1990b; Bondy and Pestka, 1991). Previous work done in our laboratory has shown that VT acts at the Peyer's patch (PP)

level by enhancing terminal differentiation to IgA-secreting cells (Pestka *et al.*, 1990a; Pestka *et al.*, 1990b; Bondy and Pestka, 1991). Altered lymphocyte profiles including increased IgA+ cells, T cells, CD4+ cells, and the CD4+:CD8+ ratio (Pestka *et al.*, 1990a) have been observed in VT-fed mice that suggest that T cells may play a role in IgA dysregulation. Here, IgA coproantibodies were quantitated to determine if mature IgA-producing cells from the Peyer's patches homed to the lamina propria and thereby increased luminal secretion of IgA. There was a significant increase in fecal IgA in the males fed 10 and 25 ppm VT, but not in the female treatment groups. The greatest increase seen in fecal IgA quantities relative to control was approximately 2-fold , whereas serum IgA increases were much higher with a maximum of 10-fold. These results suggest that increased IgA secreting cells remain primarily in the systemic compartment rather than localize in the lamina propria. Furthermore, they imply that there is no loss in luminal IgA secretion as a result of VT ingestion.

The model described here mimics human IgA nephropathy (Pestka *et al.*, 1989; Pestka and Bondy, 1990; Dong *et al.*, 1991; Dong and Pestka, 1993), which occurs 2-5 times more frequently in males than in females (Schena, 1990; Emancipator and Lamm, 1989; Bene and Faure, 1987). All male treatment groups exhibited a marked elevation in urinary erythrocytes as early as 4 wks that seemed to be dose-related. In contrast, females fed 10 and 25 ppm VT did not exhibit hematuria until the latter stages of the study. Increased mesangial deposition of IgA and $C_3$ was dose-related and was observed in male and female treatment groups at the 10 and 25 ppm doses, and the 2 ppm dose in the males only. The results presented here are similar to our previous report where we found that mesangial $C_3$ appeared to increase marginally in the B6C3F1 females and

to a much larger extent in the B6C3F1 males and C3H/HeJ females (Greene *et al.*, 1994b) and in agreement with several other experimental animal studies of IgA nephropathy (Emancipator and Lamm, 1989; Montinaro *et al.*, 1992). These latter investigations suggested that codeposits of IgG, IgM or $C_3$ may be responsible for the variations in glomerular function that occur in this glomerulonephritis (Emancipator *et al.*, 1987). It should be noted that in previous reports we did not observe significant mesangial $C_3$ deposition in VT fed B6C3F1 females (Dong *et al.*, 1991; Dong and Pestka, 1993). A reason for this difference may be that we used an image analysis technique to measure fluorescence of glomeruli in polygonal areas that included Bowman's capsule. Subtle differences in mesangial accumulation may not have been detectable because C3 is readily detectable in the Bowman's capsule of kidneys from control mice. In contrast, this study focused the image analysis on the mesangial area and excluded Bowman's capsule.

There are several possible mechanisms that may explain why males are more sensitive to VT-induced IgA nephropathy than females. First, endogenous sex hormones may amplify or diminish the detoxification of VT by cytochrome p450 (Cote *et al.*, 1985). Second, sex hormones might also regulate the immune response by affecting T cell driven mechanisms as well as production and elaboration of antibodies by B cells, and cytokines by other immune cells (Schuurs and Verheul, 1990; Forsberg, 1984; Araneo *et al.*, 1991). For example, estrogens have been shown to have the capacity to interact directly with B cells, inhibit lymphocyte proliferative responses, suppress delayed type hypersensitivity reactions and modify host responses to infection (Myers and Peterson, 1985; Forsberg, 1984; Josefsson *et al.*, 1992; Styrt and Sugarman, 1991). The

interactions of androgens with cells of the immune system are not characterized as well as those of estrogens, but have been shown to increase the effectiveness of cell mediated immunity (Schuurs and Verheul, 1990). Specifically, it has been reported that dihydrotestosterone has the ability to reduce the production of interleukins 4 and 5, as well as $\gamma$-interferon (Araneo *et al.*, 1991). Third, previous investigations of protein handling by the kidney have revealed sex differences in the rate of removal. In the rat, females are able to reabsorb more labeled protein and degrade it more quickly than males in the same amount of time (Aldin and Frith, 1991). This might explain why male mice in this study exhibited severe hematuria earlier and to a greater extent than their female counterparts.

In conclusion, the results demonstrate that male B6C3F1 mice are more susceptible to VT than females using elevated serum IgA levels, severity of hematuria and mesangial deposition of IgA and $C_3$ as indices. These observations were analogous to epidemiologic reports of a higher incidence of IgA nephropathy in human males than females. Future investigations will concentrate on characterizing the role of testosterone and estradiol in this murine model for VT-induced dysregulation of IgA production and IgA nephropathy.

# Chapter 4. Potentiating Effects of Dihydrotestosterone and Estradiol in Experimental IgA Nephropathy Induced by Vomitoxin

(To be submitted to *Fundamental and Applied Toxicology*)


## ABSTRACT

Ingestion of the trichothecene mycotoxin, vomitoxin (VT), causes an elevation of serum IgA and IgA immune complexes as well as mesangial deposition of IgA in mice that is very similar to the human glomerulonephritis, IgA nephropathy. Previous research indicates that male mice are more susceptible to these effects. In this report, the effect of castration of male and female mice and sex hormone supplementation on several immunopathologic indicators of IgA nephropathy were compared. In the first study, castrated and intact male and female B6C3F1 mice were fed control AIN76A diet or a diet containing 10 ppm VT for 12 wks. In the VT-treated mice, a greater degree of microscopic hematuria as indicated by erythrocyte counts in urine was observed as compared to controls. VT-treated, castrated females had greater hematuria than intact counterparts, and VT-treated, castrated males tended to have a lower number of erythrocytes in urine than intact counterparts. However, castrated males demonstrated a trend toward lower serum IgA levels than intact counterparts, while the castrated female mice generally exhibited higher serum IgA levels than the intact females. In a subsequent study, castrated male and female mice received controlled release implants of placebo, 5$\alpha$-dihydrotestosterone (DHT), or 17$\beta$-estradiol ($E_2$). These animals were

fed control diet or a 10 ppm VT diet for 8 wks. Castrated male and female mice treated with VT and DHT pellet exhibited more severe hematuria and significantly higher IgA levels, accompanied by greater mesangial deposition of IgA than the mice exposed to the same diet with placebo or $E_2$ pellet at wk 8. While VT-treated animals with an $E_2$ pellet exhibited hematuria at wks 4 and 8, their IgA levels were not significantly elevated over the VT-treated mice with a placebo pellet. The results of these investigations suggested that enhanced male susceptibility to VT-induced IgA nephropathy may be related to the modulation by the biologically active androgen, DHT.

# INTRODUCTION

Vomitoxin (VT) or deoxynivalenol, named for its emetic effects in swine (CAST, 1989), is a toxic *Fusarium graminearum* secondary metabolite that occurs in cereals and grains worldwide. VT and other trichothecenes are potent protein synthesis inhibitors (Ueno, 1983). Animals exposed to chronic and subchronic levels of VT experience reduced feed efficiency, voluntary feed refusal, reduced weight gain, emesis, diarrhea and intestinal irritation and inflammation (Trenholm *et al.*, 1984; Hunder *et al.*, 1991). These common clinical signs indicate that the gastrointestinal tract is one of the target organs of this compound . Our lab has shown that dietary VT is immunostimulatory to the gastrointestinal immune system as manifested by a dramatic elevation in serum IgA, an increase in the polymeric to monomeric IgA ratio and an accumulation of IgA in the mesangial region of the kidney (Pestka *et al.*, 1989; Dong and Pestka, 1993; Dong *et al.*, 1991). These effects are potentially mediated by enhanced T cell help for polyclonal IgA production in the Peyer's patch environment (Bondy and Pestka, 1991; Rasooly *et al.*, 1994a,b). These above manifestations closely parallel those associated with human IgA nephropathy (IgAN), a disease of unknown etiology (Pestka *et al.*, 1989). IgAN occurs more frequently in men than women and is considered to be the most common glomerulonephritis in the world (D'Amico, 1983). Recently, we have demonstrated that male B6C3F1 mice are more susceptible to VT-induced IgA dysregulation and IgAN than their female counterparts (Greene *et al.*, 1994a) and that this occurs in a dose response fashion (Greene *et al.*, 1994b). These latter results suggest a possible relationship between sex hormones and the capacity of VT to induce dysregulation of IgA production.

The purpose of this investigation was to examine the role of castration, and the sex hormones, 17$\beta$-estradiol and 5$\alpha$-dihydrotestosterone, on VT-induced IgAN. The results suggested that testosterone supplementation enhanced the induction of IgA nephropathy by VT.

## MATERIALS AND METHODS

Experiment 1 Design. Castrated and intact male and female B6C3F1 mice (7wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and randomized and housed (6-9 animals per group; 2 per cage) in environmentally protected cages (Nalgene, Rochester, NY) as formerly described (Pestka *et al.* 1987). Cages were kept in a room with regulated temperature and a 12 hr light and dark cycle. Animals were allowed to acclimate for 7 days before commencing with the feeding trials. Control animals were fed powdered, semipurified AIN-76A diet (ICN Biochemical, Cleveland, OH). Treatment groups were fed AIN-76A spiked with 10 ppm VT (Forsell *et al.*, 1986) purified from *Fusarium graminearum* cultures as described by Witt *et al.*, (1985). This level of toxin strongly induces IgAN in males whereas the effects in females are significantly less (Greene *et al.*, 1994b). Feeding regimens were sustained for 12 wks with new food provided every 3-4 days and water dispensed *ad libitum*.

Experiment 2 Design. Castrated male and female B6C3F1 mice (6 wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were randomized, housed, and acclimated as in Exp. 1. At 7 wks of age, animals were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL) and a 60-day controlled release pellet (Innovative Research of America, Toledo, OH) containing 17$\beta$-estradiol (1.5 mg/pellet), 5$\alpha$-dihydrotestosterone (15 mg/pellet), or placebo (1.5 or 15 mg/pellet) was implanted subcutaneously on the back of the neck. Hormone levels were selected to provide physiological doses daily (Beamer *et al.*,

1983; Gottardis *et al.*, 1988a; Gottardis *et al.*, 1988b). One week after pellet implantation, treatment group animals were switched to AIN76-A diet spiked with 10 ppm VT, while control mice were kept on the clean diet. Feeding trials were carried out for 8 wks, with new food provided every 3-4 days and water dispensed *ad libitum*.

Ig Quantitation. At 4 wk intervals, serum samples were collected from the retro-orbital plexus of ether-anesthetized animals. Serum IgA, IgG, and IgM levels were quantitated by enzyme-linked immunosorbent assay (ELISA) (Pestka *et al.*, 1990; Bondy and Pestka, 1991) using standard mouse reference serum (ICN Immunobiologicals, Lisle, IL), heavy-chain specific goat, anti-mouse IgA, G or M as a capture antibody with corresponding peroxidase conjugates (Cappel, Malvern, PA) as detection antibodies.

Hematuria. Mice were placed in metabolic units and urine collected overnight at wks 4 and 12 for Exp. 1 and wks 4 and 8 for Exp. 2. Samples were centrifuged at 500 x g for 10 min and sediment was examined microscopically for erythrocytes per microscopic field (x45)(Dong *et al.*, 1991).

Quantitation of Mesangial IgA, IgG and $C_3$. At the termination of both experiments, mice were euthanized with $CO_2$ and kidneys were removed. They were separated into halves, mounted on cork, and immediately frozen in liquid nitrogen. These were sectioned to 7 $\mu$m on a cryostat (Reichert-Jung, Cambridge Instruments, Buffalo, NY) and stained for Ig or complement deposition with FITC-labeled goat anti-mouse IgA, IgG or $C_3$ as previously described by Valenzuela and Deodhar (1981). Sections from each animal were observed under a Nikon epifluorescence

microscope. Fluorescence intensities in ten glomeruli from each section were measured using an ITM Densitometric Video Camera (Waltham, MA) and JAVA image analysis system (Jandel Scientific, San Rafael, CA). In this program, a quantitative value is generated by circling the area of interest (immunofluorescent stained glomerulus) in a frozen frame. Once circled, the program calculates an average brightness for that area based on each pixel of the screen included in the circled region. The individual pixels that make up the area of interest are measured on a grayness scale that ranges from completely black, with a value of 0, or completely white, with a value of 255. All shades of gray in between the two extremes are also measured per pixel. Camera sensitivity was adjusted separately for $C_3$, IgA and IgG to obtain maximal range. Representative photographs were taken on the same microscope using Kodak Tri-X Pan B & W film.

At the termination of experiment 2, one kidney was treated as in Exp. 1. The cortices were removed from the other kidneys and cut into 1 mm x 1 mm squares. Squares were fixed in glutaraldehyde and processed by routine methods for electron microscopy. Samples were then fixed in 1% osmium tetraoxide and rinsed in buffer before staining en bloc with 2% uranyl acetate. Samples were subsequently dehydrated through a series of graded ethanols followed by propylene oxide with infiltration and embedding in Polybed-Araldite resin. Samples were polymerized for 2 days at 74°C. One micron sections were processed using an LKB Ultrotome and stained with 1% toluidine blue for examination by light microscopy. Areas selected for ultramicrotomy included a minimum of three representative glomeruli per animal. Thin sections (70-90 nm) were cut with diamond knives and placed on 300 mesh

copper grids. Sections were stained with saturated aqueous uranyl acetate (1 hr) and lead citrate (2-3 min), respectively. All sections were examined at 60kV on a Philips 301 electron microscope.

Statistical analysis. Data were analyzed using SigmaStat (Jandel Scientific, San Rafael, CA). Significant differences ($p < 0.05$) between control and treatment groups were analyzed by Kruskal-Wallis and Dunn's method for multiple comparisons or Mann Whitney for comparison of two independent groups of nonparametric data. Parametric data were analyzed by ANOVA and Student-Newman-Keul for multiple comparisons and t-test for two independent groups.

# RESULTS

Experiment 1. Castrated and intact male and female mice were fed control AIN-76A diet or 10 ppm VT for 12 wks. The effects of dietary VT on body weight gain in Exp. 1 are shown in Table 4.1. Castrated females weighed significantly more ($p<0.05$) than their intact counterparts at the beginning of the study. All animals from Exp. 1 gained weight except the intact male mice on treatment diet, however, the treatment mice weighed significantly less ($p<0.05$) than control counterparts at the termination of the study. The most noticeable weight differences when comparing control and treatment animals was observed in the intact and castrated male animals. In both groups, the respective treatment mice weighed approximately 9 g less than controls.

Throughout the feeding trial, several immunopathologic parameters indicative of IgA nephropathy were evaluated. Blood was collected at 4 wk intervals to examine the effects of VT treatment in conjunction with castration or hormone supplementation on normal serum immunoglobulin profiles. Intact male animals eating the VT spiked diet exhibited a noticeable increase in serum IgA as early as 4 wks in comparison to control and other treatment groups, however, this was not significant ($p<0.05$). While other treatment groups also demonstrated trends toward increased IgA by 8 wks, the greatest IgA levels were seen in the VT-treated intact male animals (Figure 4.1). In contrast, serum IgG was significantly decreased in the intact and castrated females as compared to controls at wk 8 (Figure 4.2). However, these effects were not observed in the male groups. Consistent effects on serum IgM were not

Table 4.1. Effect of dietary vomitoxin on body weight changes in male and female mice.*

| | Weight Change (g)[a] | |
|---|---|---|
| | Initial wt. | Final wt. |
| Int ♀ ctrl | 20.8±0.4 | 27.0±0.9 |
| Int ♀ trt | 20.1±0.6 | 22.7±0.7[b] |
| Cas'd ♀ ctrl | 25.0±1.0[c] | 35.0±1.3[c] |
| Cas'd ♀ trt | 24.8±0.8[c] | 27.7±1.0[b,c] |
| Int ♂ ctrl | 25.2±0.4 | 34.4±0.7 |
| Int ♂ trt | 25.8±0.9 | 25.4±0.6[b] |
| Cas'd ♂ ctrl | 24.2±0.5 | 34.5±1.0 |
| Cas'd ♂ trt | 24.5±0.8 | 25.7±0.8[b] |

* n=5-8 mice, except intact male control at 8 wks, where n=4
[a] Reported as mean weight at initiation and 8 wks of study
[b] Indicates significantly different ($p<0.05$) from respective control
[c] Indicates significantly different ($p<0.05$) from intact counterpart

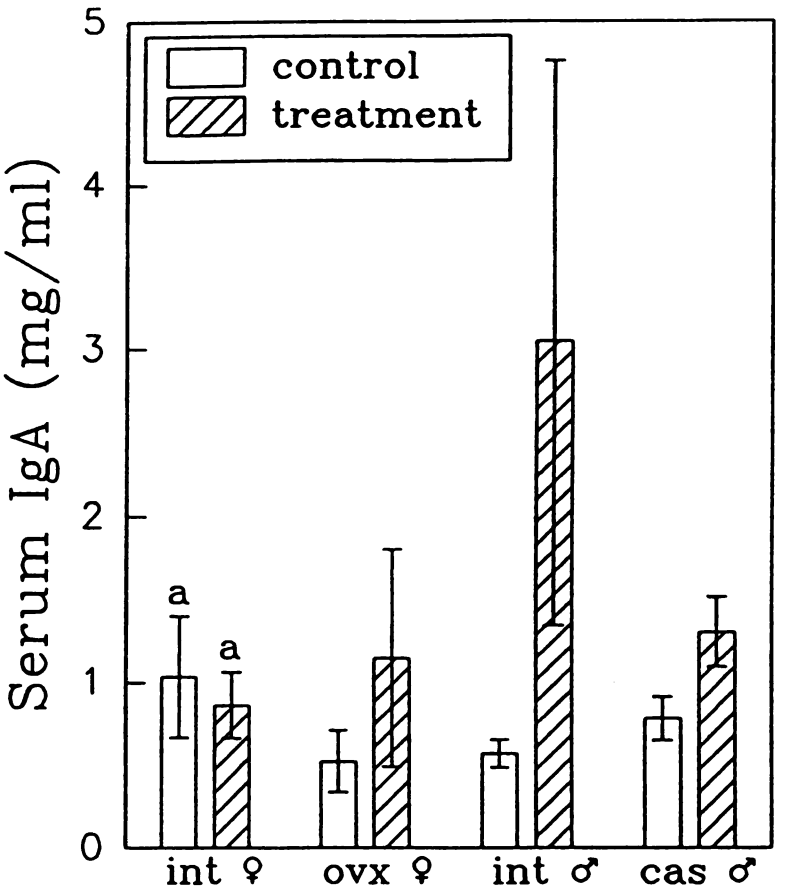



**Figure 4.1. Effect of vomitoxin and castration on serum IgA levels in B6C3F1 male and female mice. Open and hatched bars indicate control and treatment, respectively. Data are means ± SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

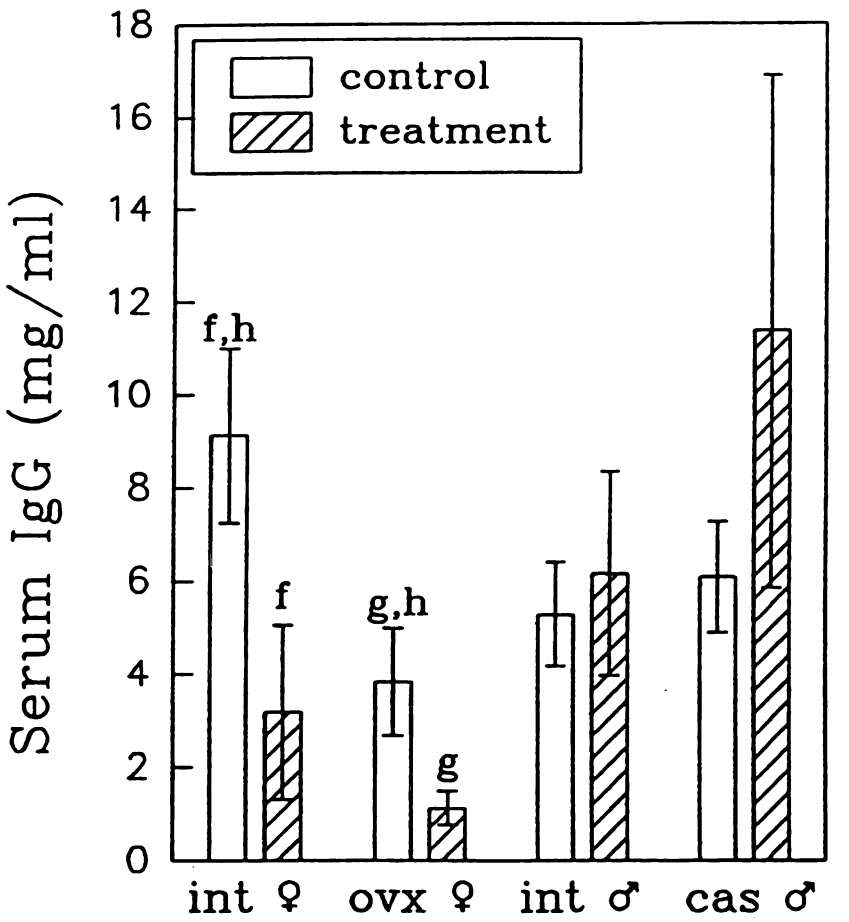



**Figure 4.2. Effect of vomitoxin and castration on serum IgG levels in B6C3F1 male and female mice. Open and hatched bars indicate control and treatment, respectively. Data are means ± SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

observed throughout the study (Figure 4.3).

Induction of glomerulonephritis was monitored at 4 and 12 wks using microscopic hematuria as an indicator (Figure 4.4). As early as 4 wks VT-treated, castrated female mice had significant urinary erythrocyte counts when compared to either controls or to intact counterparts. At wk 4, VT-treated, intact and castrated males had an increase in the number of erythrocytes in their urine as compared to control, however, this was significant only in the latter. At wk 12, all treatment groups exhibited significantly increased erythrocytes in urine as compared to controls, with the castrated females and intact males presenting the highest counts. Intact male mice treated exposed to VT had a higher average number of erythrocytes in urine than their female counterparts.

At the termination of the study, immunofluorescence microscopy and image analysis were used to detect mesangial deposition of IgA, IgG and $C_3$. Kidneys from treated animals generally had increased mesangial IgA, IgG, and $C_3$ deposition when compared to kidneys from control mice (Table 4.2; Figure 4.5). IgG deposition in the kidneys of VT-exposed mice was also affected by castration and VT feeding. Castrated male treatment mice had IgG deposition that was significantly greater than their respective control and intact, female counterparts. Similarly, castrated female treatment mice had IgG deposits that were significantly greater in frequency than in their respective control and intact counterparts. In addition, mesangial $C_3$ deposition was affected by castration and VT exposure with significant increases observed in all treatment groups.

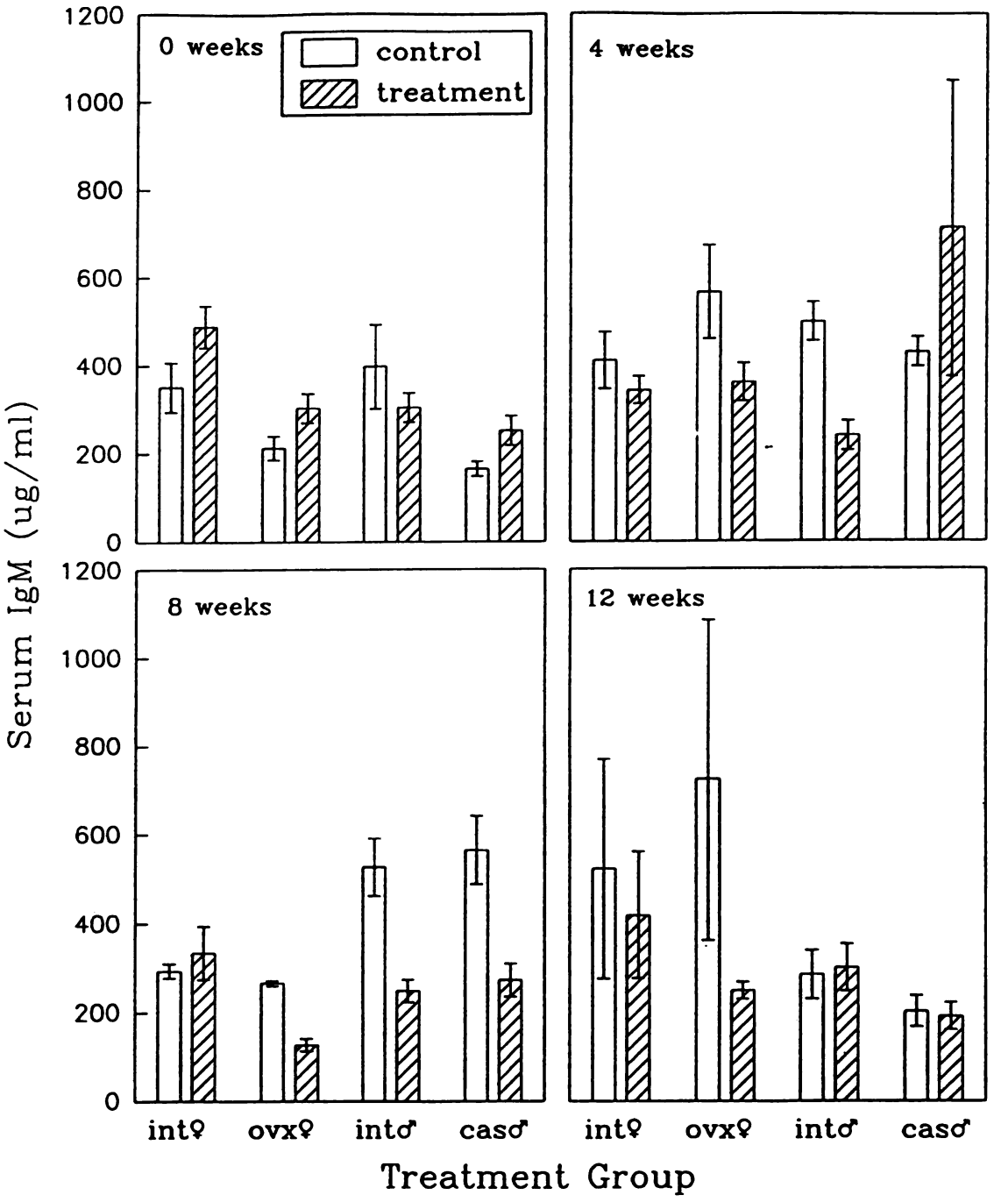



**Figure 4.3. Effect of vomitoxin and castration on serum IgM levels in B6C3F1 male and female mice. Open and hatched bars indicate control and treatment, respectively. Data are means ± SEM.**

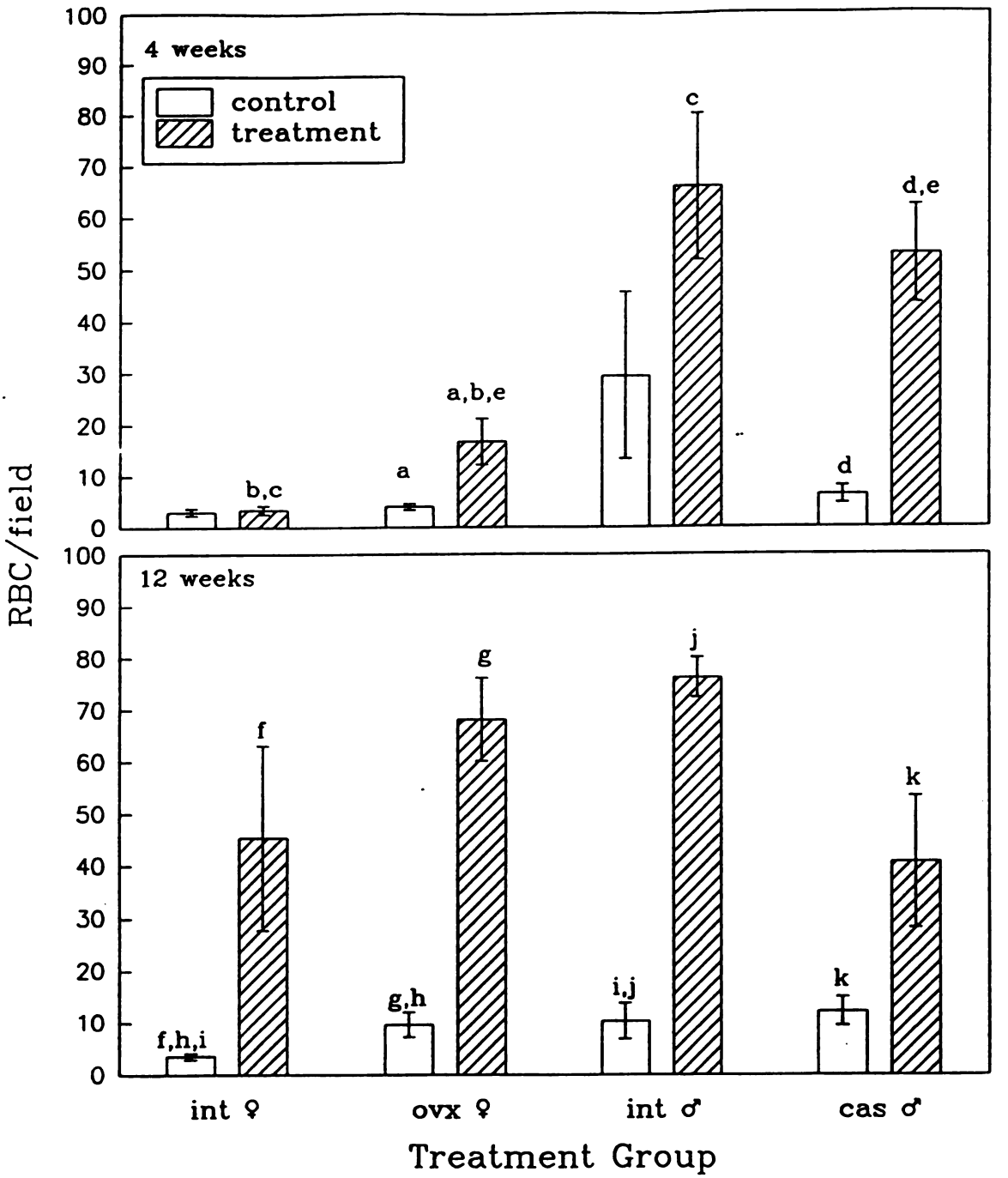



**Figure 4.4. Effect of VT and castration on hematuria in male and female B6C3F1 mice. Data are means ± SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

Table 4.2. Effect of castration and dietary vomitoxin on IgA, IgG and $C_3$ deposition in the kidney of the B6C3F1 mouse.*

| | | CONTROL | TREATMENT |
|---|---|---|---|
| IgA | intact♀ | 71±2 | 127±2[a] |
| | cas♀ | 81±2 | 158±2[a,b] |
| | intact♂ | 83±2[c] | 167±3[a,c] |
| | cas♂ | 87±2[c] | 156±2[a] |
| IgG | intact | 147±2 | 101±2[a] |
| | cas♀ | 145±4 | 160±1[a,b] |
| | intact♂ | 112±3[c] | 172±3[a,c] |
| | cas♂ | 123±2[c] | 193±2[a,b,c] |
| $C_3$ | intact♀ | 94±2 | 173±4[a] |
| | cas♀ | 134±3[a] | 198±2[a,b] |
| | intact♂ | 123±4[c] | 200±2[a,c] |
| | cas♂ | 154±4[c] | 142±2[a,c] |

10 glomeruli measured per mouse; n=4-8 mice per group

* Data reported as means ± SEM on a grayness scale of 0-255 per pixel (as described in Materials and Methods); camera settings adjusted separately for $C_3$, IgG and IgA for maximum range

[a] Significantly different ($p<0.05$) from respective control

[b] Significantly different ($p<0.05$) from intact counterpart

[c] Significantly different ($p<0.05$) from corresponding female value

Figure 4.5. Effect of 12 wk vomitoxin exposure on mesangial deposition of IgA. (a) intact ♀ control (b) intact ♀ treatment (c) castrated ♀ control (d) castrated ♀ treatment (e) intact ♂ control (f) intact ♂ treatment (g) castrated ♂ control (h) castrated ♂ treatment

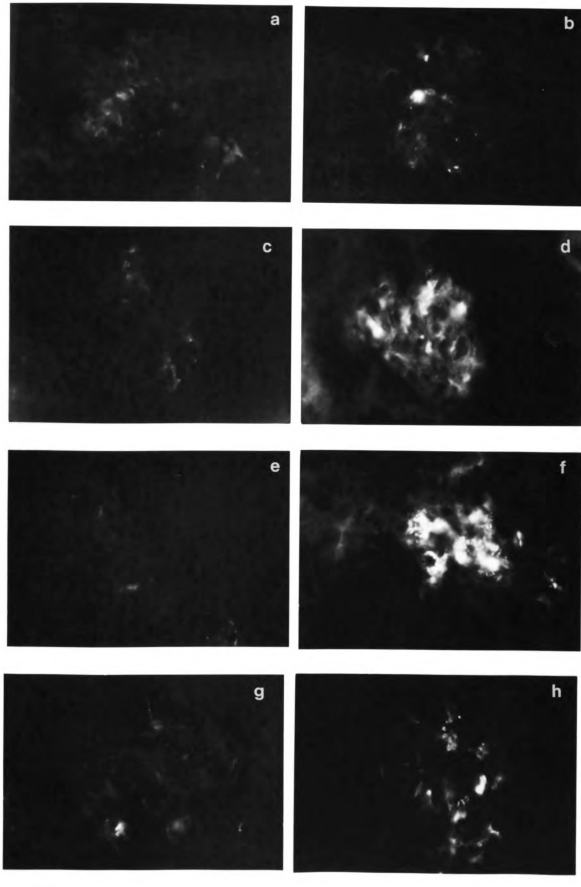

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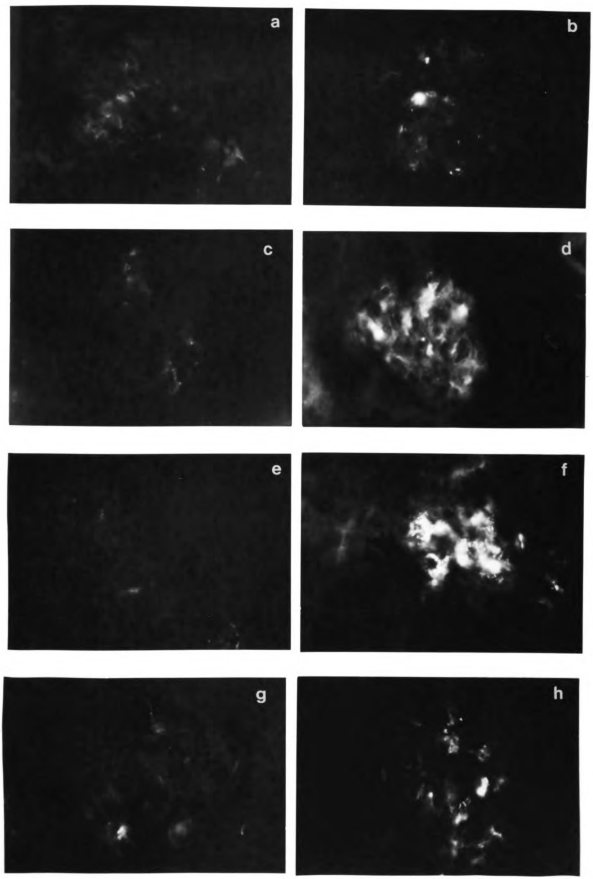
Figure 4.5. Effect of 12 wk vomitoxin exposure on mesangial deposition of IgA. (a) intact ♀ control (b) intact ♀ treatment (c) castrated ♀ control (d) castrated ♀ treatment (e) intact ♂ control (f) intact ♂ treatment (g) castrated ♂ control (h) castrated ♂ treatment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Dana M. Greene/Effects of Castration & Hormone Implantation on Toxicity of DON (VT) in the B6C3F1 Mouse Model

Figure . Mesangial IgA Deposition

Experiment 2. Castrated animals with a placebo, $E_2$, or DHT pellet were fed VT or control diet to test the effects of hormones. Similar trends as seen in Exp. 1 were observed for Exp. 2; all animals gained weight, however, the treatment mice again tended to have lower body weights in comparison to their respective controls (Table 4.3). Significantly decreased ($p<0.05$) weight gains were observed in the male treatment animals that were fed VT and given either a placebo, $E_2$, or DHT pellet. However, significant increases ($p<0.05$) in the treated female mice were observed only in those fed VT and given a placebo or DHT pellet. Decreased body weights as a result of hormone-treatment were not observed.

Elevated serum IgA levels were also found in treatment mice (Figure 4.6). The 8 wk serum collection revealed that all treatment groups had a significantly higher ($p<0.05$) serum IgA level than their respective control except the female mice with a placebo pellet. Notably, IgA levels for DHT-treated male and female mice were significantly elevated over their placebo and $E_2$ counterparts ($p<0.05$). Increases for DHT-treated male and female mice were 4 fold and 3 fold greater than for their $E_2$ counterpart, respectively, and 5 fold and 7.5 fold greater than for their placebo counterparts, respectively. It is important to note that DHT treatment alone seemed to increase serum IgA levels in control animals.

The normal serum IgG profile was also altered in the treated mice from Exp. 2. The greatest increase in IgG was seen at 8 wks in the male mice treated with VT and a DHT pellet (Figure 4.7). These levels were significantly elevated over those of the respective controls as were the levels in female mice treated with VT and a DHT pellet. Female mice exposed to VT and $E_2$ exhibited serum IgG levels that were

Table 4.3. Effect of dietary vomitoxin on body weight changes in placebo, 17β-estradiol, or 5α-dihydrotestosterone treated male and female mice.*

| | Weight Change (g)[a] | | | |
|---|---|---|---|---|
| | Female | | Male | |
| CONTROL | Initial wt. | Final wt. | Initial wt. | Final wt. |
| placebo | 22±0.6 | 35±1.0 | 27±0.8 | 33±0.8 |
| $E_2$ | 22±0.3 | 27±2.3[c] | 28±0.7 | 35±1.9 |
| DHT | 24±0.5[c,d] | 30±1.1[c] | 28±0.7 | 33±1.1 |
| TREATMENT | | | | |
| placebo | 22±0.4 | 26±0.5[b] | 26±0.2 | 27±0.5[b] |
| $E_2$ | 21±0.3[b] | 23±0.8[c] | 26±0.6 | 27±0.7[b] |
| DHT | 22±0.4[b] | 25±0.6[b] | 27±0.7 | 26±0.7[b] |

* n = 6-10 mice
[a] Reported as mean weight ± SEM at initiation and termination of study
[b] indicates significantly different ($p<0.05$) from respective control
[c] indicates significantly different ($p<0.05$) from corresponding placebo value
[d] indicates significantly different ($p<0.05$) from corresponding $E_2$ value

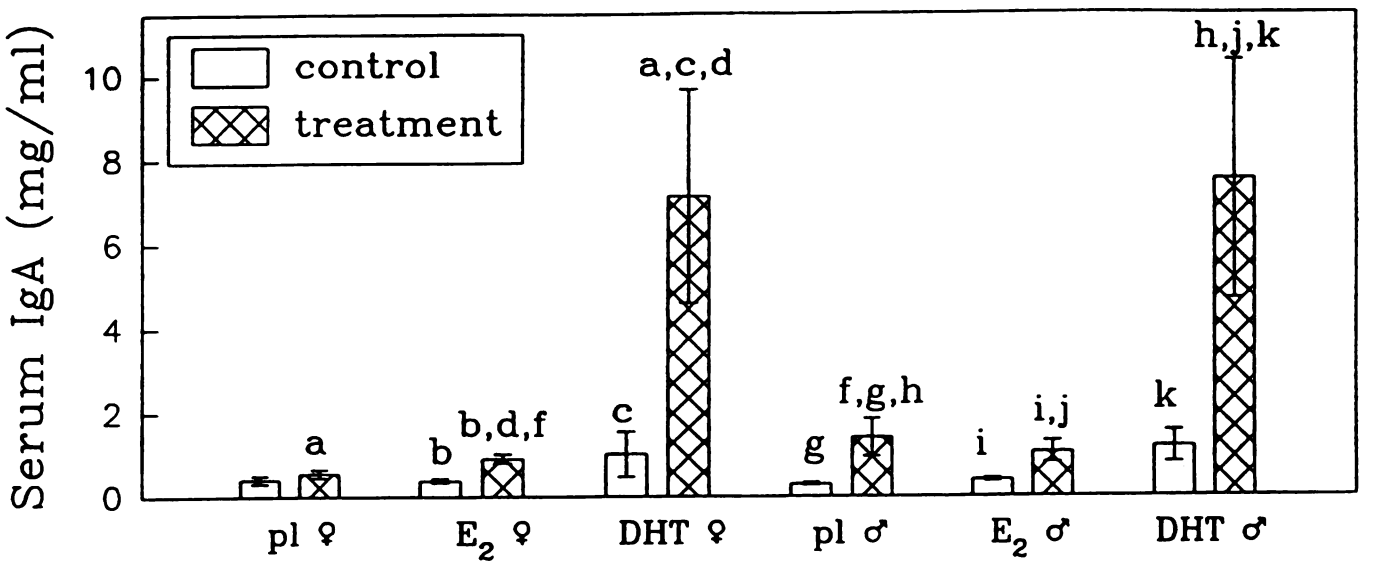



**Figure 4.6. Effect of 17$\beta$-estradiol and 5$\alpha$-dihydrotestosterone on serum IgA levels in the B6C3F1 mouse. Open and hatched bars indicate control and treatment, respectively. Data are means $\pm$ SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

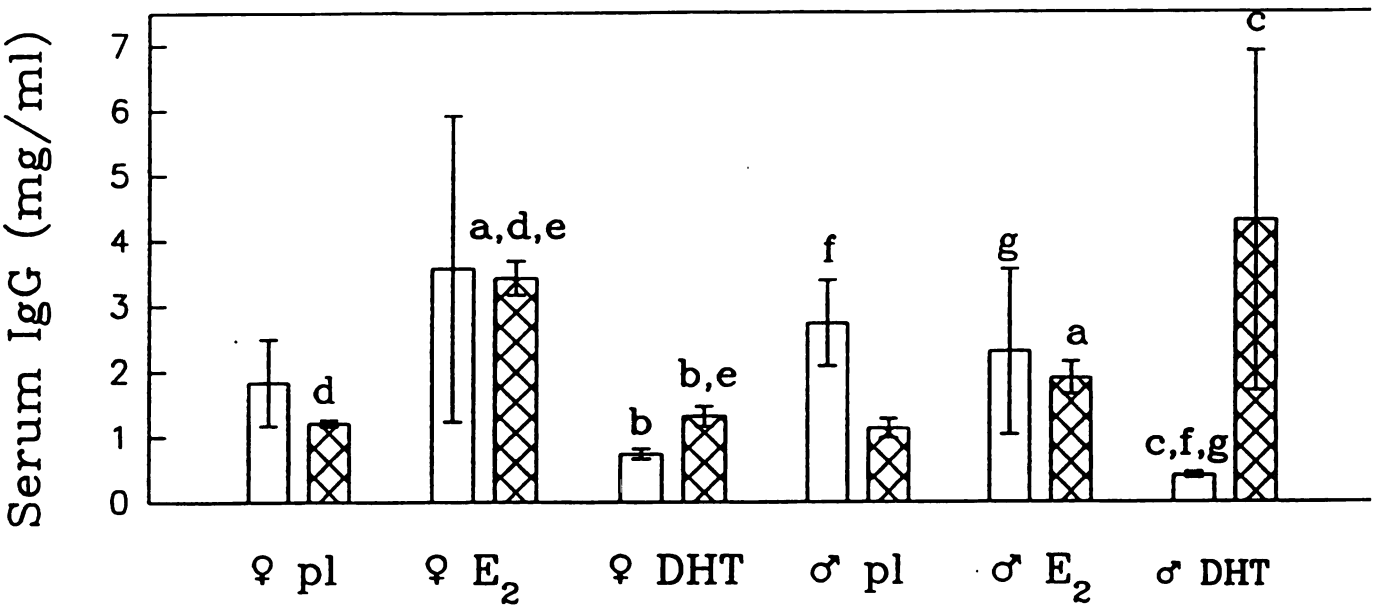



Figure 4.7. Effect of 17$\beta$-estradiol and 5$\alpha$-dihydrotestosterone on serum IgG levels in the B6C3F1 mouse. Open and hatched bars indicate control and treatment, respectively. Data are means $\pm$ SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.

unaffected by the treatment when compared to controls. Consistent trends for IgM across treatment groups were not observed, while IgM seemed to follow decreasing trends (Figure 4.8). Similarly, DHT alone seemed to decrease serum IgG levels.

Hematuria was observed in all treatment animals at early as 4 wks (Figure 4.9). Notably, wk 4 male and female mice treated with VT and DHT exhibited a significant increase in erythrocytes in urine as compared to placebo counterparts, and for the former, as compared to the $E_2$ counterpart as well. This was again seen at 8 wks when the mice treated with VT and a DHT pellet had the greatest number of erythrocytes in urine ($p<0.05$) in comparison to their VT-treated, placebo, and $E_2$ counterparts. Furthermore, VT-treated male mice with an $E_2$ or DHT pellet had significantly increased numbers of erythrocytes in the urine than did their female counterparts. Hormone treatment alone did not appear to affect microhematuria.

Kidneys analyzed from treatment mice in Exp. 2 generally exhibited elevated IgA and $C_3$ deposition when compared to control (Table 4.4). Male and female mice treated with VT and a DHT pellet exhibited the greatest IgA deposition (Figure 4.10). Those mice treated with VT and an $E_2$ pellet also exhibited significantly increased deposition of IgA as compared to placebo counterparts. Relative to IgG, consistent trends across the treatments were not identified (Table 4.4). The VT-treated mice with a DHT pellet had significantly elevated IgG deposits as compared to their respective control and placebo counterpart, while those mice treated with VT and a placebo pellet demonstrated a significantly lower frequency of IgG deposits than their respective controls. In addition, $C_3$ deposition increased in all treatment mice as compared to controls. It is important to note that IgA deposition appeared to increase

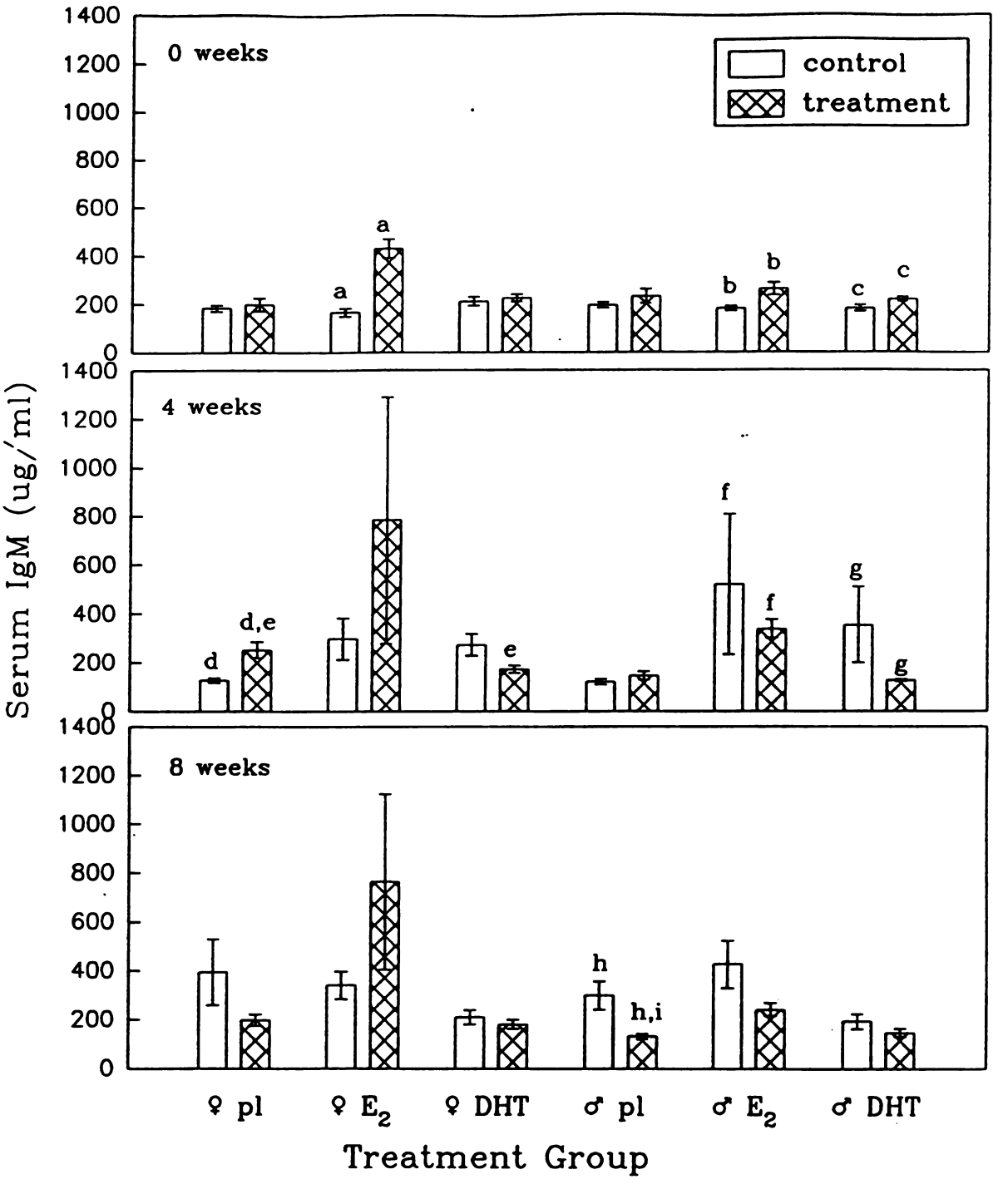



**Figure 4.8. Effect of 17β-estradiol and 5α-dihydrotestosterone on serum IgM levels in the B6C3F1 mouse. Open and hatched bars indicate control and treatment, respectively. Data are means ± SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

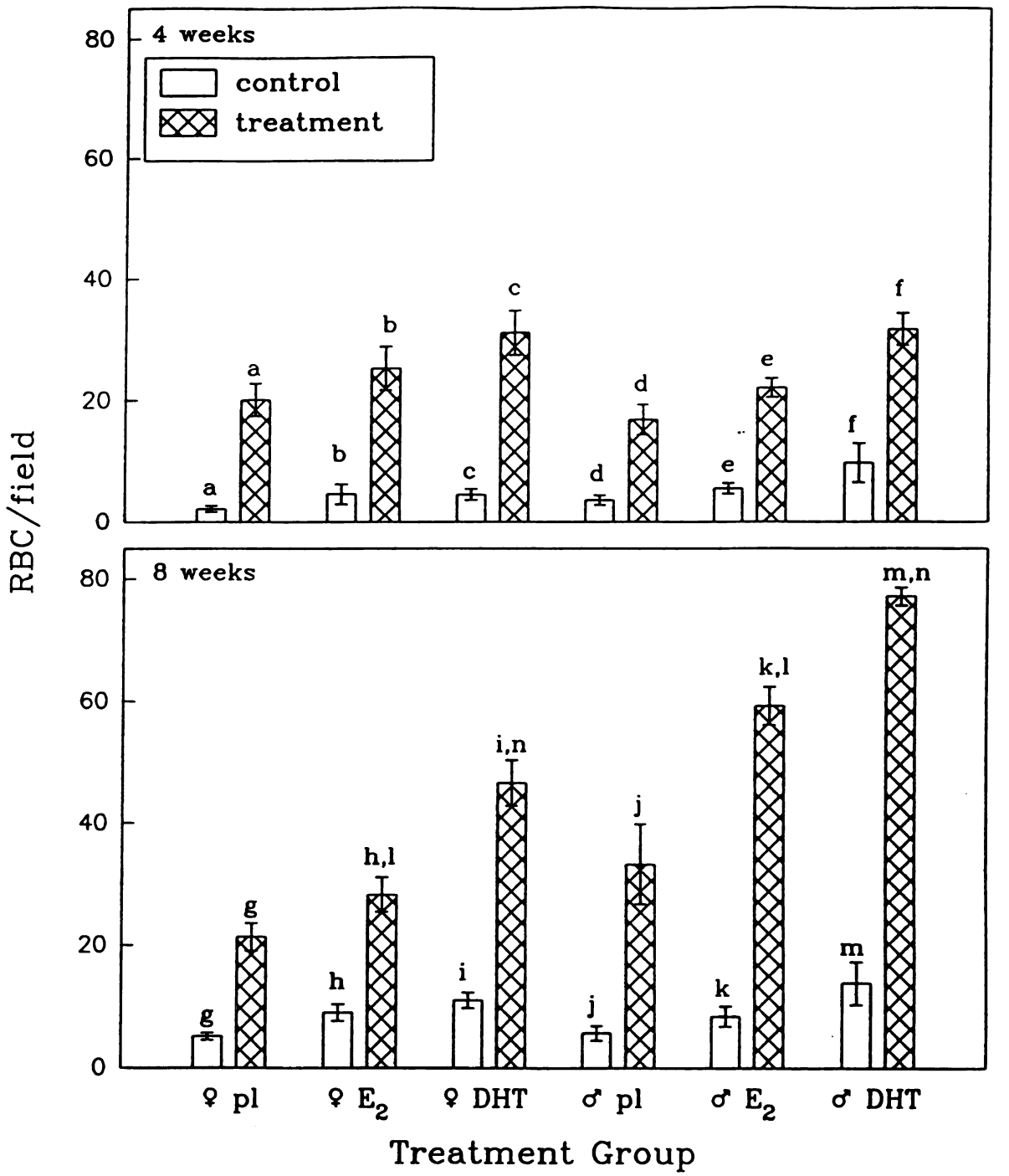



**Figure 4.9. Effect of 17β-estradiol and 5α-dihydrotestosterone on hematuria in the B6C3F1 mouse. Open and hatched bars indicate control and treatment, respectively. Data are means ± SEM. Bars with the same letter are significantly different ($p<0.05$) from one another.**

Table 4.4. Effect of 17β-estradiol, 5α-dihydrotestosterone, or placebo and dietary vomitoxin on IgA, IgG and $C_3$ deposition in the kidney of the B6C3F1 mouse.*

| | | CONTROL | TREATMENT |
|---|---|---|---|
| IgA | pl♀ | 69±1 | 89±1[a] |
| | pl♂ | 92±1[c] | 113±11[a,c] |
| | $E_2$♀ | 105±1[b] | 129±4[a,b] |
| | $E_2$♂ | 95±2[c] | 126±4[a,b] |
| | DHT♀ | 104±1[b] | 151±2[a,b] |
| | DHT♂ | 110±3[b,c] | 172±2[a,b,c] |
| $C_3$ | pl♀ | 129±2 | 159±4[a] |
| | pl♂ | 148±2 | 177±3[a,c] |
| | $E_2$♀ | 147±3[b] | 172±4[a] |
| | $E_2$♂ | 136±4[b] | 149±2[a,c] |
| | DHT♀ | 149±2[b] | 176±3[a] |
| | DHT♂ | 148±3 | 162±3[a,c] |
| IgG | pl♀ | 148±2 | 97±2[a] |
| | pl♂ | 138±2[c] | 111±2[a] |
| | $E_2$♀ | 150±2 | 154±3[b] |
| | $E_2$♂ | 155±3[b] | 123±2[a,c] |
| | DHT♀ | 151±2 | 172±2[a,b] |
| | DHT♂ | 143±3 | 181±2[a,b,c] |

10 glomeruli measured per mouse; n=6-10 mice per group

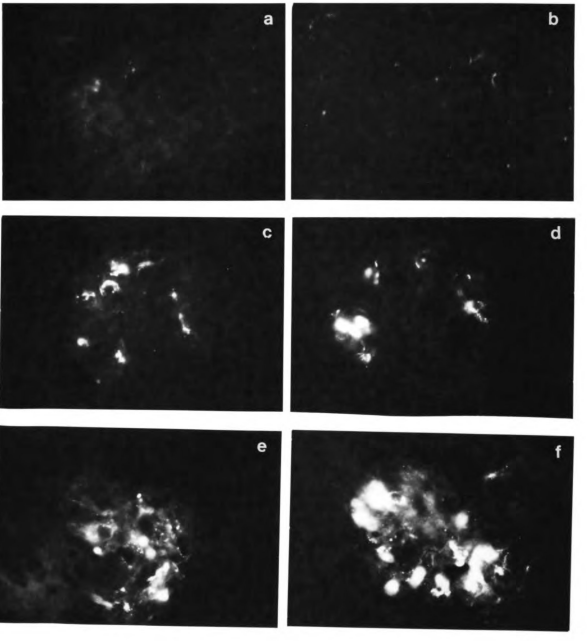
* Data reported as means ± SEM on a grayness scale of 0-255 per pixel (as described in Materials and Methods); camera settings adjusted separately for $C_3$, IgG and IgA for maximum range

[a] Significantly different ($p<0.05$) from respective control

[b] Significantly different ($p<0.05$) from corresponding placebo

[c] Significantly different ($p<0.05$) from female counterpart

Figure 4.10. Effect of 8 wk vomitoxin exposure on mesangial deposition of IgA. (a) control female with placebo pellet (b) control male with placebo pellet (c) treatment female with placebo pellet (d) treatment male with placebo pellet (e) treatment female with DHT pellet (f) treatment male with DHT pellet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DANA M. GREENE/Effects of Castration and Hormone Implantation on Toxicity of DON (VT) in the B6C3F1 Mouse Model--Sex Hormones as Cofactors FIGURE 6. IgA dep.

significantly with DHT treatment alone.

# DISCUSSION

Human IgA nephropathy is 2-5 times more common in men than women (Bene and Faure, 1987), and the model described herein mimics this disease not only in pathophysiologic and immunopathologic parameters, but also in the increased susceptibility of males. Glomerular deposits that characterize this disease have been suggested to be circulating IgA immune complexes (IgA-IC) and/or macromolecular IgA that may be antibodies against an exogenous or self-antigen. The latter implicates a possible autoimmune mechanism that may initiate the accumulation of IgA in the mesangium (Hiki *et al.*, 1991). In previous studies, we have reported that there is a predilection for VT-induced IgA nephropathy in male B6C3F1 mice (Greene *et al.*,1994a,b). The results reported here suggest that the biologically active sex hormones, 5$\alpha$-dihydrotestosterone and possibly 17$\beta$-estradiol have the capacity to act as cofactors with VT and exacerbate immunopathology associated with IgA-mediated glomerulonephritis.

Animals in these studies survived the feeding trials with relatively low or no mortality. A common result of dietary VT exposure is decreased body weight gain (Forsell *et al.*, 1986; Dong and Pestka, 1993; Rotter *et al.*, 1992). In both experiments and in agreement with previous reports (Forsell *et al.*, 1986; Greene *et al.*, 1994a,b), most animals gained weight at the 10 ppm VT dose, however, the treatment animals exhibited lower body weights than their control counterparts. Decrease in body weight gain was most likely a consequence of feed refusal and/or a reduction in feed conversion efficiency (Forsell et al, 1986; Morrissey and Norred,

1985; Arnold *et al.*, 1986; Rotter *et al.*, 1992). Notably, hormone implantation did not significantly alter weight gain in control or treatment mice when compared to placebo. Estrogens reportedly have the capacity to enhance antibody production (IgG, IgM and IgA)(Carlsten *et al.*, 1991; Ahmed *et al.*, 1989; Styrt and Sugarman, 1991) and to decrease IL-2 production (Pung *et al.*, 1985). However, in this study ovariectomized mice exhibited serum IgA levels that were significantly elevated over their intact counterparts. Testosterone reportedly inhibits the antibody response in mammals, and in some investigations it has been demonstrated that following gonadectomy, males experience enhanced immune responses or augmented resistance to infections (Rifkind, 1972; Cohn, 1979; Slater and Schreck, 1993; Castro, 1974; Aboudkhil *et al.*, 1991). Interestingly, the intact males, which had higher circulating quantities of testosterone, also had higher serum IgA levels than their castrated counterparts.

VT-induced IgA dysregulation appears to involve the mucosal as well as the systemic compartments of the immune system (Bondy and Pestka, 1991). In earlier investigations, our laboratory has demonstrated that this compound operates at the level of the Peyer's patches by enhancing the processes of terminal differentiation of B-cells into IgA-secreting cells. In addition, increases in $IgA^+$, $CD4^+$, and T cell populations as well as an increase in the $CD^+$:$CD8^+$ ratio in VT-exposed mice convincingly suggest a role for T cells in IgA dysregulation. Of particular interest were the increased IgA levels observed in male and female mice treated with VT and DHT. These mice exhibited the highest IgA levels that were not only significantly greater than IgA levels in those mice treated with VT and a placebo, but also greater

than IgA levels in those treated with VT and $E_2$. It is thus possible that testosterone positively modulates the process of terminal differentiation. This possibility contrasts with the observation that biologically active DHT down-regulates T cell production of cytokines *in vivo* and *in vitro*, particularly interleukin-4 (IL-4) and IL-5, that actively participate in the stimulation of B cells for growth, antibody production, and class switching (Araneo *et al.*, 1991; Daynes and Araneo, 1992; Daynes *et al.*, 1991). Serum profiles of IgG and IgM were altered in treated animals from both experiments which may indicate a role for isotype switching as a contributor to the increase in serum IgA quantities. In previous research efforts, a lack of VT-induced effects on serum IgG and IgM have been observed (Pestka *et al.*, 1990; Dong and Pestka, 1993; Greene *et al.*, 1994b).

In both experiments, treatment mice exhibited microhematuria by 8 wks. Notably, the treated, ovariectomized females and intact males from Exp. 1 had a higher number of red blood cells in their urine samples than did their intact and castrated counterparts, respectively. Interestingly, hematuria results were compatible with the trends established for serum IgA and also with the mesangial deposition patterns observed. These consistencies support a direct association between serum IgA levels, glomerular IgA deposition, and ultimately disease severity. Several previous investigations have presented data that support a positive relationship between elevated serum IgA levels and increased glomerular deposition (Gonzalez *et al.*, 1989; Gonzalez-Cabrero *et al.*, 1990; Muso *et al.*, 1991). Similarly, the VT- and DHT-treated mice from Exp. 2 presented the greatest quantities of erythrocytes in urine in comparison to placebo and $E_2$ counterparts.

Here again, the hematuria results were consistent with patterns established for serum IgA and mesangial IgA deposition further implying an immediate connection between serum IgA levels and mesangial deposition, as well as disease severity. These findings were also consistent with those from Exp. 1, in which the intact male mice which have the greatest levels of circulating testosterone exhibited similar patterns. The increases in mesangial IgA were in agreement with earlier studies (Emancipator and Lamm, 1989; Dong and Pestka, 1993; Greene *et al.*, 1994a,b). In human IgA nephropathy, mesangial deposition of IgA is often accompanied by $C_3$ deposition. In these studies, an increase in C3 was detected in glomeruli of kidneys from treated mice in both experiments, which is consistent with numerous reports (Emancipator *et al.*, 1987; Jessen *et al.*, 1986).

Deposition was observed by electron microscopy in kidney samples prepared from VT-treated male and female mice with a DHT pellet (Figures 4.11 and 4.12). Of particular interest were several of the control females with an $E_2$ pellet. These mice had cutaneous ulcerations of the ventral abdomen as a result of urine scalding similar to those seen in male mice with mouse urologic syndrome (MUS)(Figure 4.13)(Bendele and Carlton,1986). MUS is characterized as an obstructive infirmity of the urinary tract which usually affects male mice of a variety of strains. Mice suffering with an acute form of the syndrome are usually found dead before any signs of illness are recognized (Bendele and Carlton, 1986).

The mice from this study that appeared to have MUS were necropsied at which time marked distention of the bladder and splenomegaly were observed (Figure 4.14a,b). In some cases, the bladder contained irregular calculi which was consistent

Figure 4.11. Effect of 5$\alpha$-dihydrotestosterone on mesangial IgA deposition in the male B6C3F1 mouse as determined by electron microscopy. Deposits are indicated by "d" and arrows; mesangial cells represented by "mes"

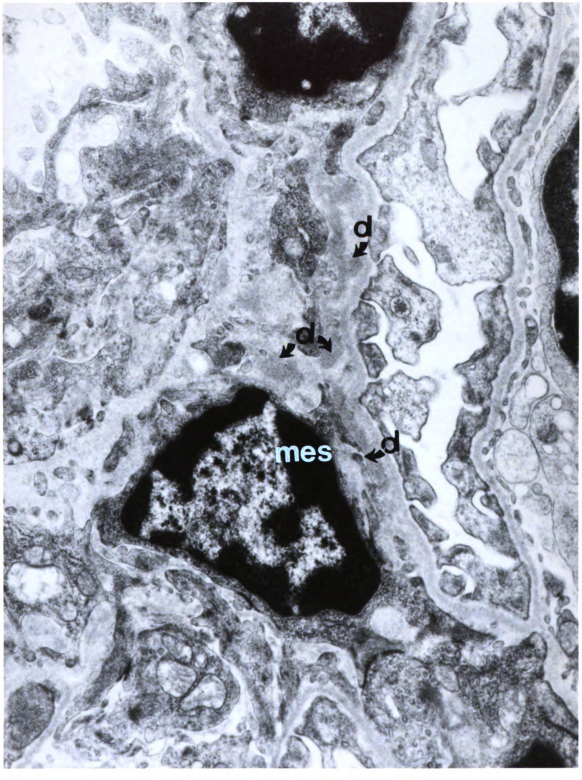

d
d
mes
d

Figure 4.12. Effect of 5$\alpha$-dihydrotestosterone on mesangial IgA deposition in the female B6C3F1 mouse as determined by electron microscopy. Deposits are indicated by "d" and arrows; mesangial cells represented by "mes"

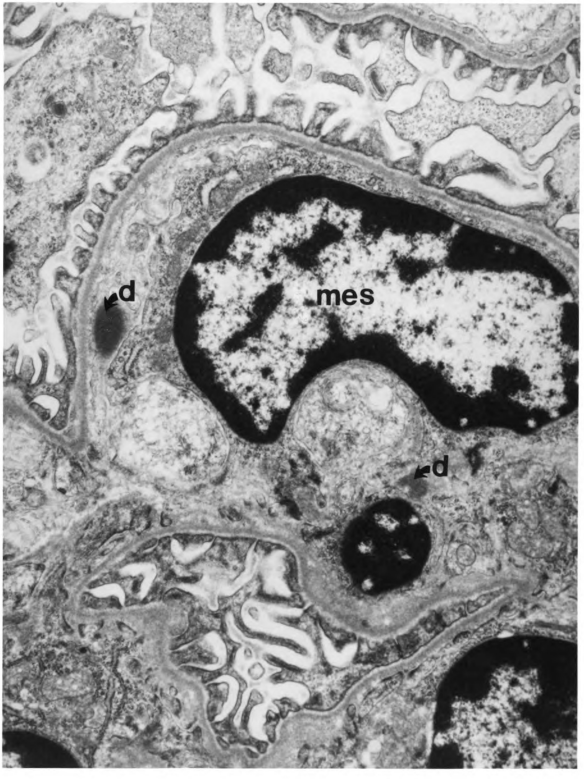
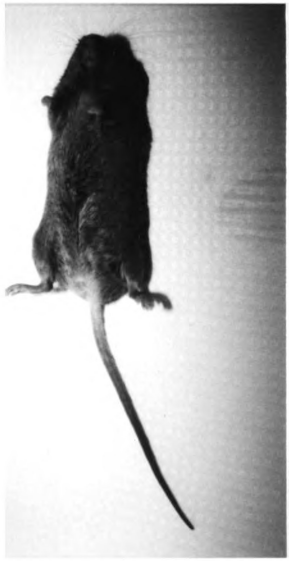

d
mes
d

Figure 4.13. Urologic syndrome in the B6C3F1 female mouse treated with estradiol. (a) normal mouse without syndrome (b)cutaneous ulceration consistent with syndrome.

a



b

Figure 4.14. Mouse urologic syndrome in B6C3F1 mouse with estradiol pellet. (a) bladder distention (b) splenomegaly (c) enlarged seminal vesicles

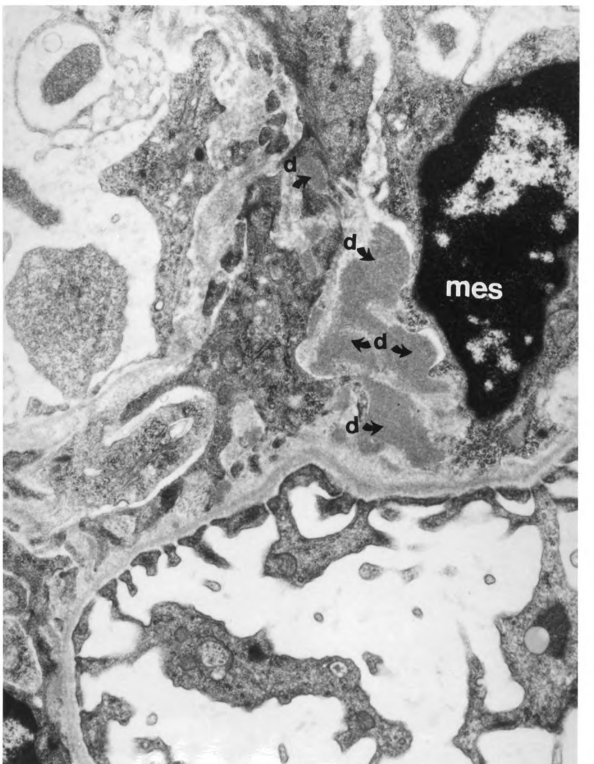
a

with gross alterations of the syndrome (Bendele and Carlton, 1986). There was alsosignificant deposition in the mesangial region of kidneys taken from these mice (Figure 4.15). Notably, male mice with the $E_2$ pellet did not experience the urine scalding, but did have distended bladder and enlarged seminal vesicles (Figure 4.14c).

The results presented herein support that 5$\alpha$-dihydrotestosterone has a greater capacity to modulate VT-induced dysregulation of IgA production than estradiol. High levels of circulating estrogens or abnormal estrogen metabolism have been previously shown to play a detrimental role in the development of diseases such as systemic lupus erythematosus and rheumatoid arthritis (Styrt and Sugarman, 1991; Okuyama *et al.*, 1992; Sthoeger *et al.*, 1988). However, female mice and those with estradiol supplements in these studies developed a milder form of IgAN. VT-induced glomerulonephritis in those mice treated with DHT appeared to be more rapid and severe as indicated by the following immunopathologic markers: (1) serum IgA, (2) hematuria, (3) mesangial IgA deposition, and (4) mesangial $C_3$ deposition. Our observations conflict with previous evidence that estradiol is capable of increasing IgG, IgA and IgM production through enhancing proliferation of antibody producing cells (Ahmed *et al.*, 1989; Myers and Petersen, 1985).

In addition, estrogens have the capacity to decrease cellular immune functions such as reducing delayed type hypersensitivity or the activity of natural killer cells (Myers *et al.*, 1986). The less severe IgA dysregulation in the female mice and those with an $E_2$ supplement may be due to stimulation of the reticuloendothelial system (RES) by enhanced hepatic macrophage phagocytosis caused by $E_2$ (Luster *et al.*, 1984). In a previous investigation, a research group in Japan formed an experimental

Figure 4.15. Mesangial deposition in kidney of mouse with urologic syndrome as determined by electron microscopy. Deposits are indicated by "d" and arrows; mesangial cells represented by "mes".


d
d
mes
d
d

model that scrutinized the role of a dysfunctioning reticuloendothelialsystem (RES) in the progression of the disease (Sato *et al.*, 1986). The authors made a direct association between decreased phagocytosis and a dysfunctioning or blocked RES which contributed significantly to IgAN.

DHT causes a depression of LPS-stimulated mitogenesis through suppression of antibody responses (Luster *et al.*, 1984). Furthermore, previous studies of protein handling by the kidney have revealed sex differences in the rate of removal. In rats, females are capable of reabsorbing more labeled protein and degrading it more rapidly than males in the same allotted time period (Aldin and Frith, 1991). The heterogeneity of immune responses in males and females has recently been attributed to variations in the patterns of cytokine production, which can occur qualitatively, quantitatively, and/or temporally (Daynes and Araneo, 1992). It has been reported that VT induces elevation of cytokine gene expression (Dong *et al.*, 1994), therefore, it seems that DHT has the capacity to antagonize this process by reprogramming T cells to produce altered levels of specific cytokines (Araneo *et al.*, 1991; Daynes and Araneo, 1992). In addition, we can not rule out the possibility that endogenous sex hormones may amplify or diminish the detoxification of VT by cytochrome p450 (Cote *et al.*, 1985; Roy and Liehr, 1992). Recently, distinct forms of cytochrome P450 have been demonstrated to have sex-related properties such as male- or female-specificity or male- or female-dominance in their expressions; levels of these enzymes are regulated by hormones (Kato and Yamazoe, 1992).

In conclusion, the results presented from these studies rendered compelling proof that supports the proposal that sex hormones, specifically

5αdihydrotestosterone, contribute an integral modulatory factor in VT toxicity. Furthermore, the results confirm increased male sensitivity to VT irrespective of hormone treatment, an observation consistent with epidemiologic reports of a greater incidence of IgAN in men than women.

# Chapter 5. Summary

The investigations conducted in this thesis yielded the following significant findings:

(1) Strains other than the B6C3F1 mouse were susceptible to VT-induced IgA dysregulation and IgA nephropathy

(2) B6C3F1 male mice were more susceptible to the effects of VT than their female counterparts based on comparison of latency, threshold dose and/or severity of the following immunopathologic markers: (a) serum IgA, (b) hematuria, (c) mesangial IgA deposition, and (d) mesangial $C_3$ deposition

(3) The optimal concentration of VT that elicited maximal IgA production in male B6C3F1 mice was 10 ppm

(4) The sex hormones, 17$\beta$-estradiol and 5$\alpha$-dihydrotestosterone have the capacity to alter the extent and severity of VT-induced IgA dysregulation and IgA nephropathy. More specifically, DHT increases the severity of VT-toxicity.

As previously discussed, VT-induced IgA dysregulation is a result of protein synthesis inhibition that prevents elongation-termination by obstructing formation of peptide bonds. VT also has the capacity to inhibit DNA and RNA synthesis at higher concentrations; however, this occurs to a much lesser extent than protein synthesis inhibition (Kiessling,1986; Ueno, 1985). Hyperproduction of IgA resulting from VT

exposure occurs most probably at the PP level where terminal differentiation of IgA-secreting plasma cells is enhanced by increased cytokine production and T cell help (Bondy and Pestka, 1991; Warner *et al.*, 1994; Dong *et al.*, 1994).

The most remarkable finding in the investigations reported herein was that B6C3F1 male mice exhibited increased susceptiblity. It is notable that previous findings in this VT IgA nephropathy model directly parallel the human disease. It is particularly critical to note that IgA nephropathy occurs much more frequently in men than in women. However, we had not formerly shown a male predilection in our animal model. Since the gender aspect of our model mimics the human disease relative to male frequency and severity, it can be utilized as a possible mechanistic model for the human disease.

Increased male susceptibility was consistent in all four *in vivo* studies. In the initial study, we observed heightened male sensitivity to VT as indicated by the very elevated levels of serum IgA, a more severe hematuria and increased deposition of IgA in the mesangium. Based on these findings, a follow-up dose response and male predilection study was conducted which showed males were more sensitive in that they were affected by a lower dose (2ppm) than females, VT effects were seen at an earlier time point and the males displayed more severe VT-induced conditions.

Having established increased male sensitivity to the toxin, and in order to more directly address the hypothesis, castrated male and female mice were employed to further identify the role of sex hormones in VT-toxicity. By castrating the mice, and thus, decreasing the levels of the active hormones in circulation, we observed trends that supported a limited, but protective role for $E_2$ in that the intact females

were inclined to have lower IgA levels than their castrated counterparts. Similarly, a possible harmful function of testosterone, specifically DHT, was identified in that castrated males tended to have lower IgA levels than their intact analogues.

The purpose of the subsequent *in vivo* study yielded two important findings. First, it demonstrated that DHT was actually modulating VT-toxicity by showing the harmful effects of this hormone in males and females. In addition, it showed that $E_2$ also has the capacity to regulate VT toxicity, but to a lesser degree than its androgenic analogue. Secondly, increased male susceptibility to VT-induced IgA dysregulation and IgAN was illustrated again, in that male animals with any of the pellet implants displayed higher IgA levels, greater mesangial deposition of IgA and more severe hematuria than their female counterparts. Although in each instance the increase was not always significant, it was notable nonetheless.

An explanation for increased sensitivity of male mice to VT is not yet possible, however, there are several possible mechanisms. There is the possibility that endogenous steroid hormones, specifically estrogens and androgens, enhance the cytochrome P-450 detoxification of VT (Cote *et al.*, 1985). Recently, it has been demonstrated that specific forms of cytochrome P450 have sex-related properties such as male- or female-specificity or male- or female-dominance in their expressions. It has also been shown that levels of these enzymes are regulated by hormones (Kato and Yamazoe, 1992). However, in the case of VT, it is probably unlikely that P-450 metabolism is responsible for the differences as evidenced by a 1987 study (Cote *et al.*, 1987). In this report, the investigators found that VT is neither bioactivated to a more toxic intermediate nor oxidized to a less toxic product by the mixed function

oxidase system (Cote *et al.*, 1987).

High levels of circulating estrogens or abnormal estrogen metabolism have been previously shown to exacerbate development of diseases such as systemic lupus erythematosus and rheumatoid arthritis (Styrt and Sugarman, 1991; Okuyama *et al.*, 1992; Sthoeger *et al.*, 1988). Estrogens can inhibit lymphocyte proliferative responses (Forsberg, 1984), affect the quality and quantity of lymphocytes and monocytes (Luster *et al.*, 1984), and depress macrophage activation by lymphokines (Pfeifer and Patterson, 1985). Sex hormones might also regulate the immune response by affecting T cell driven mechanisms as well as production and elaboration of antibodies by B cells, and cytokines by other immune cells (Schuurs and Verheul, 1990; Forsberg, 1984; Araneo *et al.*, 1991). For example, estrogens have been shown to have the capacity to interact directly with B cells, suppress delayed type hypersensitivity reactions and modify host responses to infection (Myers and Peterson, 1985; Forsberg, 1984; Josefsson *et al.*, 1992; Styrt and Sugarman, 1991).

The less severe IgA dysregulation in the female mice and those with an $E_2$ supplements may be due to stimulation of the reticuloendothelial system (RES) by enhanced hepatic macrophage phagocytosis caused by $E_2$ (Luster *et al.*, 1984). In previous investigation, a research group in Japan formed an experimental model that evaluated the role of a dysfunctioning reticuloendothelial system (RES) in the progression of experimental IgAN (Sato *et al.*, 1986). The authors made a direct association between decreased phagocytosis and a dysfunctional or blocked RES which contributed significantly to IgAN.

There is extensive documentation that $E_2$ depresses the function of thymic

lymphocytes (Matsushita *et al.*, 1993; Grossman, 1984, 1985; Luster *et al.*, 1984). Thus, it is possible that in these studies, female animals and those supplemented with $E_2$ experienced a reduction in the $CD4^+/CD8^+$ ratio in lymphoid organs (Matsushita *et al.*, 1993). It has been shown that estrogens can affect intrathymic differentiation of T cells most probably by altering or causing an imbalance in thymocyte populations resulting in depletion of double positive thymocytes, an increase in the population of single positive and double negative thymoctyes (Screpanti *et al.*, 1991). Furthermore, estradiol treatment prompts thymic atrophy at low and high replacement doses. Since VT-induced IgA dysregulation occurs at the PP level and has been shown to be modulated by $CD4^+$ lymphocytes (Bondy and Pestka, 1991; Pestka *et al.*, 1990a,b), it is conceivable that the mice with higher estrogen concentrations had depleted $CD4^+$ populations, and thus, experienced less severe hyperproduction of IgA.

The interactions of androgens such as testosterone with cells of the immune system are not characterized as well as those of estrogens, but have been shown to increase the effectiveness of cell mediated immunity (Schuurs and Verheul, 1990). For example, androgens have been shown to increase spleen and lymph node $CD8^+$ populations in the mouse in *in vivo* studies (Homo-Delarche,*et al.*, 1991). Dihydrotestosterone has the ability to reduce the production of interleukins 4 and 5, as well as $\gamma$-interferon *in vitro* (Araneo *et al.*, 1991). It is possible that DHT intensifies the effects of VT by altering T cell-driven B cell expansion and differentiation to IgA secreting cells by suppressing production of specific cytokines (Araneo *et al.*, 1991).

Related to a second possibility, previous investigations of protein handling by the kidney have revealed sex differences in the rate of removal. In the rat, females

are able to reabsorb more labeled protein and degrade it more quickly than males in the same amount of time (Aldin and Frith, 1991). This might explain why male mice in these studies exhibited severe hematuria earlier and to a greater extent than their female counterparts. A third possibility is different metabolism between males and females. Testosterone has been shown to increase the rate of acetylation of certain compounds to mutagenic intermediates in the kidneys of male mice as compared to their female counterparts (Hultin and Weber, 1986). Kidney microsomes taken from male mice have a greater capacity to activate specific compounds (2-aminofluorene to 2-acetylaminofluorene, a carcinogen) to their mutagenic forms than those taken from female kidneys (Smolen *et al.*, 1993; Brusick *et al.*, 1976).

Preliminary *in vitro* studies with purified and mixed lymphocyte populations cultured in the presence of mitogen and varying concentrations of 17$\beta$-estradiol, dihydrotestosterone, and VT were conducted in this investigation in an attempt to identify the role of sex hormones in VT-toxicity at the cytokine level. While consistent trends for IL-4, IL-5 and IL-2 production as influenced by the hormones were not identified, these investigations yielded data, that with the optimization of the system utilized, will provide important information necessary to answer many of the questions surrounding increased male susceptibility.

In conclusion, the findings of this thesis suggest that male B6C3F1 mice, in particular, were more sensitive to VT-toxicity as indicated by comparison of latency, threshold dose and severity of immunopathologic markers consistent with IgA nephropathy and that strains other than the B6C3F1 mouse were susceptible to the harmful effects of VT-toxicity following oral exposure to VT. A modulatory role for

the sex hormones in VT-toxicity has been shown. Specifically, increased male susceptibility to VT-induced IgA dysregulation and IgA nephropathy appears to be directly linked to the presence of circulating 5$\alpha$-dihydrotestosterone. Mechanisms that need to be studied in the future include: (1) the influence of varying concentrations of sex hormones on T cell regulation of Ig production by cultured PP lymphocytes; (2) effect of sex hormones on IL production by T-helper cells ($CD4^+$); (3) whether hormones act in synergy with cytokines and requisite T:B cell cognate interactions that increase IgA production .

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