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EARLY AND LATE MECHANISMS FOR VOMITOXIN-INDUCED IgA NEPHROPATHY

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EARLY AND LATE MECHANISMS FOR VOMITOXIN-INDUCED IgA NEPHROPATHY

By

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ABSTRACT

EARLY AND LATE MECHANISMS FOR VOMITOXIN-INDUCED IgA NEPHROPATHY

By

Ding Yan

Prolonged oral vomitoxin (VT) exposure in mice results in increased cytokine gene expression, elevated production of IgA and IgA nephropathy (IgAN). In this study, both early and late mechanisms for VT-induced IgAN were examined. To assess the possible role of cytokines in this IgA dysregulation, the effects of a single oral VT exposure to mice on production of IgA and cytokines in Peyer's patch (PP) and spleen cell cultures were evaluated. The results indicated that PP cells exhibited an enhanced capacity for production of IgA, IL-5 and IL-6 as early as 2 hr and as late as 24 hr after VT exposure. Both control and VT-induced IgA production were inhibited by IL-5 and IL-6 neutralizing antibodies. Subsequently, to determine the potential role of macrophages (Mϕ) on regulation of IgA production, the effects of Mϕ on in vitro IgA and IL-6 production after oral VT exposure in mice were assessed. The results demonstrated that both IgA and IL-6 production by VT-treated and control PP and spleen cultures were diminished after Mϕ depletion. Enhanced secretion of IL-6 occurred concurrently with increased IgA production in Mϕ-depleted PP cultures reconstituted with VT-treated peritoneal Mϕ. PP B cells from control animals produced significantly more IgA when co-cultured with VT-treated Mϕ and, to a lesser extent, with VT-treated $CD4^+$ T cells. Both soluble mediators and cognate interactions between Mϕ and the lymphocyte

populations appeared to be necessary for increased IgA production in PP cultures following VT exposure. The above two studies identify early mechanisms for VT-induced IgAN. Finally, to assess the role of IgA in inducing the experimental nephropathy, both B6C3F1 and BALB/C mice were injected i.p. with VT-induced monoclonal IgA Abs and several immunopathologic markers were monitored. When treated with IgAs both strains of mice showed a marked elevation of serum IgA, IgA immune complexes (IC), IgG, IgM concentrations and mesangial IgA, IgG, C3 deposition and hematuria. Formation of IgA and IgG-casein complexes was detectable in B6C3F1 mice after injection suggesting that dietary casein might participate in IC-mediated pathogenesis of nephropathy. Except for IgG elevation, these same immunopathologic effects were observed in BALB/C mice after injection of IgA-secreting hybridoma cells. The final study identifies late mechanisms for VT-induced IgAN. Taken together, theses studies suggest that: (1) superinduction of IL-5 and IL-6 expression contribute to upregulation of IgA production in mice exposed orally to VT, (2) soluble mediators and cognate help from Mϕ are responsible, in part, for upregulation of IgA production in mice exposed orally to VT, and (3) nephropathy in mice could be induced by direct injection of IgAs encountered after VT feeding.

Dedicated to my wife Wenqi Fan, my daughters Youfang Yan and Wendy Yan, my mother Zhongling Bao, my father Zhongshen Yan, and my sister Pan-Pan Yan for their love, encouragement, patience, understanding, and support.

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LIST OF ABBREVIATIONS

Ab ----------antibody

Ag ---------- antigen

ANOVA ---------- analysis of variance

BSA ---------- bovine serum albumin

CHX ---------- cycloheximide

C3 ---------- complement 3

CT----------cholera toxin

DAS ---------- diacetoxyscirpenol

DNP ---------- dinitrophenyl

Con A ---------- concanavalin A

DMEM ----------Dulbecco's modified Eagle medium

ELISA ---------- enzyme-linked immunosorbent assay

FBS ----------fetal bovine serum

IC ---------- immune complexes

IFN-γ ---------- interferon-gamma

Ig ---------- immunoglobulin

IgAN ---------- immunoglobulin A nephropathy

IL ---------- interleukin

ION ---------- ionomycin

LPS ----------lipopolysaccharide

Mϕ ----------macrophages

NIV ----------nivalenol

PBS ---------- phosphate-buffered saline

PC ----------phosphorylcholin

PMA ---------- phorbol 12-myristate-13-acetate

PP ---------- Peyer's patch

SRBC ---------- sheep red blood cells

T-2 ----------T-2 toxin

TGF-β ----------transforming growth factor-beta

TNF-α ---------- tumour necrosis factor-alpha

TNP ---------- trinitrophenyl

VT ---------- vomitoxin

INTRODUCTION

Vomitoxin (VT) is a fungal secondary metabolite that belongs to a family of mycotoxins referred to as trichothecenes (Tanaka et al., 1988). This toxin is frequently found in cereal grains as well as other food and agricultural products (Abouzied et al., 1991; Rotter et al., 1996). The toxicological effects of VT as well as other trichothecene toxins include digestive disorders, skin inflammation, hemorrhagic syndrome, destruction of bone marrow, and nerve disorders (Ueno, 1983). One important toxicological effect of VT is its capacity to modulate immune function. VT can be both immunostimulatory and immunosuppressive in a variety of animal and cell culture models depending on dose, experimental animal, and targeted immune function (Pestka and Bondy, 1994). In mice, dietary VT exposure induces extremely high levels of serum IgA (Forsell et al., 1986; Pestka et al., 1989), increases the circulating IgA immune complexes (IgA-IC), and causes glomerular IgA deposition and hematuria (Pestka et al., 1989; Pestka and Bondy, 1990; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b). These symptoms are very similar, clinically, to human IgA nephropathy (Berger's Disease), which is the most common type of human glomerulonephritis world wide. Increases in percentages of membrane IgA^+ cells and IgA-secreting cells in Peyer's patch (PP) and spleens of VT-fed mice occur concurrently with these effects (Pestka et al., 1990a; Bondy and Pestka, 1991), suggesting that VT can stimulate IgA secretion. Furthermore, polyspecificity and autoreactivity of monoclonal IgA antibodies (Abs), typically have been identified in

hybridomas derived from PP of VT-fed mice (Rasooly et al., 1994). Notably, elevation of mesangial IgG and C3 deposition and hematuria was observed in mice after injection of VT-induced monoclonal IgA Abs, thus suggesting that these polyreactive IgAs might be nephritogenic in the mouse (Rasooly et al., 1994).

Cytokines are soluble protein or glycoprotein mediators that have marked regulatory effects in immune system. Cytokines influence B cell activation, class-switching, proliferation, and terminal differentiation to IgA-producing plasma cells (McGhee et al., 1989). T cells are one major source of helper cytokines for regulating IgA production (McGhee et al., 1989; Beagley and Elson, 1992; McGhee and Kiyono, 1993; Kihira and Kawanishi, 1995). Previous studies have shown that exposure to VT in vitro superinduces interleukin (IL)-2, IL-4, IL-5 and IL-6 mRNA expression in murine splenic $CD4^+$ T cells stimulated with concanavalin A (Con A) or phorbol myristate acetate (PMA) (Ouyang et al., 1995; 1996a; Azcona-Olivera et al., 1995a; Warner et al., 1994) as well as PMA-stimulated EL-4 thymoma cultures (Dong et al., 1994). These cytokines have previously been shown to enhance differentiation of B cells to IgA secretion (Lebman et al., 1990a; 1990b; Coffman et al., 1987; 1988; Beagley et al., 1988; 1989; Pockley and Montgomery, 1991a; Dieli et al., 1995). Recently, Azcona-Olivera et al., (1995b) demonstrated that acute oral VT exposure to mice elevates cytokine mRNA levels with maximal effects occurring within 2 hr in the 25 mg/kg BW groups. VT has been previously shown to elevate the percentages of T cells, $CD4^+$ T cells and $CD4^+/CD8^+$ T cell ratios in PP and spleens of VT-fed mice (Pestka et al., 1990a). In vitro studies have

demonstrated that VT plus $CD4^+$ T cells can significantly increase IgA production in B cells (Warner et al., 1994). In these investigations, VT also increased IL-6 secretion in Con A-stimulated $CD4^+$ T cells. Significantly elevated IgA production was also observed when PP T cells isolated from VT-fed mice were co-cultured with B cells (Bondy and Pestka, 1991). These findings suggest that T cells might play a role in regulating VT-induced IgA production.

Macrophages (Mϕ) are another important source of cytokines, some of which are capable of modulating T and B cell responses. Mϕ have been demonstrated to be major producers of IL-1, IL-6 and TNF-α within the immune system (Bauer et al., 1988; Bauer, 1989). Recently, VanCott et al., (1996) suggested that IL-6 secreted by Mϕ may contribute to development of mucosal IgA responses. VT has been previously shown to stimulate IL-1 release from peritoneal Mϕ in vitro. Azcona-Olivera et al., (1995b) and Zhou et al., (1997) recently demonstrated that acute oral VT exposure in mice hyperelevates proinflammatory cytokines IL-6, IL-1β, TNF-α and IFN-γ mRNA expression in spleen and PP. Wong et al., (1997) have recently demonstrated that VT can superinduce IL-6 and TNF-α in a cloned murine Mϕ cell line. Thus it is reasonable to suggest that VT might alter regulation of IgA.

Based on the observations of elevation cytokine gene expression, IgA production and IgA nephropathy after oral VT exposure in mice, we hypothesized that: (1) VT induces the specific stimulatory effect on IgA production via superinduction of helper cytokines; (2) VT stimulation of IgA production is mediated via the Mϕ; and (3) polyreactive IgA Abs induced by VT contribute to experimental

nephropathy. The objectives of my research were as follows:

1. To assess the effects of acute oral VT exposure on IgA and cytokine production in PP and spleen cell cultures in vitro.
2. To assess the effects of cytokine-specific neutralizing monoclonal Abs on IgA production in PP and spleen cultures isolated from mice exposed orally to VT.
3. To determine the effects of Mϕ depletion on in vitro IgA and IL-6 production by PP and spleen cultures following acute oral VT exposure in mice.
4. To compare the effects of acute oral VT exposure on IL-6 secretion by Mϕ and $CD4^+$ T cells and determine their capacity to increase IgA production.
5. To assess whether cognate interactions and/or soluble factors contribute to Mϕ-mediated enhancement of IgA production following acute oral VT exposure in mice.
6. To induce experimental nephropathy by injection of VT-induced IgA Abs into mice.

The dissertation is composed of five chapters. Chapter 1 is a review of the literature on VT and other trichothecenes, IgA nephropathy, and cytokines and IgA production. Chapter 2 describes the potential role for cytokines in enhanced IgA secretion by PP cells isolated from mice acutely exposed to VT. Chapter 3 describes the role of Mϕ in elevated IgA and IL-6 production by PP cultures

following acute oral VT exposure. The results from both chapter 2 and 3 also identify the early mechanisms for VT-induced IgA nephropathy. Chapter 4 describes the induction of nephropathy by injection with dietary VT-induced IgA monoclonal Abs into mice and identifies the late mechanisms for VT-induced IgA nephropathy. Finally, chapter 5 summarizes these inter-related studies and makes suggestions for future studies.

CHAPTER 1

LITERATURE REVIEW

1.1 Deoxynivalenol (vomitoxin) and other trichothecenes

1.1.1 History

Deoxynivalenol (vomitoxin) is a fungal secondary metabolite that belongs to a family of mycotoxins referred to as trichothecenes (Tanaka et al., 1988). Deoxynivalenol (DON) was first identified in Japanese barley contaminated with Fusarium spp and given the trivial name Rd toxin (Morooka et al., 1972). Subsequently, it was named vomitoxin (VT) due to emesis (vomiting) in swine caused by Fusarium-infected corn (Vesonder et al., 1973). This toxin was also detected in culture of Fusarium graminearum by Yoshizawa and Morooka, (1973). VT was structurally identified as a trichothecene (Vesonder et al., 1973) and given the chemical name DON (Yoshizawa and Morooka, 1973). VT is important economically due to the prevalence of grain and cereal products contamination by this toxin (Scott, 1989) and is a cause for a concern to human and animal health.

1.1.2 Chemical structure

Trichothecenes consist of oxygen, hydrogen, and carbon elements and they possess a tricyclic, epoxide ring system (Figure 1.1). All natural occurring trichothecenes contain a double bond at position C-9, 10 and an epoxy group at position C-12, 13 which are basic requirements for toxicity and biological activity (Ueno, 1980; Bamburg, 1983). The biological activity is completely lost after the epoxide group is reductively removed (Patterson, 1973). The trichothecenes

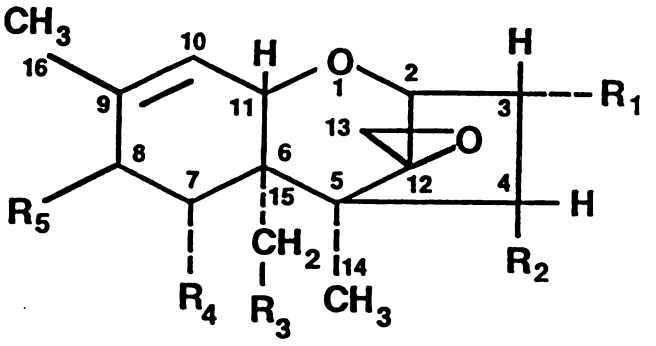


Figure 1.1. Structure of trichothecene

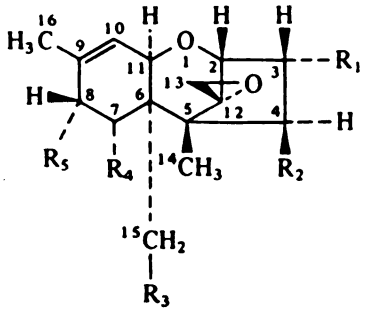
bear oxygen-containing substituents located at one or more of positions 3, 4, 7, 8 and 15. These substituents may be hydroxyl, esterified hydroxyl, keto (position 8 only), or epoxide (position 7 and 8 only) groups or combinations thereof (Ueno, 1980).

The trichothecenes have been classified into four groups according to their structural characteristics (Ueno, 1980) (Figure 1.2). VT belongs to group B of the trichothecenes and possesses a carbonyl group at position C-8 (Ueno, 1980). VT has structural similarities to several other trichothecene toxins such as T-2, nivalenol (NIV), and diacetoxyscirpenol (DAS) produced by strains of Fusarium, whose chemical structure is shown in Figure 1.3.

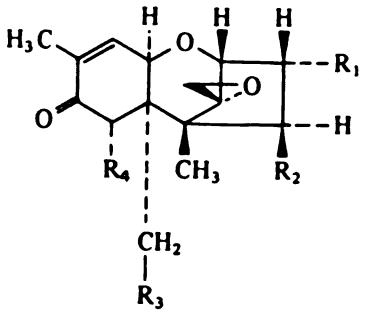
The natural trichothecenes are colorless, mostly crystalline, optically active solids which are generally soluble in moderately polar organic solvents but only very slightly soluble in water (Ueno, 1980). Alcohol derivatives have a higher solubility in water than their esterified homologue (Betina, 1989a). These compounds are stable in the solid state but can undergo reactions in solution. For example, esters are saponified by treatment with alkali and the 12-13 epoxide is opened by strong mineral acid (Ueno, 1977b).

1.1.3 Natural occurrence

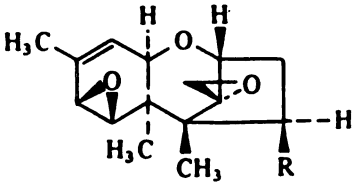
Each year, about 25% of the world's food crops are contaminated by mycotoxins (Mannon and Johnson, 1985). Currently, over 148 trichothecenes have been isolated and identified (Scott, 1990; Buck and Cote, 1991), and the most



Group A



Group B



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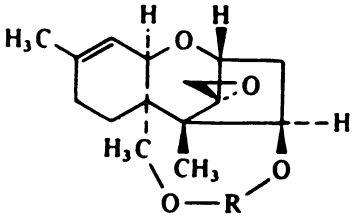
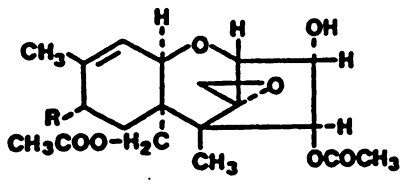
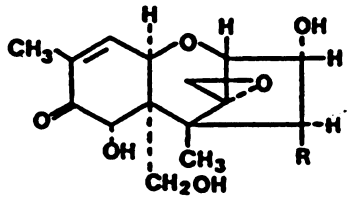


Group D

Figure 1.2. Classification of trichothecenes based on their chemical structure (from Ueno, 1980).



Group A



Group B

Trichothecenes	Group	R
T-2 toxin	A	$(CH_3)_2CHCH_2COO$-
Diacetoxyscirpenol	A	H
Nivalenol	B	OH
Deoxynivalenol (vomitoxin)	B	H

Figure 1.3. Examples of some naturally identified trichothecenes (from Scott, 1990).

commonly encountered by animals and humans are VT and T-2 toxin (Council for Agriculture Science and Technology, 1989). In both U.S. and Canada, VT is the most important trichothecene in cereal grains (Scott, 1990). VT is a naturally occurring metabolite and produced predominantly by Fusarium graminearum which is a principal cause of head blight in wheat (Sutton, 1982). They are frequently found in cereal grains as well as other food and agricultural products (Abouzied et al., 1991; Rotter et al., 1996). A number of reports have indicated that VT is the major toxicant in grains in Italy, Austria, South Africa, England, Canada, and United States (Vesonder and Ciegler, 1979; Bottalico et al., 1984; Ueno, 1984). Wide-spread occurrence of VT in cereal products has been also reported in France, West Germany, Japan, China, Taiwan and Russia (Jemmali et al., 1978; Blaas et al., 1984; Tanaka et al., 1985; Ueno et al., 1986). Thus, VT has been widely detected throughout the world in wheat, barley, corn, rice, mixed feed, and other products (Mirocha et al., 1977; Jemmali et al., 1978; Yoshizawa et al., 1979).

1.1.4 Toxin production and toxicity

The genus Fusarium contains important mycotoxin-producing species (Marasas et al., 1985). Under optional conditions of temperature and humidity, preharvest cereal grains, especially barley, wheat and corn, can be invaded by Fusarium graminearum (Vesonder et al., 1973; Vesonder and Ciegler, 1979; Yoshizawa et al., 1979). Cool and wet conditions favor fungal growth and toxin production (Pathre and Mirocha, 1979; Bamburg, 1983). The production of VT is

enhanced during cold and wet weather due to delayed harvest and extended growth of the fungus on the crop (Vesonder et al., 1978). VT can also be produced during warm, very humid weather (Richardson et al., 1985). Production of VT can continue during storage (Trenholm et al., 1981). These findings indicated that VT contamination is primarily confined to temperate climate zones of the world and is predominantly a result of field infection rather than storage development.

Once VT is produced by the fungus, it persists and cannot be decreased with feed additives, nor does it disappear with time (Wyllie and Morehouse, 1977). VT is heat stable and likely to survive during baking processes (Patey and Gilbert, 1989). It has been demonstrated that VT was not destroyed in the bread baked from naturally contaminated whole wheat flour (Abbas et al., 1985). This evidence suggests that VT is not inactivated during milling and processing of cereal grain products (Scott et al., 1983; Young et al., 1984) and detoxification may be difficult. A single report has indicated that soaking contaminated corn in 10% sodium bisulfite may detoxify VT completely (Hamilton, 1983). In general, detection and diversion of VT contamination during early stages are important. In the past, the methods that may be particularly useful in detection and identification of VT were based on thin layer or gas chromatography and mass spectroscopy. However, an immunochemical assay which is also effective in determining this toxin has subsequently been developed and applied (Pestka, 1988; Pestka and Casale, 1990; Abouzied et al., 1991).

The occurrence of VT in food and feeds creates a risk for humans and

animals that ingest the contaminated products (Ueno, 1977a). It has been reported in one study that 60% of breakfast cereals contained VT (Trucksess et al., 1986). In another survey, the mean concentration of VT was > 4.0 μg/g in all positive samples that come from various wheat and corn products including oat cereals, mixed grain cereals, and oat- and rice-based products (Abouzied et al., 1991). Due to the toxicity and wide occurrence of VT, some countries have set limits for this toxin in cereal grains. For example, in the U.S., an advisory level for VT (which has no legal force) is 2.0 μg/g in wheat and wheat products for milling process; 1.0 μg/g in finished wheat products for human consumption; and 4.0 μg/g for wheat and wheat milling by-products used in animal feed (Wood, 1992). Russia has an official tolerance limit of 0.5 -1.0 μg/g for VT in wheat. In Canada, the guideline level for VT is 2.0 μg/g in uncleaned soft wheat (Van-Egmond, 1989). However, in Romania, a tolerance limit for VT is only 0.005 μg/g in feeds (Van-Egmond, 1989).

The toxicological effects of VT as well as other trichothecene toxins (T-2, NIV and DAS) include digestive disorders, skin inflammation, hemorrhagic syndrome, destruction of bone marrow, and nerve disorders (Ueno, 1983). A study on the acute toxicity of VT in mice revealed extensive necrosis in the gastrointestinal tract, bone marrow and lymphoid tissue, as well as focal lesions in kidney and cardiac tissue (Forsell et al., 1987). Since ingestion is the usual route of exposure to VT, it has been suggested that the gastrointestinal tract is one of the major target tissues for this toxin (Hunder et al., 1991). LD_{50} values of VT in mice are 70 mg/kg i.p. (intraperitoneal) and 78 mg/kg orally (Forsell et al., 1987), which is less acutely

toxic than other trichothecene toxins such as NIV, T-2 and DAS (Ueno, 1977b). The LD_{50} of NIV, T-2 and DAS in the mice (i.p.) is 4.1 mg/kg, 5.2 mg/kg and 23 mg/kg, respectively (Ueno, 1977b).

Notably, farm animals have different sensitivities to VT. Swine are most sensitive to VT, refusing feed containing < 2 ppm VT (Trenholm et al., 1984). On the other hand, cattle and poultry may be relatively tolerant to VT and have been shown to tolerate ≥ 20 ppm VT (Trenholm et al., 1984; Hamilton et al., 1985). A possible reason for cattle being less susceptible to VT is the ability of rumen microorganisms that metabolize/detoxify this toxin (King et al., 1984). Furthermore, there is a sex-associated susceptibility to VT in that male animals appear to be more sensitive than female animals (Iverson et al., 1995; Greene et al., 1994a; 1994b; Rotter et al., 1994).

The half-life (t 1/2) of VT ranges from 2.08 to 3.65 hr in swine which receive VT intravenously (Coppock et al., 1985). Prelusky et al., (1984) observed that the half-life of VT is about 4 hr following oral exposure of VT to dairy cows. Clearance of VT from turkey plasma was very rapid with the t ½ of 44 min after VT exposure (Gauvreau, 1991). Recently, Azcona-Olivera et al., (1995b) reported that VT is rapidly absorbed from gut with peak levels being detected in plasma at ≤ 30 min after mice received 25 mg/kg BW VT. Notably, at this dose, the initial and terminal elimination half-life were 0.56 and 88.9 hr, respectively.

Since VT is often found in corn and wheat infected by Fusarium graminearum, these grains are high risk as an animal feeds. VT-contaminated

feeds has been associated with many cases of sublethal toxicosis in animals resulting in feed refusal, reduced weight gain, emesis and diarrhea (Yoshizawa and Morooka ,1974; Vesonder et al., 1976; Yoshizawa et al., 1978). There is also evidence to show that an increase in hepatic neoplastic nodules occurs in rats fed Fusarium-contaminated corn (Wilson et al., 1985). However, some other results suggest that VT does not have carcinogenic potential and does not have mutagenic activity (Wehner et al., 1978; Rogers and Heroux-Metcalf, 1983; Lambert et al., 1995).

Clinical symptoms such as emesis and diarrhea were also observed in humans who ingested VT in India in 1987 (Bhat et al., 1989) and similar reportes of human illness were associated with VT in China (Luo, 1988). Several countries have reported outbreaks of wheat toxicoses in humans due to the consumption of VT in the food infected by Fusarium graminearum (Ueno, 1983), but this toxicosis rarely causes death (Luo, 1988). Some reports indicated that human esophageal cancer may be associated with high levels of VT in Africa (Marasas et al., 1979) and China (Luo et al., 1990). These outbreaks of disease indicate a need for caution, especially with regard to the potential health effects of chronic exposure to VT.

Taken together, the above studies support the hypothesis that trichothecene mycotoxins might be the causative toxicants in some food borne intoxications such as alimentary toxic aleukia (ATA) in Russia, mold corn toxicosis (most frequently associated with VT) in the United States, and red mold disease in Japan (Ueno et

al., 1972a; 1972b). Therefore, contamination of VT in cereal grain products is a considerable food safety concern in many countries.

1.1.5 Immunotoxicity

VT and other trichothecenes are potent protein synthesis inhibitors (Ueno, 1985; Betina, 1989b; Pestka and Casale, 1990) that can significantly alter cell-mediated immunity, humoral immunity and host resistance in animal models (Pestka et al., 1987; Pestka and Bondy, 1990). VT may inhibit elongation steps of protein synthesis by interaction with the 60S ribosomal subunit and suppression of peptidyl transferase activity (Bamburg, 1983; Kiessling, 1986). Robbana-Barnat et al., (1985) reported that cardiac protein synthesis was decreased about 30% after intraperitoneal administration of VT. Recently, Azcona-Olivera et al., (1995b) observed that more than 70% of protein synthesis is inhibited for as long as 9 hr in all tissue of mice receiving 25 mg/kg BW VT.

Trichothecenes are acutely toxic to actively dividing cell populations in tissues such as bone marrow, thymus, lymph nodes, spleen and intestinal mucosa (Ueno, 1977b). Acute and chronic toxicities of trichothecenes cause depletion of lymphoid tissues (Ueno et al., 1972a) which induces disorders of the immune system. One interesting finding is that VT can be both immunosuppressive and immunostimulatory (Pestka et al., 1987; Pestka and Bondy, 1994). Some studies demonstrated that VT was capable of producing immunosuppressive effects in experimental animals (Tryphonas et al., 1984; 1986), which may lead to increased

incidence of infection and disease (Vanyi and Sandor, 1988). VT can decrease murine thymus weight, humoral response, and inhibit murine splenocyte as well as rat and human lymphocyte cellular proliferation (Atkinson and Miller, 1984; Robbana-Barnat et al., 1988,). It is possible that impaired immunologic responsiveness may reduce resistance to infection and therefore cause animal disease (Pier et al., 1980). For example, it has been reported that oral VT exposure to mice results in reduced ability to resist Listeria monocytogenes and significantly increased splenic Listeria counts (Tryphonas et at., 1986; Pestka et al., 1987). In addition to the suppressive effects of VT, it also has the stimulatory effects on immune response. In mice, dietary VT exposure induces extremely high levels of serum IgA (Forsell et al., 1986; Pestka, et al., 1989) and IgE (Pestka and Dong, 1994) as well as decreased IgG and IgM levels (Forsell et al., 1986; Pestka, et al., 1989). Subsequently, Pestka et al., (1989) and Greene et al., (1994a) found that the optimal VT level for serum IgA elevation was at 25 ppm. In addition to increasing total amount of serum IgA, VT can also increase the polymeric/monomeric IgA ratio in serum (Pestka et al., 1989).

A study on the effects of IgA reactivity to casein (a component of the diet), and cholera toxin (CT), indicated that VT can enhance antigen (Ag)-specific IgA and diminish Ag-specific IgG production (Pestka et al, 1990b). Casein-specific IgA was significantly increased in mice fed 25 ppm VT for 16 and 20 wks. Elevation of CT-specific IgA secretion was also found in CT-unimmunized mice fed 25 ppm VT for 16 and 20 wks but not in CT-immunized mice fed VT. Both casein and CT-specific

IgG were inhibited in VT-fed mice.

In vitro exposure to VT has been also shown to marginally stimulate IgA secretion in cloned B cell line CH12LX (Minervini et al., 1993). However, VT can not elevate IgA production in purified splenic B cell cultures (Warner et al., 1994). VT has been shown to increase the percentages of membrane IgA^+ cells, T cells and $CD4^+$ T cells as well as alter $CD4^+/CD8^+$ cell ratios in PP and spleens of mice fed 25 ppm VT (Pestka et al., 1990a; Bondy and Pestka. 1991). The effects of VT increasing $CD4^+$ T cells and $CD4^+/CD8^+$ cell ratios implied that T helper cells may play a role in VT-induced IgA secretion (Pestka et al., 1990a). Bondy and Pestka, (1991) demonstrated that significantly increased IgA production occurs when control B cells are co-cultured with PP T cells isolated from mice fed 25 ppm VT for 8 wks. This contribution from T cells is further supported by recent in vitro studies, where it was demonstrated that IgA production was significantly increased when murine splenic $CD4^+$ T cells were exposed to 50 ng/ml of VT and Con A for 24 or 48 hr and then co-cultured with B cells for 7 days with lipopolysaccharide (LPS) (Warner et al., 1994).

The T cell helper effects on elevation of IgA production may be mediated by cytokines such as IL-2, IL-4, IL-5 and IL-6. These cytokines have previously been shown to enhance differentiation of B cells to IgA secretion (Lebman et al., 1990a; 1990b; Coffman et al., 1987; 1988; Beagley et al., 1988; 1989; Pockley and Montgomery, 1991a; Dieli et al., 1995). Recent studies have shown that exposure to VT in vitro superinduces cytokine IL-2, IL-4, IL-5 and IL-6 mRNA expression in

murine splenic $CD4^+$ T cells stimulated with Con A or PMA (Ouyang et al., 1995; 1996a; Warner et al. 1994; Azcona-Olivera et al., 1995a) as well as PMA-stimulated EL-4 thymoma cultures (Dong et al., 1994). Azcona-Olivera et al., (1995b) demonstrated that acute oral VT exposure to mice elevates cytokine mRNA levels with maximal effects occurring in the 25 mg/kg BW group in as little as 2 hr. These observations suggest that VT induced a specific stimulatory effect on IgA production, possibly mediated by elevating T helper cell responses and resulting in enhancement of cytokine secretion.

Beside VT disrupting normal regulation of IgA production in the mouse, this toxin can also increase circulating IgA-IC in serum, and cause glomerular IgA deposition and hematuria in VT-fed mice (Pestka et al., 1989; Pestka and Bondy, 1990; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Green et al., 1994a; 1994b). These symptoms are very similar, clinically, to human IgA nephropathy which is the most common form of glomerulonephritis world-wide with an unclear etiology (D'Amico, 1987). This information suggests that VT may be a possible etiological factor in IgA nephropathy. Taken together, all these findings are indicative of dysregulation of IgA production by VT.

1.2 IgA nephropathy

1.2.1 History

Immunoglobulin A nephropathy (IgAN) is a form of glomerulonephritis that has now been recognized to be the most common nephritis leading to end-stage renal failure in the world (D'Amico 1987). The etiology of this disease is not well-understood. IgAN, first described by Berger and Hinglais (1968), is characterized by the presence of IgA and C3 (complement component) deposits in the mesangium. In this study, all 25 patients had a somewhat similar clinical history consisting of macroscopic hematuria and moderate proteinuria. In most patients, episodes of recurrent macroscopic (gross) hematuria were observed. Immunofluorescence microscopy revealed diffuse and global glomerular mesangial staining with IgA, with less intense mesangial staining for IgG and C3. In 10 of these original patients, mesangial deposits were also observed by electron microscopy. Thus, IgAN was also named Berger' disease. Schena, (1990) indicated that an estimated 20% to 40% patients with IgAN develop end-stage renal failure 5 to 20 years after diagnosis because of the progressive nature of the disease. Although the pathogenesis of this disease is not fully understood, high serum levels of IgA and/or polymeric IgA and several kinds of IgA-class Abs suggest that there is an aberration in the control of the mucosal immune response resulting in a hyper-immune state of IgA production in patients with IgAN (Czerkinsky et al., 1986).

1.2.2 Geographic distribution

Although IgAN appears to be a ubiquitous disorder, its reported geographical distribution is irregular. Investigations from many countries have shown that the apparent incidence of this disease has varied in studies from different countries. IgAN has been known to be very frequent in Asian-Pacific and South European countries (Schena, 1990). The disease is particularly prevalent and is one of the most common forms of glomerular disease in Australia (Clarkson et al., 1979), Spain (Navas-Palacios et al., 1981), Italy (Mandreoli et al., 1981), France (Levy et al., 1973), China (Zhou and Chen, 1986), Hong Kong (Lai et al., 1985), Singapore (Sinniah et al., 1981) and Japan (Shirai et al., 1978). In these countries, patients with IgAN may display more serve renal lesions and have a high rate of progressive renal insufficiency leading to end-stage renal failure. The frequency noted on renal biopsy examination ranges from 12 to 40 percent. However, much lower rates of incidence have been observed in the United States (McCoy et al., 1974; Lee et al., 1982), England (Sissons et al., 1975), Germany (Michalk et al., 1980), Canada (Katz et al., 1976), India (Kher et al., 1983) and the Netherlands (van-der-Peet et al., 1977) with the frequency rate ranges from 2 to 8 percent in these countries. Thus, the high frequency of IgAN in certain countries in contrast to the lesser prevalence in others suggests that some geographical, genetic, or dietary factor may be relate to the development of this disease.

1.2.3 Clinical features

The clinical presentation of IgAN covers a wide range from isolated hematuria to rapidly progressive renal failure (Nicholls et al., 1984). Most patients with this disease display mild to moderate mesangial proliferation associated with increase mesangial electron-dense deposits which are often preceded and accompanied by hematuria (Emancipator et al., 1987; Hisano et al., 1991). Kidney damage is revealed as hematuria and proteinuria. Macroscopic hematuria was thought initially to be the presenting symptom in the majority of patients with IgAN. The hematuria is painless, but is associated with systemic symptoms such as fever, malaise, fatigue, diffuse muscle aches, and abdominal pain (Walshe et al., 1984) as well as loin pain (MacDonald et al., 1975). Andreoli et al., (1986) reported that patients with heavy proteinuria have the most severe glomerular lesions and least favorable prognosis.

Notably, many reports have indicated that the clinical features of IgAN in adults and children patients are different. These include elevated frequency of proteinuria (Mina et al., 1985), serum levels of IgA (Clarkson et al., 1977), and systemic hypertension (Morel-Maroger et al., 1972; Droz et al., 1984) in adults as well as increased frequency of gross hematuria in children (Mina et al., 1985). In France, gross hematuria is the presenting symptom in 40% of adults and 80% of children (Levy et al., 1973). Furthermore, Abuelo et al., (1984) observed that 41 percent of patients with rapidly progressive form of IgAN were 16 years of age or younger, and suggested that younger patients (under 18 years of age) may be predominantly involved in this end-stage renal disease occurred.

There is a sex-related susceptibility to IgAN in that female patients appear to be associated with minor glomerular lesions as compared with males, either in adults or in children (Droz et al., 1984). This may account for the better prognosis found in females. Interestingly, IgAN is rare in the black population and is more common in black female (Jennette et al., 1985).

1.2.4 Laboratory features

Since the initial studies of IgAN were characterized by staining of glomerular mesangial regions with antisera to IgA and IgG (Berger and Hinglais, 1968), positive mesangial immunofluorescence for IgA as the predominant Ig is the diagnostic hallmark and requires renal biopsy. It has been reported that serum levels of IgA are increased in about 50% of patients with IgAN (D'Amico, 1983), and there was a persistent elevation of serum IgA in many patients during the three year follow-up period. Such enhancement of serum IgA may be contribute to the persistent deposition of IgA in the mesangial areas. The finding of high serum levels of polymeric IgA in patients with IgAN is possibly associated with these observations (Trascasa et al., 1980).

IgA is the primary Ig associated with the mucosal membrane. There, IgA is dimerized and transformed into secretory IgA by a secretory component and J chain. About 70% of the IgA eluted from renal biopsies from patients with IgAN is polymeric IgA (Monteiro et al., 1984). The importance of the presence of dimeric or polymeric IgA in the circulating IC in relation to the clinical activity of the IgAN has

been further confirmed by Valentijn et al., (1984), where it was demonstrated that glomerular IgA deposits in IgAN is mostly IgA1 and polymeric. Mestecky et al., (1987) also verified that circulating IC isolated from patients with IgAN contain polymeric IgA1. Therefore, it is possible that overproduction of polyclonal or antigen-specific IgA1 and/or its defective clearance may be associated with the pathogenesis of IgAN (Emancipator et al., 1989).

IgA containing IC have been well demonstrated in the circulation of patients with IgAN (Woodroffe et al., 1980; Lesavre et al., 1982; Czerkinsky et al., 1986), and they suggest that IgA-IC may play a important role in the pathogenesis of IgAN. Woodroffe et al., (1980) indicated that dysregulation of IgA production against dietary Ag or pathogens leads to form circulating IgA-IC and subsequent deposition in the kidney result in glomerular damage. Circulating IC were present intermittently and corresponded with episodes of gross hematuria. Woodroffe et al., (1980) had demonstrated that IC also contains the IgG class. The mixed IgA-IgG IC have been detected in more than 40% of serum from IgAN patients (Czerkinsky et al., 1986). The circulating IC are intermediate in size (9 to 17s) and contained IgA, IgG and, less commonly, IgM (Woodroffe et al., 1980; Lesavre et al., 1982). Hall et al., (1983) indicated that IgA-IC are more prevalent in the early stages of the disease, and suggested that the initial immunologic insult to the glomerulus occurs most frequently in childhood and adolescence.

Although the early reports from Berger and Hinglais (1968) indicated that characterization of IgAN is predominant deposition of IgA in the renal mesangium,

there other Igs are associated with this disease. According to the statistical results from different countries by D'Amico, (1983), IgG is found in combination with IgA in more than 50 percent of the IgAN patients examined in most countries. About 25 to 30 percent of biopsy specimens from patients with IgAN contain IgM class. The Southwest Pediatric Nephropathy Study Group, (1982) proposed that IgM deposits may occur as a secondary process deposits.

C3 deposition parallels IgA in both distribution and intensity in most patients (more than 80%) with IgAN (D'Amico, 1983). However, the classical pathway complement components, C1 and C4, are usually absent or present in a lower percent (10% of patients) and exhibit low intensity staining. On the other hand, properdin is often present in 50% to 100% of patients with IgAN (Katz et al., 1976). C3 and properdin in the glomerular immune deposits have been assumed to indicate a predominant role for the alternative complement activation pathway in this disease (McCoy et al., 1974). A previous study has demonstrated that IgA-IC can activate the alternative complement pathway (Gotze and Muller-Eberhard, 1971). Julian et al., (1983) also observed that certain types of IgA aggregates or IgA myeloma complexes can activate complement in vitro. All these observations suggest that IC (including IgA-IC) may activate C3 through the alternative complement pathway, resulting in generation of activation fragments with the potential for mediating inflammatory injury in the glomeruli.

Sakai (1988) suggested that cytokines may play an important role in the etiology and pathogenesis of IgAN. One interesting observation is that IgAN

patients have high urinary IL-6 activity (Dohi et al., 1991). Other studies have demonstrated that there is an increase in IL-5, IL-6 and TGF-β mRNA levels in $CD4^+$ T cells (de-Caestecker et al., 1993; Lai et al., 1994a; 1994b) as well as IL-4, IL-5 and IL-6 mRNA levels in peripheral blood mononuclear cells of IgAN patients (Ichinose et al., 1996).

1.2.5 Animal models

Several animal models of IgAN can elicit clinical and morphologic features which closely resemble the human syndrome. The first experimental model of IgAN employed IC of the hapten-specific mouse myeloma IgA anti-dinitrophenyl derived from MOPC 315 plasmacytoma and dinitrophenyl hapten conjugated to bovine serum albumin (DNP-BSA) in BALB/C mice (Rifai et al., 1979). In this study, passive injection of IC of IgA and DNP-BSA, or separate injections of these two compounds, resulted in deposition of IgA and DNP-BSA in the mesangium. Similar depositions but more severe pathological changes were observed upon injection of DNP-BSA alone into MOPC 315 tumor-bearing mice. The degree of deposition was associated with the size and dose of complexes. Furthermore, Rifai and Millard, (1985) further demonstrated that IC induce C3 deposits and elicit hematuria. Complexes prepared with dimeric or polymeric IgA regularly generated mesangial deposits, whereas monomeric IgA at the same dose and degree of Ag excess failed to cause deposition. This may be related to the predominance of polymeric IgA-IC occurring in glomerular deposits of this disease. Thus, Rifai and

colleagues reproduced the clinical and pathologic observations most frequently found in patients with IgAN. They proposed that the human disease is IC in nature, the Ab being predominantly of the IgA class.

Induction of glomerular IgA deposits (IgA/IgA-IC) was also observed in mice following administration of IgA anti-DNP (Ab) and DNP-conjugated IgA anti-phosphorylcholine (as an Ag) (Montinaro et al., 1991). This evidence demonstrated that the Ag plays a critical role in development of glomerulonephritis associated with IgA-IC. Some other studies have demonstrated that glomerular IgA, C3 and, with less amounts, IgM and IgG deposits were induced in Swiss Webster mice (Isaacs et al., 1981; Isaacs and Miller, 1982) or in Lewis rats (Fornasieri et al., 1993) by active injection of different sized (10, 70 and 500 kD) and different charged dextrans (neutral dextran, dextran sulfate and diethylaminoethyl-dextran). Both treatment mice and rats also developed hematuria. These observations suggest that size and charge of IgA-IC may be important factors in formation of glomerular deposits.

Emancipator et al., (1983) developed another murine model of IgAN by active oral immunization with proteins. In this experiment, after protracted oral immunization with three different protein Ags (bovine gamma globulin, ovalbumin and ferritin) for 14 wks, mice exhibited a heightened mucosal Ab response to these Ags with significant increases in serum IgA Ab and mesangial deposits of IgA. The immunofluorescence observations and ultrastructural appearance of glomeruli closely resemble those from patients with IgAN. These observations suggest that

prolonged mucosal immunization can induce a specific IgA response that leads to IgA-containing IC deposits in the mesangium. Emancipator et al., (1987) further demonstrated that serum IgA, IgG and IgM Abs were significantly elevated after oral immunization with protein Ags such as bovine gamma globulin to mice. In addition to mesangial deposition of IgA and oral immunogen, codeposition of IgG, IgM and C3 as well as microhematuria were also detected in these treatment mice. The investigators suggested that IgG and IgM codeposits in murine IgAN can induce the deposition of complement, which is turn contributes to glomerular injury. Subsequently, a similar model was developed in Wistar and Lewis rats (Gesualdo et al., 1992). In this study, Lewis rats showed predominantly glomerular IgA deposits with lesser IgG and C3 after 8 wks of continuous oral immunization with bovine gamma globulin. These immunized rats also developed microhematuria with negligible proteinuria. Other investigators also reported that oral immunization with gluten or ferritin resulted in elevation of serum IgA and mesangial IgA deposition as well as increase of serum anti-gluten or anti-ferritin Abs in BALB/C mice (Coppo et al., 1989) or in C3H/HeJ mice (Genin et al., 1986).

Jessen et al., (1987) have induced an experimental model of IgAN by injection of Sendal virus into mice since viral Ags have been implicated in the pathogenesis of human IgAN (Tomino et al., 1989). Immunization of mice with live viruses or viral protein extract resulted in a prevalent serum IgA (and IgG) anti-viral immune response, associated with mesangial deposition of IgA, C3 and viral Ag. Subsquently, Jessen et al., (1992) further demonstrated that immunized mice

challenged with either live or dead virions showed a high incidence of hematuria. These results suggest that this animal model may be useful to probe infection-related IgAN.

Interestingly, a spontaneous animal model for primary IgAN has been reported by Imai et al. (1985) in ddY mice. ddY mice older than 40 wks have a marked accumulation of IgA and C3 in their glomeruli and elevated serum IgA levels. In contrast to other animal models, the glomerulopathy and glomerular immune deposits developed without the deliberate administration of exogenous Ag. The investigators suggest that mesangial IgA deposits might associate with the onset of retrovirus-induced tumors spontaneously occurring in ddY mice. This contention is supported by the observation where the murine retroviral envelope glycoprotein, gp70, was deposited in the glomerular mesangial areas in ddY mice (Takeuchi et al., 1989).

As mentioned earlier, Pestka and colleagues have observed that dysregulation of IgA production and IgAN were induced by feeding the trichothecene VT in the murine model. Pestka et al., (1989) reported that increase serum IgA production after feeding 25 ppm VT into mice with beginning at 4 wks of exposure and peaking at 24 wks. Immunofluorescence staining showed marked accumulation of mesangial IgA and electron microscopy revealed electron-dense deposits in the glomeruli of VT- fed mice. Subsequently, Dong et al., (1991), Dong and Pestka, (1993), Rasooly and Pestka, (1994), and Greene et al., (1994a; 1994b) further demonstrated that dietary VT exposure induces elevation of serum IgA and

circulating IgA-IC as well as causes glomerular IgA deposition and hematuria in mice. All these observations suggest that dietary exposure VT to mice can dysregulate IgA production and induce glomerular IgA deposition in the experimental IgAN.

1.3 Cytokines and IgA production

1.3.1 Characteristics of cytokines

Cytokines can be defined as the soluble protein or glycoprotein mediators that act as intercellular signals and mediate their effects via interaction with specific cell surface receptors on sensitive target cells (Callard, 1990). Cytokine production is transient and the action radius is usually short. Cytokines can induce the synthesis and release of both positive and negative regulatory cytokines from their target cells. Generally, a cytokine may exhibit autocrine action, binding to the same cell that secreted it. An example is IL-2 secretion and utilization by an inflammatory T cells, or they may exhibit paracrine action, binding to nearby cells. In a few cases, cytokines can also act systemically in an endocrine manner, binding to a distant cell (Arai et al., 1990).

Cytokines have marked regulatory effects in immune system and stimulate and inhibit the growth, proliferation, differentiation of a wide variety of target cells or their secretion of Abs or other cytokines (Arai et al., 1990). Most cytokines have multiple biologic activities that overlap, and there is considerable redundancy, synergy, and antagonism effects during their actions (Paul and Ohara, 1987). Synergistic interactions are likely to occur between cytokines. For example, a combination of IL-5 and IL-6 has been shown to enhance IgA secretion in PP B cells cultures (Kunimoto et al., 1989). There are also many examples of antagonistic interactions among cytokines. One example of this effect is the actions of IL-4 and IFN-γ on the synthesis of Ig subclasses in B cells (Snapper et al.,

1988b). All these actions permit cytokines to regulate cellular activity in a coordinated interactive way.

Cytokine-producing cells are often physically located immediately adjacent to the responder cells (Metcalf, 1991) and generally secrete very small quantities of cytokine and, in some cases, this is directed towards the responder cells (Poo et al., 1988). Many cytokines bind to elements of the extracellular matrix around responder cells and increase their bioavailability to the responder cells (Gordon, 1991). An example of this bound localization is the cell surface cytokines which presumably require cell-cell interaction for their action (Gordon, 1991).

1.3.2 Regulation of IgA production by T cell-secreted cytokines

1.3.2.1 T lymphocytes

It is now clear that an appropriate Ig response to Ag requires both B and T lymphocytes and Ag presenting cells. T lymphocytes have a "control role" in immune responses and have been divided into two groups of cells expressing either CD4 or CD8 on their cell surfaces. "Helper" function is carried out by $CD4^+$ T cells, whereas $CD8^+$ T cells are cytolytic to Ag-bearing target cells. $CD4^+$ T cells have been subdivided further into two distinct categories based on their cytokine secretion profiles. T helper 1 (Th1) cells produce IFN-γ, IL-2 and TNF-β but not IL-4, IL-5, IL-6, or IL-10, and are responsible for cell-mediated immunity, and delayed type hypersensitivity (DTH) responses. In contrast, T helper 2 (Th2) cells produce IL-4, IL-5, IL-6 and IL-10 but not IFN-γ or IL-2, and can provide "helper activity" for

humoral immune responses including Ig isotype switching and secretion (Cherwinski et al., 1987; Mosmann and Coffman, 1989). Finally, T helper 0 (Th0) cells are characterized by IL-2, IFN-γ and IL-4 production, and are thought to be precursors of Th1 and Th2 cells (Street et al., 1990).

Cytokines are critically important since they influence B cell activation, proliferation, and terminal differentiation as well as class switching from other isotypes (McGhee et al., 1989). It has been reported that regulation of isotype switching during B cells development may be controlled by cytokines secreted during an immune response (Rizzo et al., 1995). For example, IL-4, TGF-β and IL-5, and IFN-γ help switching of IgM to IgE and IgG1, IgA, and IgG2a, respectively (Bond et al., 1987; Coffman et al., 1988; Snapper et al., 1988a).

1.3.2.2 IL-2

IL-2 is derived from activated Th1 cells although it functions principally as a T cell growth factor, but it also exerts a range of effects on activated B lymphocytes (Callard, 1990). IL-2 was shown to act on previously activated B cells and support the growth and differentiation (Zubler et al., 1984). B cells growth and Ig production can be promoted by IL-2 (Jelinek and Lipsky, 1987). IL-2 has been observed to induce secretion of IgA from tonsil B cells (Le-thi-Bich-Thuy and Fauci, 1985). Recent studies also show that induction of IL-2-secreting Th1 cells results in induction of high tetanus toxoid-specific IgA responses in the murine intestinal tract (VanCott et al., 1996). This result suggests that IL-2 may contribute a replacement

signal for the induction of IgA B cell responses. IL-2 was shown to increase IgA synthesis, but this ability was less efficient when compared with IL-5 or IL-6 (Coffman et al., 1991). Although TGF-β has been shown to be a switching factor for murine IgA class (Coffman et al., 1988; Lebman et al., 1990a), it has been reported that the addition of IL-2 to B cell cultures with TGF-β results in increased IgA secretion (Lebman et al., 1990b; Iwasato et al., 1994). Nonoyama et al., (1994) also reported that IL-2 significantly elevated the secretion of IgA by anti-CD40-activated B cells cultured in the presence of IL-10. These findings indicate that IL-2 is capable of inducing the synthesis of IgA when used combination with other cytokines. This contention is supported by recent studies that IL-2 greatly increases IgA production in B cells when in combination with IL-5 (Beagley et al., 1995) or with TGF-β (Min et al., 1996).

1.3.2.3 IL-4

IL-4 is a pleiotropic cytokine derived from Th2 cells with multiple biological effects on B cells (Ohara, 1989). In the mouse, B cell proliferation is augmented by IL-4 (Paul and Ohara, 1987). This cytokine acts by elevating the frequency of B cells responses following T cells help (Noelle et al., 1991). IL-4 is known to accelerate Ig secretion from activated B cells (Jelinek and Lipsky, 1988). Although IL-4 is well known as a switching factor for IgE and IgG1 (Bond et al., 1987), it also enhances IgA class switching in splenic B cells and other two B cell lines, I.29u and CH12. LX (Shockett and Stavnezer, 1991; Whitmore et al., 1991; McIntyre et al.,

1995). IL-4 itself plays a dominant role in developing the differentiation of native T cells into Th2-like cell type in vitro and in vivo (Swain et al., 1990; Kopf et al., 1993). This role was also demonstrated by results that switching T cells to an IL-5 producing phenotype by IL-4 which is required for the induction of lung Th2 mucosal immunity (Coyle et al., 1995). These Th2 cells are important regulators of humoral immunity.

1.3.2.4 IL-5

IL-5 is produced primarily by stimulated but not resting T cells (Altman 1990) and plays a major role in T cell-dependent IgA production in murine system (Harriman et al., 1988). Under the absence of other signals, a small number of apparently resting B cells can be activated to secrete Ig by IL-5 (Lernhardt et al., 1987). Karasuyama et al., (1988) demonstrated that murine B cells directly mature into Ig-producing cells upon exposure to IL-5. A critical role for IL-5 is supported by fact that neutralizing anti-IL-5 Ab can inhibit the polyclonal Ab response induced by T cell clones on B cells (Rasmussen et al., 1988). The ability of IL-5 to elevate IgA production has been demonstrated by Bond et al., (1987) and Coffman et al., (1987). IL-5 also increases the release of IgA from activated human mucosal B cells (Schoenbeck et al., 1989) and stimulates IgA synthesis through promotion of maturation of postswitch surface IgA^+ B cells into IgA-producing cells (Sonoda et al., 1992). This evidence is consistent with a recent report of increasing Ag-specific IgA secretion by in vitro addition or in vivo injection of recombinant IL-5 (Dieli et al.,

1995).

The early finding that IL-5 increases IgA secretion suggested that this cytokine might be an important element of mucosal immunity (Harriman and Strober, 1987) and may ultimately represent a specific switching factor for IgA secretion (McGhee et al., 1989). In agreement with such a notion, an in vivo study by Ramsay and Kohonen-Corish, (1993) demonstrated that murine specific mucosal IgA responses are markedly elevated following intranasal immunization with recombinant vaccinia virus vector that expressed IL-5 in lung. This effect was inhibited by treating with neutralizing anti-IL-5 Ab in mice. This further confirmed that IL-5 plays a critical role for selectively increasing the development of mucosal IgA responses.

Interestingly, it has been reported that IL-5 alone, or together with IL-2 appears to augment IgM J chain mRNA synthesis (Matsui et al., 1989). In addition, B cells cultured with LPS plus IL-5 show increased levels of mRNA expression for the secreted form of IgA heavy chain (alpha) (Takatsu et al., 1988). These activities help to explain the potential for IL-5 to increase IgA secretion

Early studies indicated that IL-5 could elevate IgA production in the presence of LPS and may act as a possible IgA switching factor (Bond et al., 1987; Coffman et al., 1987; Murray et al., 1987). However, several investigations have subsequently demonstrated that IL-5 enhanced the IgA secretion by acting on surface IgA^+ B cells that are presumably already committed to IgA synthesis. Thus, IL-5 appears to serve more as a terminal differentiation factor than as an isotype

switching factor (Beagley et al., 1988; 1989; Schoenbeck et al., 1989).

Studies of the effect of IL-5 on murine B cells revealed that IL-5 stimulates the secretion of Abs when used combination with other cytokines such as IL-2 (McHeyzer-Williams, 1989), IL-4 (Murray et al., 1987), and TGF-β (Sonoda et al., 1989). Investigations by Beagley et al., (1995) indicated that IL-5 alone, or in combination with IL-2, greatly increased IgA secretion in murine B cells. IL-4 and IL-5 have been shown to be required for Ig class switching and secretion (Hodgkin et al., 1991; Noelle et al., 1991). This was supported by experiments in which IL-4 induced the differentiation of membrane IgM^+ cells to membrane IgA^+ types and IL-5 promoted the secretion of IgA by membrane IgA^+ cell types (Kunimoto et al., 1988). It was also found that interaction between IL-4 and IL-5 produced by PP T cells can increase IgA and IgG1 production (Coffman et al., 1987; Murray et al., 1987). Production of IgA can also be induced by the addition of IL-5 to TGF-β pretreated B cells (Sonoda et al., 1989). These results suggest that TGF-β is required for early activity while IL-5 appears only to act late in these B cell cultures (Sonoda et al., 1989). Recently, McIntyre et al., (1995) reported that cytokines, IL-4, IL-5 and TGF-β are required for high rates of IgA class switching to occur. This requirement involved T and B cell interaction.

1.3.2.5 IL-6

IL-6 is a pleiotropic multi-functional cytokine with regulative effects in immune response, inflammation, acute phase response and hematopoiesis (Van-

Snick, 1990). IL-6 is produced by T cells as well as a variety of other cell types including Mϕ, B cells, endothelial cells, fibroblasts, and epithelial cells under varied conditions (Van-Snick, 1990). IL-6, as a B cell stimulatory factor, induces terminal proliferation and differentiation of mitogen- or Ag-activated B cells into Ig secreting cells (Kishimoto and Hirano, 1988; Kishimoto, 1989). When IL-6 was added to PP B cell cultures, IgA secretion was increased (Beagley et al., 1989; 1991). In addition, IL-6-induced IgA production is abrogated by specific neutralizing anti-IL-6 Ab in pokeweed mitogen (PWM)-activated B cells (Muraguchi et al., 1988). Of particular interest are recent observations that reduction of mucosal IgA-secreting cells and IgA responses in IL-6-deficient mice (Ramsay et al., 1994b), and that mucosal IgA responses were restored by intranasal inoculation with recombinant vaccinia viruses directed to express IL-6 in lung (Ramsay et al., 1994b). These studies demonstrate that IL-6 is important for the development of mucosal IgA responses.

Purified IL-6 has been shown to elevate Ig secretion without bias to particular isotypes (Muraguchi et al., 1988). IL-6 did not induce release of IgA in membrane-bound IgA-negative B cells. This effect suggests that IL-6 is not an isotype switching factor (Beagley et al., 1989), but acts as a relatively late acting cytokine whose main function is directing terminal B cell differentiation (Kishimoto, 1989). Thus, it would appear that both IL-5 and IL-6 are integrally involved in the final differentiation of IgA-committed B cells into IgA-secreting plasma cells in the mucosal tissue. This has been confirmed by elevation of mucosal IgA responses

due to IL-5 and IL-6 (Ramsay et al., 1994a; Xu-Amano et al.,1994) and decrease number of mucosal IgA-producing cells observed in IL-6 knock-out mice (Ramsay et al., 1994b). Several reports have suggested that IL-6 has strong activity on B cells in the presence of other cytokines. When PP B cells are stimulated by either cytokine alone, IgA secretion is only modestly increased, but is greatly enhanced by IL-5 and IL-6 in combination (Kunimoto et al., 1989). Some other in vivo studies revealed that IL-5 and IL-6 in combination with Ag can markedly elevate tear IgA response following ocular-topical route administration in the rat (Pockley and Montgomery, 1991a). TGF-β and IL-5, combined with IL-6, were demonstrated to upregulate IgA production in both rat lacrimal and salivary gland cultures (Pockley and Montgomery, 1991b; Rafferty and Montgomery, 1995). These findings offer the prospect of using cytokines IL-5 and IL-6 as immune modulators in mucosal tissues.

1.3.2.6 TGF-β

TGF-β is produced by a variety of cell types including activated T cells (Kehrl et al., 1986a), B cells (Kehrl et al., 1986b) and Mϕ (Assoian et al., 1987). TGF-β has been shown to specifically induce surface IgA^- cells to switch to IgA production (Coffman et al., 1988). Co-incubation of TGF-β with LPS-driven splenic B cells enhances the number of surface IgA^+ cells (Lebman et al., 1990a). Thus, TGF-β appears to be an IgA switch factor as opposed to a terminal differentiation factor such as IL-5. Kunimoto et al., (1992) has demonstrated that TGF-β alone, or in combination with IL-4, can induce membrane IgA expression in CH12LX B cell

lines. Furthermore, increased production of IgA by TGF-β is further enhanced by either IL-2 (Lebman et al., 1990b; Iwasato et al., 1994; Rafferty and Montgomery, 1995; Min et al., 1996), IL-5 (Sonoda et al., 1989; Rafferty and Montgomery, 1995), IL-6 (Rafferty and Montgomery, 1995) or IL-4 and IL-5 (McIntyre et al., 1995), and IL-5 and IL-6 (Rafferty and Montgomery, 1995).

1.3.2.7 IFN-γ

IFN-γ is a T cell derived cytokine with important immunomodulatory properties in addition to its anti-viral and anti-proliferative activities (Farrar and Schreiber, 1993). IFN-γ is one of the natural B cell differentiation factors and displays positive effects on murine B cells. Previous in vitro or in vivo studies have demonstrated that IFN-γ could induce Ig production (Snapper et al., 1988a; Finkelman et al., 1988). Prabhala and Wira, (1991) reported that in vivo treatment with IFN-γ stimulates the mucosal immune system in the female rats reproductive tract by increasing secretory component and IgA levels in the uterine lumen. Recently, Snapper et al., (1995) demonstrated that IFN-γ could increase IgA production in the presence of IL-2 by TGF-β-induced membrane IgA^+ cells. Furthermore, a combination of IFN-γ and IL-4 has been shown to synergistically elevate polymeric IgA receptor levels in human intestinal epithelial cell line HT29 (Denning, 1996).

1.3.2.8 Th2 cells and mucosal IgA immune responses

It is generally agreed that common mucosal humoral immune responses are predominantly of the IgA isotype (McNabb and Tomasi, 1981). IgA is considered to participate in the primary defense of the host to exogenous pathogens (Kilian and Russell, 1994). The PP in the gut-associated lymphoid tissue are thought be a major source of B cell precursors for IgA-secreting plasma cells (Craig and Cebra, 1971). PP are believed to be major inductive sites where B cells are activated and committed to IgA secretion (McGhee et al., 1989; Beagley and Elson, 1992). These activated B cells migrate to distant mucosal effector sites, such as lamina propria, where further stimulation and terminal differentiation into IgA producing cell takes place. The preferential switching of mucosal B cells from IgM^+ to IgA^+ phenotypes and differentiation to IgA secretion are governed by Th cells which comprise a characteristic and dominant T cell subset in the PP (Kawanishi et el., 1982; 1983; Kawanishi and Mirabella, 1988; McGhee and Kiyono, 1993; Kihira and Kawanishi, 1995). These data suggest that PP contains T cells which specifically regulate the secretion of IgA by B cells (Kiyono et al., 1982). In support of this contention, in vitro studies have demonstrated that PP T cells can elevate IgA synthesis in LPS-stimulated B cell cultures (Elson et al., 1979). Furthermore, $CD4^+$ T cell involvement in regulation of IgA production is likely based on the observation that IgA plasma cells are significantly decreased after chronic treatment of mice with anti-$CD4^+$ T cell monoclonal Ab (Mega et al., 1992). Recent studies indicated that autoreactive gut PP $CD4^+$ T cells can regulate IgA B cell heavy-chain switching and terminal

differentiation during gut mucosal B cell development (Kihira and Kawanishi, 1995).

It seems reasonable that IgA responses are highly dependent on T cells (Clough et al., 1971) because Th1 type cytokines IL-2 and IFN-γ as well as Th2 type cytokines IL-4, IL-5 and IL-6 have been shown to enhance B cell responses and IgA synthesis. Although both Th1 and Th2 cells can regulate IgA synthesis, the Th2 cells may have the predominant effects (Coffman et al., 1988). Cytokine IL-4 and IL-5 secretion is high in lamina propria T cells of non-human primates and is important to the helper activity of these cells (James et al., 1990). Xu-Amano et al., (1992b) reported that when mice were immunized orally with sheep red blood cells (SRBC), PP cells in mucosa-associated tissue exhibited a Th2-type response, whereas systemic administration of SRBC elicited a TH1-type response in systemic spleen tissue. Therefore, it was assumed that Th2 cells responses would predominate at the mucosal site (McGhee et al., 1989). This contention was supported by several studies demonstrating that Th2 cells are more abundant than Th1 cells at the mucosal site (Xu-Amano et al., 1992a; 1992b; 1993). In contrast, Th1-type responses occur mainly in systemic lymphoid tissue such as the spleen (Xu-Amano et al., 1992a; 1992b; 1993). Furthermore, it was found that high numbers of IgA plasma and IL-5-producing Th2 cells are present in IgA effector sites, especially in the intestinal lamina propria (Taguchi et al., 1990). Recent studies have also demonstrated that oral immunization with tetanus toxoid and CT results in enhanced Th2 cells and IgA responses in mucosal tissues (Xu-Amano et al., 1993; 1994; VanCott et al., 1996).

Many studies have shown that in vitro incubation of IL-5 or IL-6 with PP B cells or LPS-stimulated splenic B cells results in the induction of IgA synthesis (Coffman et al., 1987; Harriman et al., 1988; Beagley et al., 1988; 1989). In addition, both murine and human recombinant IL-6 have been reported to induce terminal differentiation of surface IgA^+ ($sIgA^+$) B cells from gut-associated lymphoid tissue to IgA-producing plasma cells (Fujihashi et al., 1991). The production of IL-6 from local intestinal mucosal has also been reported (Bao et al., 1993). Mucosal production of IL-6 is important because this cytokine may regulate a number of local and systemic immune responses, including IgA secretion, Mϕ differentiation and T cell proliferation (Akira et al., 1990). This conclusion is supported by a recent study demonstrating that the numbers of mucosal IgA-producing cells were dramatically decreased in IL-6 knock-out mice and suggesting that mucosal immunity may be critically affected in the absence of IL-6 (Ramsay et al., 1994b). Thus, above observations suggest that Th2 type cytokines such as IL-5 and IL-6 are essential for inducing $sIgA^+$ B cells differentiation and increased rates of IgA secretion in mucosal immunity.

1.3.3 Macrophage-secreted cytokines

Although significant attention has been paid to cytokine-mediated IgA production by Th cells, other cell types producing cytokines may also contribute to Ig secretion. In addition to T lymphocytes, Mϕ are considered to be an important source of cytokines and are capable of manipulating the T and B cell responses as

well as regulating the immune response. Mϕ are required accessory cells in the activation of T lymphocytes (Rosenwasser and Rosenthal, 1978) and can be stimulated directly by endotoxin (LPS) (Oppenheim et al., 1980; Raetz et al., 1991; Verstovsek et al., 1994). Alternatively, Mϕ can be indirectly stimulated to secrete cytokine by contact with Ag (Farr et al., 1977) or mitogen activated lymphocytes (Mizel et al., 1978) and by cytokines such as IFN-γ secreted from activated lymphocytes (Meltzer and Oppenheim, 1977; Young and Hardy, 1995)).

It has been reported that Mϕ are required for the induction of Ag-specific helper T cells in vitro (Erb and Feldmann, 1975a). Pierce et al., (1977) also proposed that Ag-presentation by Mϕ is necessary to Th cells for optimal activity during induction of Ab responses. A notable observation is that transplantation of peritoneal Mϕ from adult mice to neonatal mice resulted in an enhanced Ig response to simultaneously injected SRBC (Argyris, 1968). This author suggested that a critical ratio of Mϕ to immune competent cells may be important for stimulating Ig synthesis. Erb and Feldmann, (1975b) demonstrated that optimal Th cell induction required the admixture of about 3% Mϕ obtained from peritoneal exudate.

Mϕ, in response to LPS stimulation, are able to produce cytokines such as IL-1, IL-6, TNF-α and TGF-β which play important roles in immune activities (Cavaillon and Haeffner-Cavaillon, 1990; Libermann and Baltimore, 1990; Trinchieri, 1991; Ayala et al., 1993). Their roles include Ag-presentation, co-stimulation of T and B cells, anti-tumor activity and anti-infection action. Some in

vitro experiments have shown that a single stimulation by endotoxin results in a dose-dependent increase in the release of IL-1, IL-6, and TNF by murine peritoneal Mϕ (West et al., 1992; 1993). TNF-α, as a important inflammatory cytokine, is produced mainly by Mϕ and has been shown to stimulate B cells increase Ig secretion (Kehrl et al 1987).

The most important sources of IL-6 in vivo are believed to be Mϕ which, in fact, has been demonstrated to be major producer of IL-6 within the immune system (Bauer et al., 1988; Bauer, 1989). Recently, VanCott et al., (1996) reported that Mϕ secretion of IL-6 was increased in murine PP and spleen cultures after oral exposure to Salmonella expressing fragment C of tetanus toxin, and that this IL-6 could increase plasma cell production of IgA at the mucosal effector site. Thus, this evidence suggests that IL-6 secreted by Mϕ may contribute the signals for development of mucosal IgA responses in the lack of classical Th2 cytokine IL-4, IL-5 and IL-6.

Taken together, these findings indicate that IgA synthesis and responses are modulated by cytokines from certain immune cells. Mediators such as IL-4, IL-5 and IL-6 from Th2 cells or Mϕ provide "helper" function for B cells and may participate in the upregulation of IgA secretion in mucosal system.

The effects of cytokines on B cells activation and IgA production are summarized in Table 1.1.

Table 1.1 Cytokines contributing to differentiation of IgA secreting cells

CYTOKINE	EFFECTS	REFERENCE
IL-2	Activate B cells to secrete Ig	Jelinek and Lipsky, 1987
	Increase limited IgA synthesis	Coffman et al., 1991
	Increase IgA production (with IL-5)	Beagley et al., 1995
	Elevate IgA secretion (with IL-10)	Nonoyama et al., 1994
	Increase IgA secretion (with TGF-β)	Lebman et al., 1990b; Iwasato et al., 1994; Rafferty and Montgomery, 1995; Min et al., 1996
IL-4	Activate B cells to secrete Ig	Jelinek and Lipsky, 1988
	Induce $mIgM^+$ cells to $mIgA^+$ cells	Kunimoto et al., 198ε
	Increase IgA production (with IL-5)	Murray et al., 1987; Coffman et al., 1988
	Increase rates of IgA class switching (with IL-5 and TGF-β)	McIntyre et al., 1995
IL-5	Activate B cells to secrete Ig	Lemhardt et al., 1987
	Increase IgA production	Bond et al., 1987; Coffman et al., 1987; Beagley et al., 1988; Harriman et al., 1988
	Increase IgA production (with IL-2)	Beagley et al., 1995
	Increase IgA production (with IL-4)	Murray et al., 1987; Coffman et al., 1987
	Increase IgA production (with IL-6)	Kunimoto et al., 1989
	Increase IgA production (with TGF-β)	Sonoda et al., 1989; 1992; Rafferty and Montgomery, 1995
	Increase rates of IgA class switching (with IL-4 and TGF-β)	McIntyre et al., 1995
	Elevate mucosal IgA responses	Ramsay et al., 1994a; Xu-Amano et al., 1994
	Increase IgA plasma cell numbers	Taguchi et al., 1990
	A terminal differentiation factor for IgA secretion	Beagley et al., 1988; 1989; Schoenbeck et al., 1989

Table 1.1 Continued

CYTOKINE	EFFECTS	REFERENCE
IL-6	Activate B cells to secrete Ig	Kishimoto et al., 1988; Kishimoto, 1989
	Increase IgA secretion	Coffman et al., 1987; Beagley et al., 1989; 1991
	Increase IgA production (with IL-5)	Kunimoto et al., 1989
	Increase IgA production (with TGF-β)	Rafferty and Montgomery, 1995
	Elevate mucosal IgA responses	Ramsay et al., 1994a; Xu-Amano et al., 1994
	Induce sIgA$^+$ B cells to IgA-producing plasma cells	Fujihashi et al., 1991
	Decrease IgA-producing cells following disruption of IL-6 gene	Ramsay et al., 1994b
TGF-β	Switch factor for IgA class	Coffman et al., 1988; Lebman et al., 1990a
	Enhance sIgA$^+$ cell numbers	Lebman et al., 1990a
	Increase IgA secretion (with IL-2)	Lebman et al., 1990b; Iwasato et al., 1994; Rafferty and Montgomery, 1995; Min et al., 1996
	Induce mIgA expression (with IL-4)	Kunimoto et al., 1992
	Increase IgA production (with IL-5)	Sonoda et al., 1989; 1992; Rafferty and Montgomery, 1995
	Increase IgA production (with IL-6)	Rafferty and Montgomery, 1995
	Increase rates of IgA class switching (with IL-4 and IL-5)	McIntyre et al., 1995
	Increase IgA production (with IL-5 and IL-6)	Rafferty and Montgomery, 1995
IFN-γ	Increase IgA production	Prabhala and Wira, 1991
	Increase IgA production (with IL-2 and TGF-β)	Snapper et al., 1995
	Elevate polymeric IgA receptor levels	Denning, 1996

CHAPTER 2

POTENTIAL ROLE FOR CYTOKINES IN ENHANCED IgA SECRETION BY PEYER'S PATCH CELLS ISOLATED FROM MICE ACUTELY EXPOSED TO VOMITOXIN

2.1 ABSTRACT

Dietary exposure to VT results in hyperelevated serum IgA and IgAN in mice. To assess the possible role of cytokines in this IgA dysregulation, the effects of a single oral exposure in B6C3F1 male mice to 0, 5 or 25 mg/kg BW VT on production of IgA and cytokines in PP and spleen cell cultures were evaluated. IgA levels were increased significantly in PP cell cultures prepared from mice at 2 or 24 hr after oral exposure to VT and subsequently stimulated with PMA and ionomycin (ION) or with LPS. Significant effects on IgA production were not observed in spleen cell cultures. Since cytokines such as IL-2, IL-4, IL-5 and IL-6 have been shown to promote IgA production, the effect of the same VT exposure regimen on secretion of these mediators was determined in PP and spleen cultures. Supernatant IL-2 and IL-4 levels were unaffected by the prior treatment of animals with VT. In contrast, IL-5 levels were increased significantly in 7 day PP cell cultures obtained 2 hr after VT exposure both with and without PMA + ION exposure but not in other cultures. IL-6 levels were increased significantly in LPS-treated cultures prepared from PP at 2 and 24 hr following exposure to VT. IL-6 levels were also elevated significantly in both PMA + ION or LPS treated cultures from spleen isolated at 2 hr but not 24 hr post VT exposures. To determine whether cytokines may play a role in IgA hyperelevation in vitro, PP and spleen cells from mice obtained 2 hr after exposure to 25 mg/kg VT were cultured in the presence of neutralizing cytokine Abs and IgA production was monitored. Consistent with IL-5's previously documented role in IgA production, anti-IL-5 decreased IgA levels to background in cultures of both control

and VT-exposed PP or spleen cells in the presence of either PMA + ION or LPS. Similar results were seen with addition of anti-IL-6. IgA levels were decreased to a lesser extent in PP cells cultured with LPS and in spleen cells cultured with PMA + ION from VT- exposed mice to which anti-IL-2 Ab was added. The results indicated that the potential for enhanced IgA production exists in lymphocytes as early as 2 hr and as late as 24 hr after a single oral exposure to VT and that this may be related to the increased capacity to secrete helper cytokines of T cell and Mϕ origin. Taken together, the results suggest that the superinduction of cytokine expression may, in part, be responsible for upregulation of IgA secretion in mice exposed orally to VT.

2.2 INTRODUCTION

VT is a fungal secondary metabolite that belongs to a family of mycotoxins referred to as trichothecenes (Tanaka et al., 1988). This toxin is frequently found in cereal grains as well as other food and agricultural products (Abouzied et al., 1991; Rotter et al., 1996). VT and other trichothecenes are potent protein synthesis inhibitors that can cause acute and chronic toxicity (Ueno, 1985; Betina, 1989b; Pestka and Casale, 1990). Trichothecenes can be both immunostimulatory and immunosuppressive in a variety of animal and cell culture models (Pestka and Bondy, 1994). In mice, dietary VT exposure induces extremely high levels of serum IgA (Forsell et al., 1986; Pestka et al., 1989) and can increase the percentages of membrane IgA^+ cells, T cells, and $CD4^+$ T cells as well as $CD4^+/CD8^+$ T cell ratios in PP and spleen (Pestka et al., 1990a; Bondy and Pestka, 1991). The toxin also causes glomerular IgA deposition and hematuria, which are very similar, clinically, to human IgAN (Pestka et al., 1989; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b). All of these findings are indicative of dysregulation of IgA production.

Cytokines influence B cell activation, class-switching, proliferation, and terminal differentiation to IgA-producing plasma cells (McGhee et al., 1989). Previous studies in our laboratory have shown that exposure to VT in vitro superinduces IL-2, IL-4, IL-5 and IL-6 mRNA expression in murine splenic $CD4^+$ T cells stimulated with Con A or PMA (Ouyang et al., 1995; 1996a; Azcona-Olivera et al., 1995a; Warner et al., 1994) as well as PMA-stimulated EL-4 thymoma

cultures (Dong et al., 1994). These cytokines have previously been shown to enhance differentiation of B cells to IgA secretion (Lebman et al., 1990a; 1990b; Coffman et al., 1987; 1988; Beagley et al., 1988; 1989; Pockley and Montgomery, 1991a; Dieli et al., 1995). Recently, Azcona-Olivera et al., (1995b) demonstrated that acute oral VT exposure to mice elevates cytokine mRNA levels with maximal effects occurring in the 25 mg/kg BW group in as little as 2 hr.

We hypothesize that VT stimulates IgA production via superinduction of T helper cytokines. To test the hypothesis, I: (1) related the effects of acute oral VT exposure on IgA to cytokine production in mucosal and systemic lymphocytes cultured in vitro, and (2) assessed the effects of cytokine-specific neutralizing monoclonal Abs on IgA production in these cultures. The results indicate that PP lymphocytes exhibited enhanced capacity for elevated production of IgA, IL-5 and IL-6 when isolated as early as 2 hr and as late as 24 hr after VT exposure in vivo. Both control and VT-induced IgA secretion could be inhibited by neutralizing Abs for IL-5 and IL-6 and, to a lesser extent, IL-2. Taken together, these results suggest that superinduction of in vivo cytokine expression by VT may be responsible, in part, for upregulation of IgA secretion.

2.3 MATERIALS AND METHODS

2.3.1 Chemical and reagents

All chemicals were of reagent grade quality or better and obtained from Sigma Chemical (St Louis, MO) except where otherwise noted.

2.3.2 Animal and VT exposure regiment

Male B6C3F1 mice (8-9 weeks) were used because: (1) male mice are more sensitive than female to VT-induced IgA nephropathy (Greene et al., 1994a) and chronic effects (Iverson et al., 1995), and (2) males have been previously shown to be more sensitive to VT superinduced cytokine mRNAs expression (Azcona-Olivera et al., 1995b). Mice were obtained from Charles River Laboratories (Wilmington, MA) and kept in the university animal care facility room with a humidity- and temperature-controlled and a 12 hr light and dark cycle. Mice were housed in cages equipped with filter bonnets (Nalgene, Rochester, NY) and fed powdered semi-purified AIN-76A diet (ICN Nutritional Biochemical, Cleveland, OH) upon arrival. Animals were acclimated for at least one wk prior to usage. Food and water were withdrawn from cages 2 hr before toxin administration.

VT was purchased from Romer Labs (Washington, MO). Mice (4 per group) were orally gavaged with 5 or 25 mg/kg BW VT in 500 µl of 0.01M carbonate-bicarbonate buffer (pH 9.6) and control mice received 500 µl vehicle only (Azcona-Olivera et al., 1995b). Food and water were restored after gavaging. Mice were euthanized at 2 or 24 hr after gavage, and their spleens and PP were removed for isolation and culture.

2.3.3 Cell cultures

Spleens were teased apart with sterile tissue forceps in harvest buffer consisting of 0.01M phosphate buffered saline (PBS, pH 7.4), containing 2% (v/v) heat inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell suspensions were held on ice for 10 min to allow settling of tissue particles. Supernatant was removed following centrifugation at 450 x g for 10 min. Erythrocytes were lysed for 3 min at room temperature in 0.02 M Tris buffer (pH 7.65) containing 0.14M ammonium chloride. Cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 10% (v/v) FBS, 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acid and 5 x 10^{-5} M 2-mercaptoethanol, and then counted using a hemacytometer (American Optical, Buffalo, NY) (Strober, 1991).

PP were teased apart in harvest buffer, passed through a sterile 85 -mesh stainless steel screen and resuspended in the same buffer. Cells were centrifuged at 450 x g for 10 min, resuspended in supplemented RPMI-1640 medium and counted.

Cells (1 x 10^5) were cultured in 1 ml of supplemented RPMI-1640 medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific Co., Corning, NY) at 37^0 C under a 7% CO_2 in a humidified incubator. Cultures were unstimulated or stimulated with the T cell inducing agents, PMA (10 ng/ml) plus ION (500 ng/ml) (PMA + ION) or the Mϕ inducing agent, Salmonella typhimurium LPS (20 μg/ml). Supernatant was collected at 1, 4 and 7 days and stored in aliquots at -20^0 C until

analysis.

For cytokine-specific neutralizing Ab blocking studies, neutralizing rat anti-mouse cytokine Abs, (anti-IL-2 , IL-4, IL-5 and IL-6 from PharMingen, San Diego, CA) were included at a concentration of 10 μg/ml in the cultures (5 x 10^5/ml cells). Isotype matched rat-IgG Ab (PharMingen) was used as a control. Supernatant was collected at 5 days and stored in aliquots at -20^0 C until analysis.

2.3.4 IgA quantitation

IgA was measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Bondy and Pestka, 1991). Immunolon 4 Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^{0}C with 50 μl/well of heavy-chain specific goat anti-mouse IgA (Cappel Worthington, Malvern, PA) at a concentration of 10 μg/ml in 0.1M bicarbonate buffer (pH 9.6). Coated plates were washed 3 times with 0.01M PBS (pH 7.2) containing 0.2% Tween 20 (PBST) to remove excess capture Abs. plates were incubated with 300 μl of 1% (w/v) BSA in PBST (BSA-PBST) at 37^0 C for 30 min to block nonspecific protein binding, and then washed 4 times with PBST. For IgA determination, standard mouse reference serum (Bethyl Laboratories, Inc, Montgomery, TX) or samples were diluted in 10% (v/v) FBS RPMI-1640 medium and 50 μl was added to appropriate wells. Plates were incubated at 37^0 C for 60 min, washed 4 times with PBST, and then 50 μl of goat anti-mouse IgA horseradish peroxidase (α-chain specific, Cappel Worthington, Malvern, PA), diluted 1:1000 in 1% (w/v) BSA in PBS, was added to each well. Plates were incubated at 37^0 C for 30 min and washed 6

times with PBST. Bound peroxidase was determined with 2,2-azino-bis (3-ethylbenzthiazolin-6-sulfonate) (ABTS) substrate [0.4mM ABTS, 50mM citrate buffer (pH 4.0), and 1.2mM hydrogen peroxide] as described previously by Pestka et al., (1980). Absorbance was measured at 405 nm on a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) and IgA was quantitated by using Vmax Software (Molecular Devices).

2.3.5 Cytokine quantitation

Commercial mouse recombinant cytokine IL-2 (Collaborative Research Inc. Bedford, MA), IL-4 (Cellular Products Inc. Buffalo, NY), IL-5 (Genzyme, Cambridge, MA) and IL-6 (PharMingen, San Diego, CA) were used as standards for cytokine quantitation. Cytokine production was monitored by ELISA using modification of the procedure of Dong et al., (1994). Briefly, Immunolon 4 Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^0C with 50 µl/well of 1.0 µg/ml purified rat anti-mouse cytokine capture Abs (PharMingen) in 0.1M sodium bicarbonate buffer (pH 8.2). Plates were washed 3 times with PBST, blocked with 300 µl of 3% (w/v) BSA-PBST at 37^0 C for 30 min and washed 4 more times with PBST. Standard murine cytokines or samples were diluted in RPMI-1640 medium containing 10% (v/v) FBS and 50 µl aliquots were added to appropriate wells. Plates were incubated at 37^0 C for 60 min, washed 4 times with PBST, and 50 µl of biotinylated rat anti-mouse cytokine detection monoclonal Abs (1.5 µg/well; PharMingen) diluted in BSA-PBST were added to each well. After incubation at room temperature for 60 min, plates were washed 6 times with PBST, 50 µl of

streptavidin-horseradish peroxidase conjugate (1.5 μg/well in BSA-PBST) were added to each well and plates were incubated at room temperature for 60 min. The plates were then washed 10 times with PBST, and 100 μl of substrate [10mM citric-phosphate buffer (pH 5.5), containing 0.4mM tetramethylbenzidine (TMB; Fluka Chemical Corp, Ronkonkoma, NY) and 1.2mM H_2O_2] were added to each well. The reaction was stopped by adding an equal volume of 6 N H_2SO_4. Absorbance was read at 450 nm on a Vmax Kinetic Microplate Reader (Molecular Devices) and cytokine concentrations were quantitated by using Vmax Software (Molecular Devices).

2.3.6 Statistics

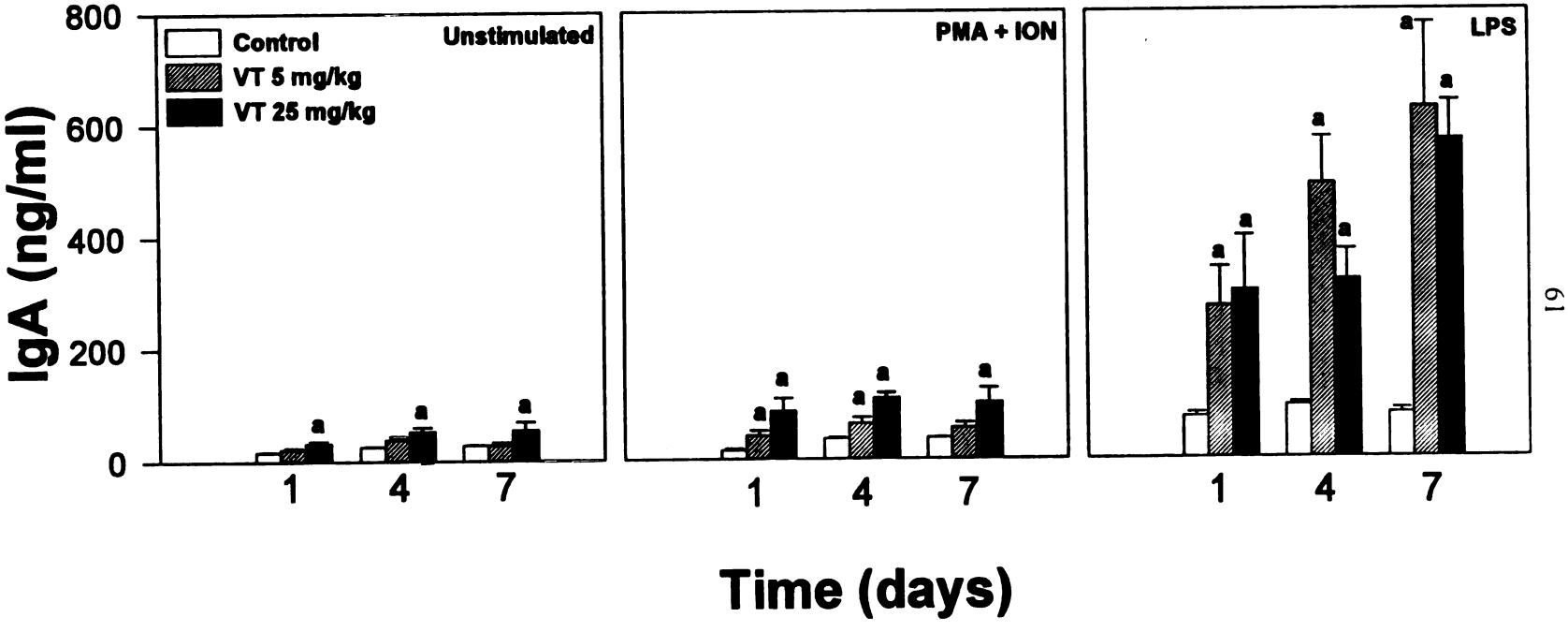
The data were analyzed by Dunnett's test following one way analysis of variance (ANOVA) using SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A *p* value of less than 0.05 was considered statistically significant.

2.4 RESULTS

The effects of single oral VT exposures of 5 or 25 mg/kg BW on IgA production in vitro were determined in cultures prepared from PP and spleens as representative of mucosal and systemic lymphoid tissues, respectively. IgA levels were increased significantly in PP cell cultures from VT-exposed mice with or without T cell activators, PMA + ION or the Mϕ activators, LPS and obtained at 2 (Figure 2.1) or 24 hr (Figure 2.2) after toxin exposure as compared to controls with the greatest effects being observed in the 25 mg/kg group. Significant effects on IgA were not observed in spleen cells (data not shown). These results suggested that oral VT administration to mice can increase the potential of PP cells to secrete IgA.

The effects of oral VT on cytokines were assessed in cultures prepared from PP and spleens. IL-2 and IL-4 supernatant levels were not affected in PP and spleen cell cultures by VT exposure. In contrast, IL-5 levels were significantly higher in 4 and 7 day PP cell cultures obtained from mice 2 hr after VT exposure but not in other cultures. IL-5 was not appreciably affected in 1, 4 and 7 day PP cultures prepared 24 hr after VT exposure (Figure 2.3) or in 1, 4 and 7 day spleen cultures prepared 2 and 24 hr after VT exposure (data not shown). IL-6 levels were significantly greater in LPS-stimulated cultures prepared from PP at 2 hr (Figure 2.4) and 24 hr (Figure 2.5) following exposure to VT. IL-6 levels were also elevated significantly in both PMA + ION- or LPS-stimulated cultures from spleen isolated at 2 hr (Figure 2.6) but not 24 hr (data not shown) post VT exposure. These results

Figure 2.1. Effect of oral exposure of mice to 0. 5 and 25 mg/kg BW VT on IgA secretion in PP cell cultures. Mice were sacrificed at 2 hr after gavage. PP cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IgA levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.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600
400
200
0
IgA (ng/ml)
Control
VT 5 mg/kg
VT 25 mg/kg
Unstimulated
PMA + ION
LPS
a
1
4
7
Time (days)

Figure 2.2. Effect of oral exposure of mice to 0, 5 and 25 mg/kg BW VT on IgA secretion in PP cell cultures. Mice were sacrificed at 24 hr after gavage. PP cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IgA levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.

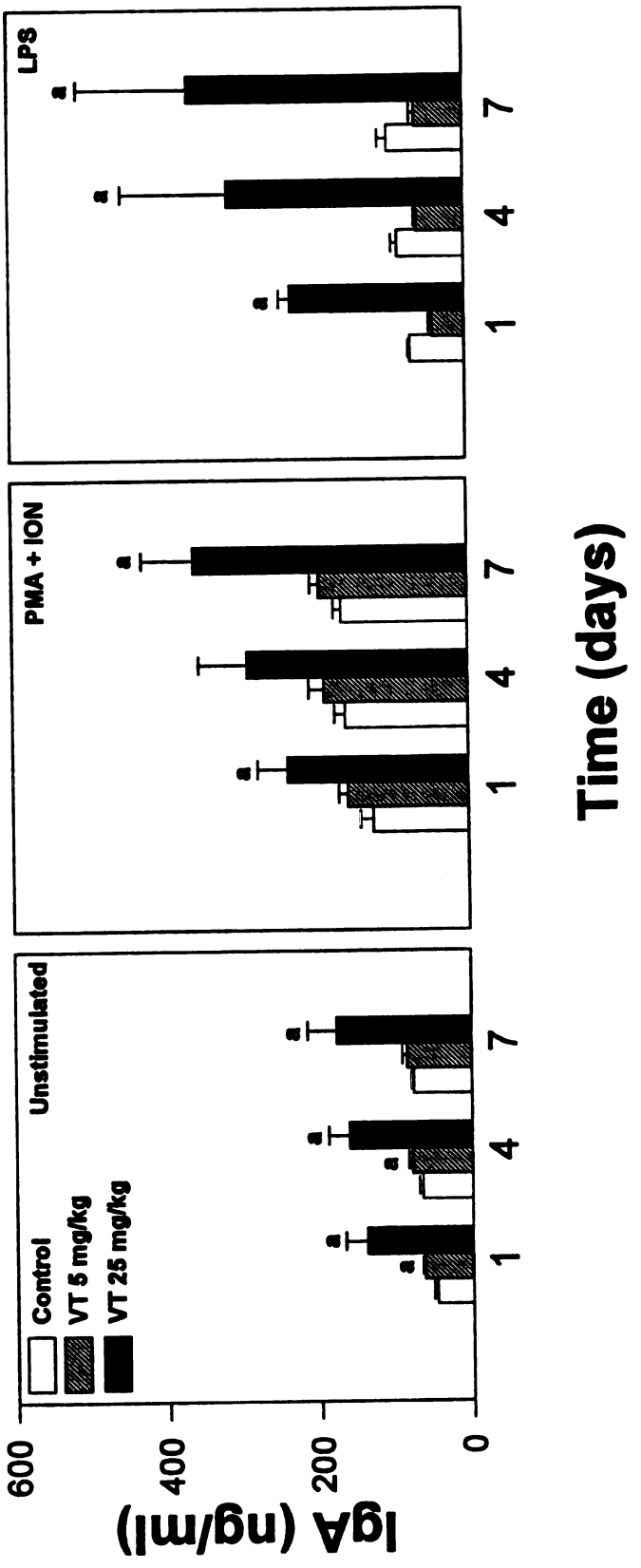

IgA (ng/ml)
600
400
200
0
Unstimulated
PMA + ION
LPS
Control
VT 5 mg/kg
VT 25 mg/kg
a
1
4
7
Time (days)

Figure 2.3. Effect of oral exposure of mice to 0, 5 and 25 mg/kg BW VT on IL-5 production in PP cell cultures. Mice were sacrificed at 2 or 24 hr after gavage. PP cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IL-5 levels were analyzed by ELISA. Data are mean ± SEM (n=4). ND indicates non-detectable. Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.

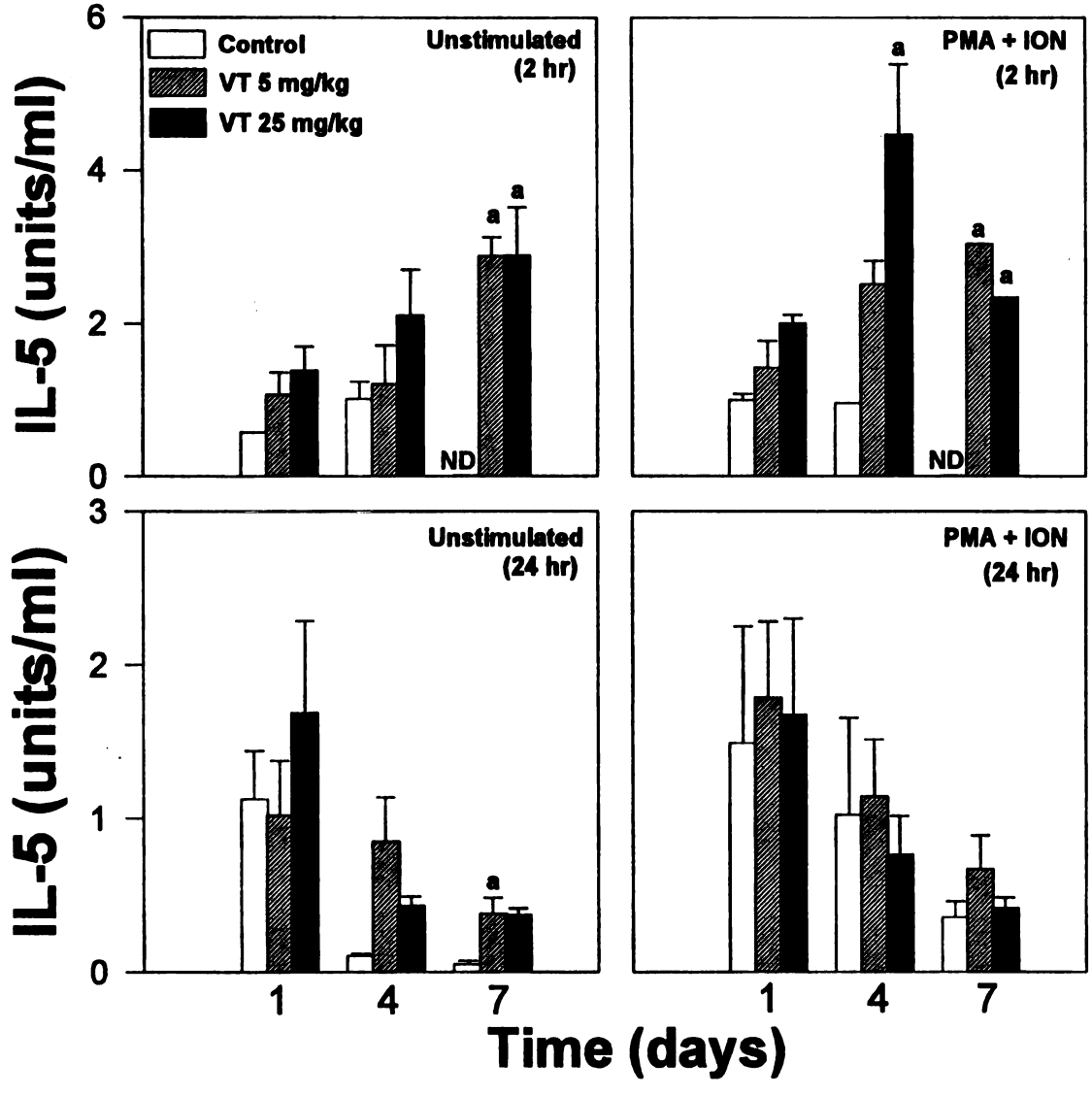

Control
VT 5 mg/kg
VT 25 mg/kg
Unstimulated (2 hr)
PMA + ION (2 hr)
Unstimulated (24 hr)
PMA + ION (24 hr)
IL-5 (units/ml)
IL-5 (units/ml)
ND
ND
a
Time (days)
1
4
7
0
2
4
6
1
2
3

Figure 2.4. Effect of oral exposure of mice to 0, 5 and 25 mg/kg BW VT on IL-6 production in PP cell cultures prepared 2 hr after gavage. PP cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.

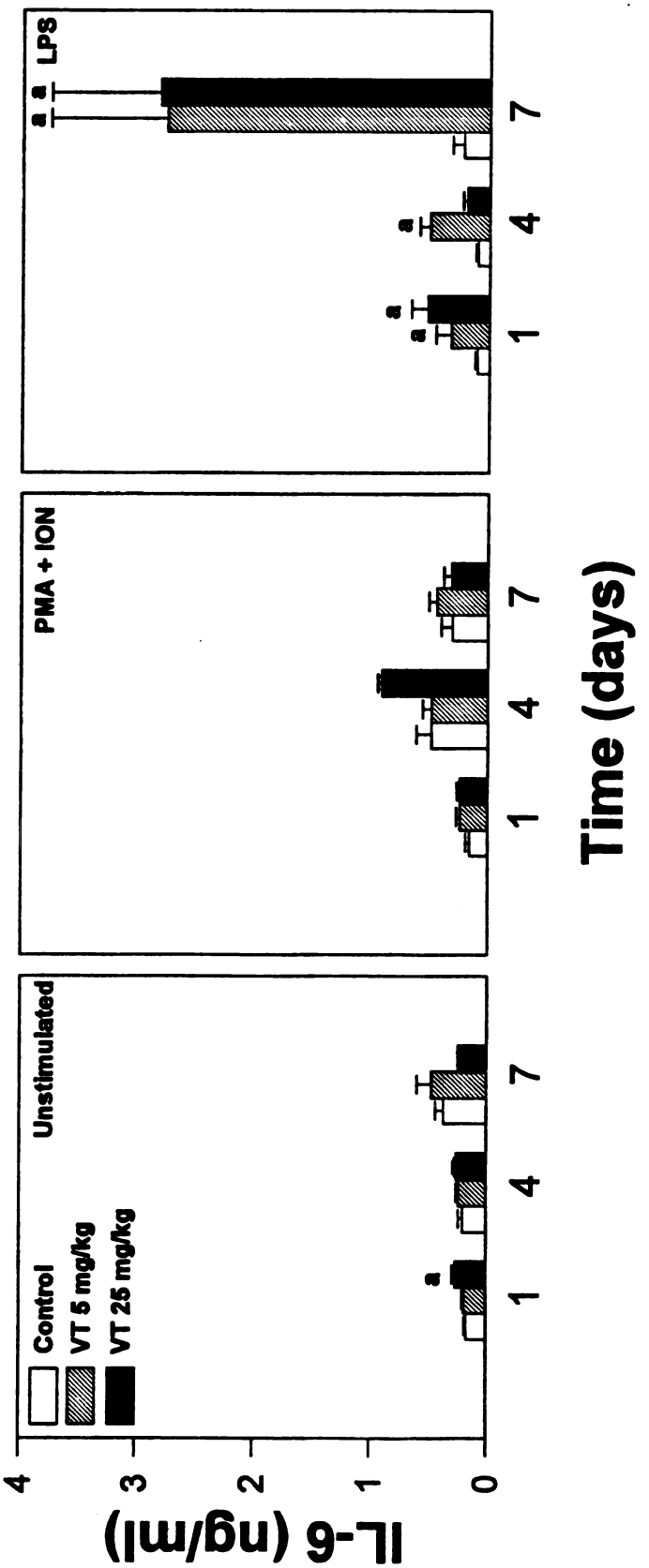

IL-6 (ng/ml)
0
1
2
3
4
Control
VT 5 mg/kg
VT 25 mg/kg
Unstimulated
PMA + ION
LPS
a
1
4
7
Time (days)

Figure 2.5. Effect of oral exposure of mice to 0, 5 and 25 mg/kg BW VT on IL-6 production in PP cell cultures prepared 24 hr after gavage. PP cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.

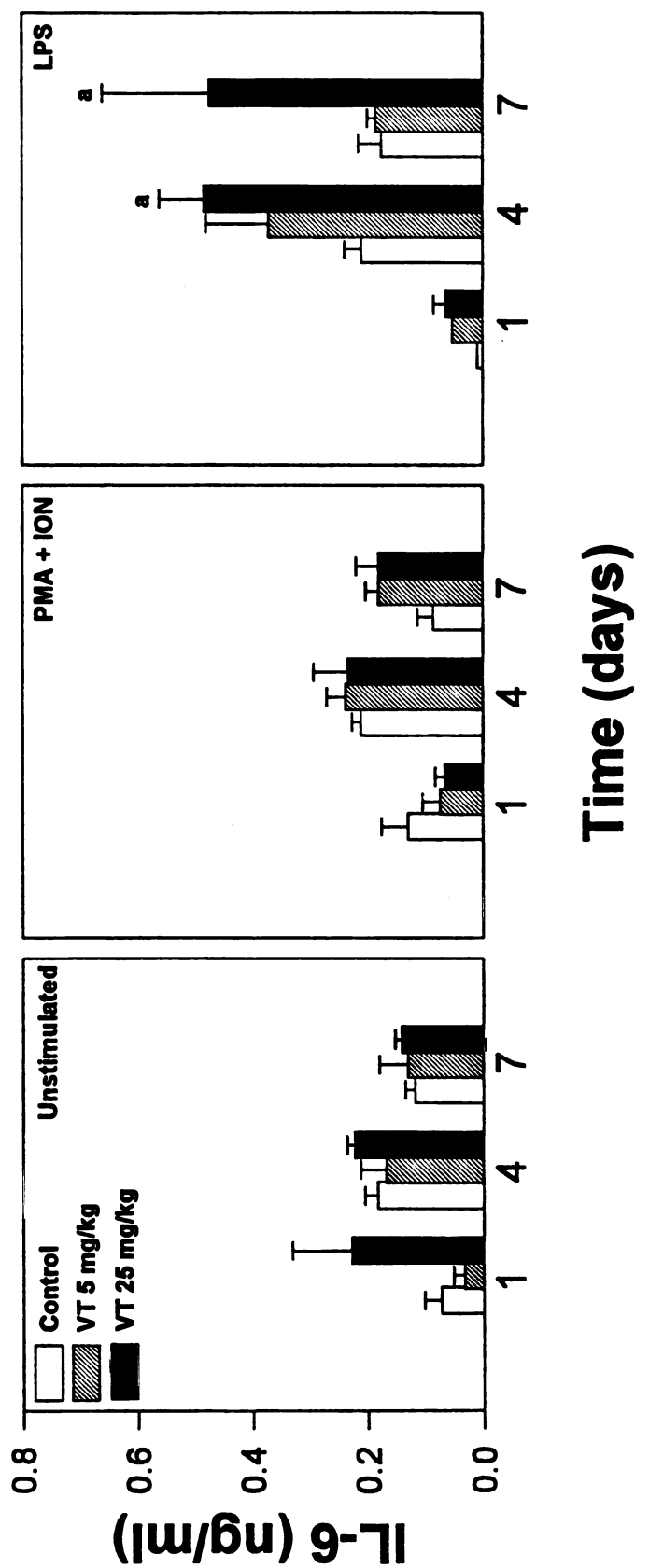
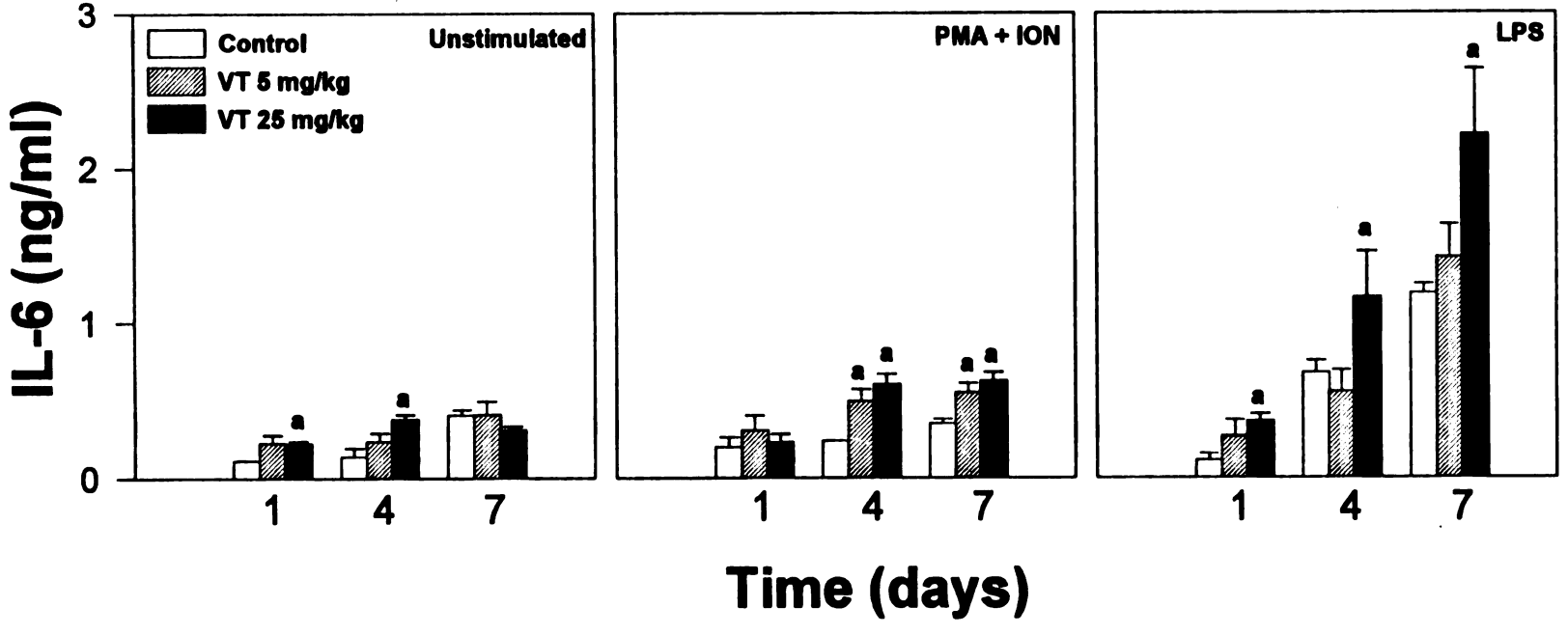

IL-6 (ng/ml)
0.0
0.2
0.4
0.6
0.8
Unstimulated
Control
VT 5 mg/kg
VT 25 mg/kg
PMA + ION
LPS
a
a
1
4
7
Time (days)

Figure 2.6. Effect of oral exposure of mice to 0, 5 and 25 mg/kg BW VT on IL-6 production in spleen cell cultures prepared 2 hr after gavage. Spleen cells ($1x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days, respectively. Supernatant IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from corresponding control (0 mg/kg) group.


IL-6 (ng/ml)
3
2
1
0
Control
VT 5 mg/kg
VT 25 mg/kg
Unstimulated
PMA + ION
LPS
a
1
4
7
Time (days)

indicated that VT exposure increased the potential for secretion of IL-5 and IL-6 by PP cells as well as IL-6 by spleen cells.

To determine the potential role of cytokines on VT-induced IgA hyperelevation, PP and spleen cells from control and treatment mice (2 hr after exposure) were cultured in the presence of cytokine neutralizing Abs and supernatant IgA monitored after 5 days. Cells were stimulated with PMA + ION or with LPS and co-cultured with anti-IL-2, IL-4, IL-5 or IL-6 singly. IgA levels were significantly lower in VT-treated PP (Figure 2.7) and spleen (Figure 2.8) cell cultures stimulated with PMA + ION or with LPS and containing anti-IL-5 or IL-6. The effects were also observed in the control animal groups (Figure 2.7 and 2.8). IgA levels were partially decreased in LPS-stimulated PP (Figure 2.7) and PMA + ION-stimulated spleen (Figure 2.8) cells from VT-exposed animals to which anti-IL-2 Ab was added . These results suggested that increased levels of both IL-5 and IL-6 and to a lesser extent, IL-2, might enhance IgA production in cultures from VT-treated mice.

Figure 2.7. Effect of cytokine-specific neutralizing Abs on mitogen-driven IgA production in PP cell cultures isolated from mice exposed to 0 and 25 mg/kg BW VT 2 hr after gavage. PP cells ($5x10^5$/ml) were cultured with PMA + ION or with LPS in 24-well plates for 5 days in the presence of anti-IL-2, IL-4, IL-5 or IL-6 singly. As a control, isotype matched rat-IgG Ab was used. Supernatant IgA levels were analyzed by ELISA. Open and solid bars indicate 0 and 25 mg/kg BW VT, respectively. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from no anti-IL Ab group and letter (**b**) are significantly different ($p < 0.05$) from isotype matched Ab group.

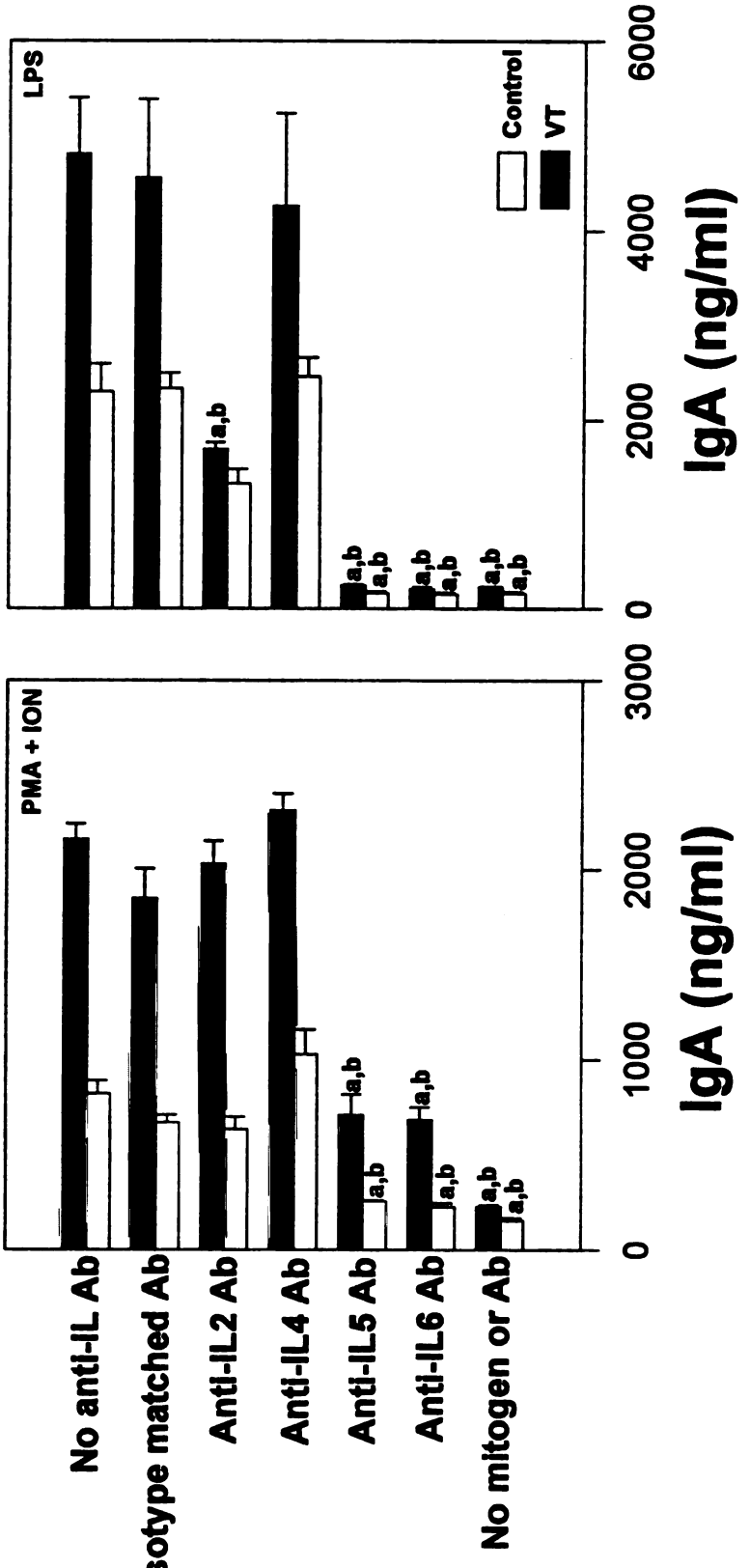
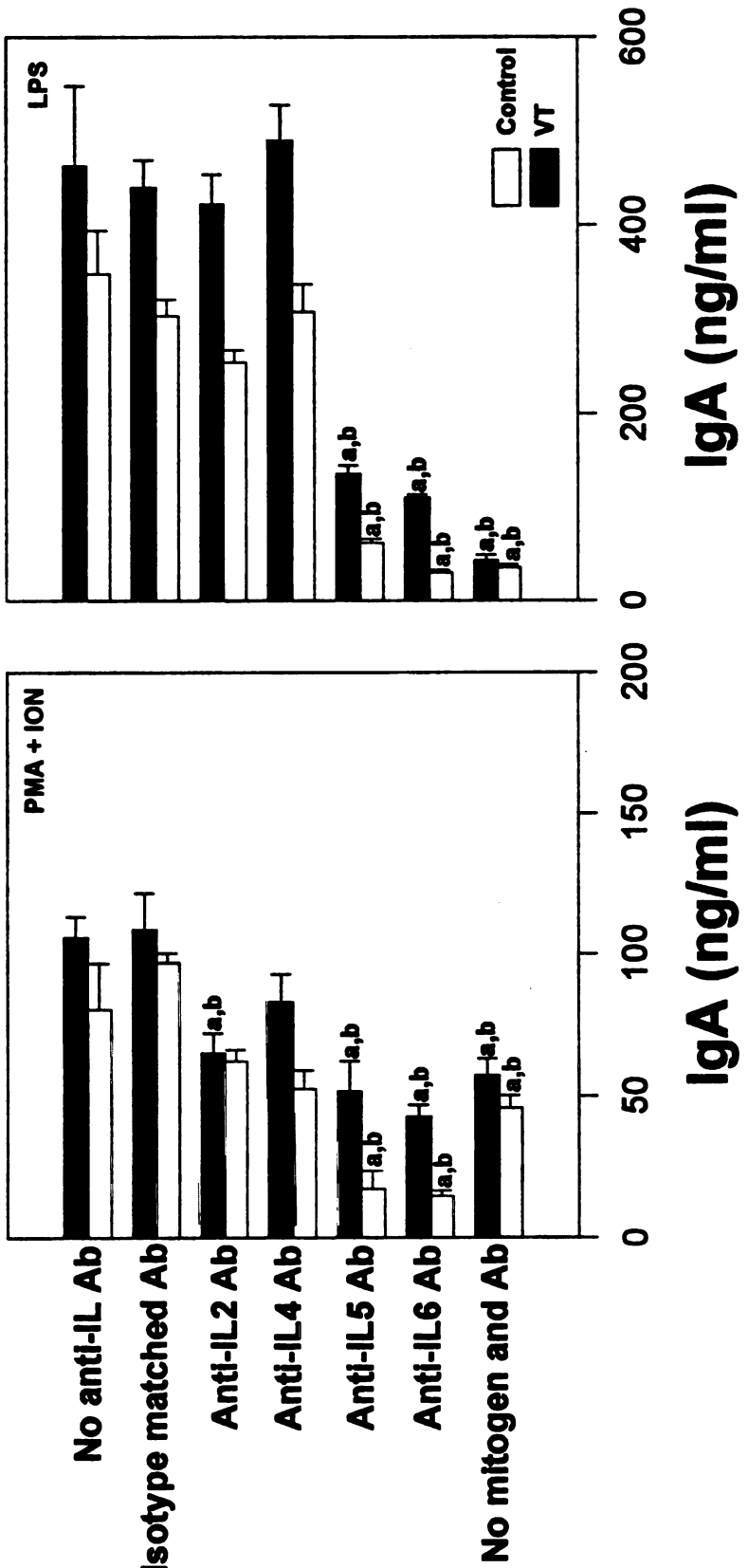

PMA + ION
LPS
Control
VT
IgA (ng/ml)
IgA (ng/ml)
0
1000
2000
3000
0
2000
4000
6000
No anti-IL Ab
Isotype matched Ab
Anti-IL2 Ab
Anti-IL4 Ab
Anti-IL5 Ab
Anti-IL6 Ab
No mitogen or Ab
a,b

Figure 2.8. Effect of cytokine-specific neutralizing Abs on mitogen-driven IgA production in spleen cell cultures isolated from mice exposed to 0 and 25 mg/kg BW VT 2 hr after gavage. Spleen cells ($5x10^5$/ml) were cultured with PMA + ION or with LPS in 24-well plates for 5 days in the presence of anti-IL-2, IL-4, IL-5 or IL-6 singly. As a control, isotype matched rat-IgG Ab was used. Supernatant IgA levels were analyzed by ELISA. Open and solid bars indicate 0 and 25 mg/kg BW VT, respectively. Data are mean ± SEM (n=4). Bars marked with letter (**a**) are significantly different ($p < 0.05$) from no anti-IL Ab group and letter (**b**) are significantly different ($p < 0.05$) from isotype matched Ab group.


PMA + ION
LPS
Control
VT
IgA (ng/ml)
IgA (ng/ml)
0
50
100
150
200
0
200
400
600
a,b
No anti-IL Ab
Isotype matched Ab
Anti-IL2 Ab
Anti-IL4 Ab
Anti-IL5 Ab
Anti-IL6 Ab
No mitogen and Ab

2.5 DISCUSSION

The aberrant elevation of serum IgA and development of IgAN (Pestka and Bondy, 1994) following chronic oral exposure to VT in mice represents a novel and isotype-specific dysregulation of the humoral immune response by a toxin. IgA has a critical role in the humoral immune response of the intestinal mucosa and is considered to participate in the primary defense of the host to exogenous pathogens (Kilian and Russell, 1994). The PP in the gut-associated lymphoid tissue is a major source of B cell precursors for IgA-secreting plasma cells (Craig and Cebra, 1971). The observations that production of IgA, IL-5 and IL-6 were affected more by VT in PP cultures than in spleen cultures suggests that VT exerts a greater effect in the gut mucosal compartment as compared to the systemic compartment. PP are believed to be major inductive sites where B cells are activated and committed to IgA secretion (McGhee et al., 1989; Beagley and Elson, 1992;). These activated B cells migrate to distant mucosal effector sites, such as lamina propria, where further stimulation and terminal differentiation into IgA-producing cells takes place. T cells and accessory cells as well as their secreted cytokines are involved in regulating the differentiation of B cells into IgA-secreting plasma cells in the PP (McGhee et al., 1989; Beagley and Elson, 1992; McGhee and Kiyono, 1993). Several major results were observed in this study suggest that PP function is a primary target for VT. First, oral VT administration to mice increased the capacity of PP cell cultures to secrete IgA and the cytokines IL-5 and IL-6. Secondly, the capacity for elevated IgA and IL-6 production was observed in lymphocytes as early

as 2 hr and as late as 24 hr after VT exposure. Third, both control and VT-induced IgA production could be inhibited by IL-5 and IL-6 neutralizing Abs. These data suggest that VT-induced IgA production may be directly related to increased levels of helper cytokines.

Many cytokines have been shown to elevate IgA production including the Th1 type cytokine IL-2 (Coffman et al., 1991) and the Th2 type cytokines IL-4 (Kunimoto et al., 1988) , IL-5 and IL-6 (Beagley et al.,1988; 1989; Beagley and Elson, 1992). Although both Th1 and Th2 cells can regulate IgA synthesis, the Th2 cells may have the predominant effects (Coffman et al., 1988). Oral immunization with tetanus toxoid and CT results in enhanced Th2 cells and IgA responses in mucosal associated tissues (Xu-Amano et al., 1993; VanCott et al., 1996). The Th2 cytokines IL-5 and IL-6 have been associated with B cell differentiation and promotion of IgA secretion (Beagley et al., 1988; 1989). Sonoda et al., (1992) determined that IL-5 can stimulate IgA synthesis through promotion of maturation of postswitch $sIgA^+$ B cells into IgA-producing cells. IL-6, as a B cell stimulatory factor, is capable of enhancing IgA production from $mIgA^+$ PP B cell cultures (Beagley et al., 1989). Synergy between IL-5 and IL-6 has been suggested based on the observations when PP B cells are stimulated by either cytokine alone, IgA secretion is only modestly increased, but is greatly enhanced by IL-5 and IL-6 in combination (Kunimoto et al., 1989). Thus, it would appear that both IL-5 and IL-6 are integrally involved in the final differentiation of IgA-committed B cells into IgA-secreting plasma cells in the mucosal tissue. This has been further confirmed by

elevation of mucosal IgA responses due to IL-5 and IL-6 (Ramsay et al., 1994a) and the decreased numbers of mucosal IgA-producing cells observed in IL-6 knock-out mice (Ramsay et al., 1994b). My findings that IgA levels were significantly decreased in both control and VT-treated PP and spleen cell cultures upon addition of anti-IL-5 or IL-6 Abs further suggest that these cytokines are critical to elevated differentiation of B cells to IgA production. This is consistent with early studies demonstrated that induction of IgA production by IL-5 and IL-6 is inhibited by treating with specific neutralizing Abs (Muraguchi et al., 1988; Ramsay and Kohonen-Corish, 1993).

The preferential switching of mucosal B cells from IgM^+ to IgA^+ phenotypes and differentiation to IgA secretion is governed by T helper ($CD4^+$) cells which comprise a characteristic and dominant T cell subset in the PP (Kawanishi et al., 1983; Kawanishi and Mirabella, 1988; McGhee and Kiyono, 1993; Kihira and Kawanishi, 1995). In support of this contention, in vitro studies have demonstrated that PP T cells can elevate IgA synthesis in LPS-stimulated B cell cultures (Elson et al., 1979). Furthermore, $CD4^+$ T cell involvement in regulation of IgA production is likely based on the observation that IgA plasma cells are significantly decreased after chronic treatment of mice with anti-$CD4^+$ T cell monoclonal Ab (Mega et al., 1992). The results provided herein support earlier findings that VT induces hyperelevated IgA production and that $CD4^+$ cells play a critical role in this effect (Forsell et al., 1986; Pestka et al., 1989; Bondy and Pestka, 1991; Dong et al., 1991; Dong and Pestka, 1993; Greene et al., 1994a; Rasooly and Pestka, 1994;

Warner et al., 1994). They are also consistent with previous observations that percentages of membrane IgA^+ cells, T cells and $CD4^+$ T cells as well as $CD4^+/CD8^+$ cell ratios are increased in PP and spleens of mice fed 25 ppm VT (Pestka et al., 1990; Bondy and Pestka, 1991). Bondy and Pestka, (1991) reported that significantly increased IgA production occurs when control B cells are co-cultured with PP T cells isolated from mice fed 25 ppm VT for 8 wks. In vitro exposure of $CD4^+$ T cells to VT can significantly increase IgA production by B cells (Warner et al., 1994). Thus, one possible interpretation of these data is that T cell dysregulation in the PP caused by VT may contribute to aberrantly increased IgA production.

It should be recognized that in addition to Th2 cells, Mϕ are major producers of IL-6 within the immune system (Bauer et al., 1988, Bauer, 1989). Recently, VanCott et al., (1996) reported that Mϕ secretion of IL-6 was increased in murine PP and spleen after oral exposure to Salmonella expressing fragment C of tetanus toxin, and that this IL-6 could elevate plasma cells production of IgA at mucosal effector sites. In this study, it is critical to note that elevation of IL-6 secretion was observed in LPS-driven PP and spleen cell cultures after oral exposure to VT. Since Mϕ are responsive to this mitogen (Raetz et al., 1991; Verstovsek et al., 1994) whereas T cells are not, it is likely that Mϕ are specifically associated with the VT-induced upregulation of IgA production via secretion of cytokine IL-6. Further investigation of the effects of VT on Mϕ in mice is thus warranted.

IL-2 has also been shown to increase IgA production by B cells when in

combination with TGF-β (Iwasato et al., 1994). Nonoyama et al., (1994) also reported that IL-2 significantly elevated the secretion of IgA by anti-CD40-activated B cells cultured in the presence of IL-10. These findings indicated that IL-2 is capable of inducing the synthesis of IgA when in combination with other cytokines. The observation that IgA secretion is reduced in the presence of anti-IL-2 suggests that IL-2 may contribute synergistically in the presence of IL-5 and IL-6 to the induction of IgA production. This contention is supported by recent studies that IL-2 greatly increases IgA production in B cells when in combination with IL-5 (Beagley et al., 1995) or with TGF-β (Min et al., 1996). Although I did not detect increases in supernatant IL-2 in my cultures, this may have resulted from binding to membrane or soluble receptors (Mohler and Butler, 1991).

In this study, the observed enhancement of IL-5 and IL-6 secretion in PP cell cultures after oral VT exposure supports previous observations that VT superinduces cytokine gene expression and secretion following exposure in vitro or in vivo (Warner et al., 1994; Dong et al., 1994; Azcona-Olivera et al., 1995a; 1995b). Superinduction of cytokine gene expression by protein synthesis inhibitors such as VT have been observed previously. For example, VT has been shown to stimulate IL-1 secretion by peritoneal macrophages in vitro (Miller and Atkinson, 1986). Furthermore, other protein synthesis inhibitors such as T-2 toxin and cycloheximide (CHX) superinduce cytokine IL-2 mRNA expression and secretion (Efrat et al., 1984; Holt et al., 1988; Zubiaga et al., 1991). One explanation for the superinduction by trichothecenes or CHX relates to the ability of these compounds

to inhibit synthesis of a labile protein repressor of cytokine mRNA expression thus leading to increased cytokine mRNA expression (Efrat et al., 1984). Recently, Ouyang et al., (1996b) have demonstrated that VT induces NF-κB/Rel binding activity particularly through inhibiting resynthesis of IκB-α in murine EL-4 and primary $CD4^+$ T cells. A second possible mechanism for VT-induced superinduction could involve to alteration of cytokine mRNA half-life. Studies of IL-2 mRNA stabilization suggest that labile RNases may be responsible for CHX-mediated stabilization of IL-2 mRNA since CHX may directly inhibit synthesis of labile RNases (Shaw et al., 1988). Based on the ability of VT to inhibit protein synthesis, it is possible that VT could inhibit such labile RNases synthesis and consequently enhance the half-life of cytokine mRNA.

It is notable that the effects of VT exposure on cytokines and IgA production were observed following tissue harvest as early as 2 hr with greatest effect being observed in the 25 mg/kg group. Consistent with this observation, Azcona-Olivera et al., (1995b) and Zhou et al., (1997) reported that VT-induced maximal effects in elevation of IL-6 as well as IL-1β, IL-2, TNF-α and IFN-γ mRNA expression in PP and spleen occurred in 2 hr after mice received 25 mg/kg VT. Interestingly, at this dose, VT is rapidly absorbed from gut with peak levels being detected in plasma at ≤ 30 min after exposure, but more than 70% protein synthesis is inhibited as long as 9 hr in all tissues of mice receiving 25 mg/kg VT (Azcona-Olivera et al., 1995b). Thus it is appears that VT superinduces IL-6 and other cytokines concurrently with impairment of protein synthesis. The observation that IL-6 effects were still

observable 24 hr after VT exposure suggests that the effects on this cytokine were long-lasting.

Sakai (1988) has indicated that cytokines may play an important role in the etiology and pathogenesis of IgAN. One interesting observation is that IgAN patients have high urinary IL-6 activity (Dohi et al., 1991). Recently, other studies have demonstrated that there is an increase in IL-5, IL-6 and TGF-β mRNA levels in $CD4^+$ T cells (de-Caestecker et al., 1993; Lai et al., 1994a; 1994b) as well as IL-4, IL-5 and IL-6 mRNA levels in peripheral blood mononuclear cells of IgAN patients (Ichinose et al., 1996). Thus, induction of cytokines may be intimately associated with immunopathologic sequelae associated with both human IgAN and experimental VT-induced IgAN.

Taken together, the results presented herein indicated that a single oral VT administration to mice can increase the capacity of PP cells to secrete IgA, IL-5 and IL-6 and that Ab neutralization of these cytokines prevented IgA secretion ex vivo. The observation that neutralization of these cytokines also depressed IgA production in control cultures, suggest that VT may elevate IgA secretion via regulatory mechanisms already existing in the PP. Thus, VT's isotype-specific effects may not be primarily a direct effect on IgA production but rather an indirect effect of altered cytokine levels which regulate IgA production. Further clarification of both the leukocyte phenotypes involved in enhanced cytokine production and the mechanisms by which this occurs are warranted.

CHAPTER 3

ROLE OF MACROPHAGES IN ELEVATED IgA AND IL-6 PRODUCTION BY PEYER'S PATCH CULTURES FOLLOWING ACUTE ORAL VOMITOXIN EXPOSURE

3.1 ABSTRACT

Oral VT exposure in mice results in elevated cytokine gene expression, increased production of IgA and IgAN. To determine the potential role of Mϕ in these effects, PP and spleen cell cultures, prepared from mice 2 hr after oral exposure to 0 or 25 mg/kg BW VT, were evaluated for IgA and cytokine IL-6 production. Both PP and spleen cells from treatment mice produced more IgA over a 7 day period than did corresponding control cells when cultured without a co-stimulus or in the presence of either the T cell activators PMA + ION or the Mϕ activator LPS. The VT effect was completely ablated in PP cultures depleted of Mϕ but not in spleen cells. In LPS-treated cultures, supernatant IL-6 was higher in the VT treatment groups as compared to controls at 7 days for PP cells and 1, 4 and 7 days for spleen cells whereas these effects were not observed in unstimulated or PMA + ION cultures. VT-induced elevation of IL-6 secretion in LPS-treated PP and spleen cells was also ablated by Mϕ depletion. A potential co-stimulatory role for Mϕ was further suggested because both IgA and IL-6 production increased when Mϕ-depleted PP cells from VT-treated animals were co-cultured with peritoneal Mϕ from VT-treated animals as compared to both: (1) control Mϕ-depleted PP cells plus control peritoneal Mϕ and (2) treatment Mϕ-depleted PP cells plus control peritoneal Mϕ. Higher IgA and IL-6 concentrations were also observed in supernatants from cultures containing VT-treated Mϕ-depleted PP cells and control peritoneal Mϕ as compared to control Mϕ-depleted PP cells plus control peritoneal Mϕ. Furthermore, PP B cells from control animals secreted elevated levels of IgA

and IL-6 when co-cultured with peritoneal Mϕ from VT-treated animals. Direct contact with the VT-treated Mϕ appeared to be necessary for an optimal stimulatory signal because the degree of IgA increase was lower in the reconstituted cell cultures where VT-treated Mϕ were separated by a semi-permeable membrane from PP cells as compared to co-cultures without a membrane. Taken together, these results suggest that Mϕ were primarily responsible for upregulation of IgA production in mice exposed orally to VT and that this was likely to involve both secretion of soluble mediators such as IL-6 and cognate cell-cell interactions.

3.2 INTRODUCTION

VT is a common fungal toxin that occurs in wheat and corn-based foods and belongs to a family of mycotoxins referred to as trichothecenes (Tanaka et al., 1988). Prolonged dietary VT exposure can induce extremely high levels of serum IgA (Forsell et al., 1986) as well as IgAN (Pestka et al., 1989; Pestka and Bondy, 1990; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b). Increases in percentages of membrane IgA^+ cells and IgA-secreting cells in PP and spleens of mice occur concurrently with these effects (Pestka et al., 1990a; Bondy and Pestka, 1991), suggesting that VT stimulates IgA secretion.

Cytokines influence B cell activation, class-switching, proliferation, and terminal differentiation to IgA-producing plasma cells (McGhee et al., 1989). Previous studies in our laboratory have shown that exposure to VT in vitro superinduces IL-2, IL-4, IL-5 and IL-6 mRNA expression in murine splenic $CD4^+$ T cells stimulated with Con A or PMA (Ouyang et al., 1995; 1996a; Azcona-Olivera et al., 1995a; Warner et al., 1994) as well as PMA-stimulated EL-4 thymoma cultures (Dong et al., 1994). These cytokines enhance differentiation of B cells to IgA secretion (Lebman et al., 1990a; 1990b; Coffman et al., 1987; 1988; Beagley et al., 1988; 1989; Pockley and Montgomery, 1991a; Dieli et al., 1995). Azcona-Olivera et al (1995b) further demonstrated that acute oral VT exposure to mice superinduces mRNA expression of the proinflammatory cytokines IL-6, IL-1β, TNF-α and IFN-γ. These effects are maximal in mice exposed to 25 mg/kg BW VT for

as little as 2 hr (Zhou et al., 1997).

Mϕ are important sources of cytokines, some of which are capable of modulating T and B cell responses. Mϕ have been demonstrated to be major producers of IL-6 within the immune system (Bauer et al., 1988; Bauer, 1989). Recently, VanCott et al., (1996) suggested that IL-6 secreted by Mϕ may contribute to development of mucosal IgA responses. T cells are another major source of helper cytokines for regulating IgA production (McGhee et al., 1989; Beagley and Elson, 1992; McGhee and Kiyono, 1993; Kihira and Kawanishi, 1995). In vitro studies have demonstrated that VT-treated $CD4^+$ T cells can significantly increase IgA production in B cells (Warner et al., 1994). Significantly elevated IgA production was also observed when PP T cells isolated from VT-fed mice were co-cultured with B cells (Bondy and Pestka, 1991).

The purpose of this study was to test the hypothesis that VT stimulation of IgA production is mediated via the Mϕ. Specific goals were to assess the potential role of Mϕ by: (1) determining the effects of Mϕ depletion on in vitro IgA and IL-6 production by PP and spleen cultures following acute oral VT exposure in mice; (2) comparing the effects of acute oral VT exposure on IL-6 secretion by Mϕ and $CD4^+$ T cells and determining their capacity to increase IgA production; and (3) assessing whether cognate interactions and/or soluble factors contribute to Mϕ-mediated enhancement of IgA production following acute oral VT exposure in mice. The results indicate that prior exposure to VT can enhance the capacity of both Mϕ and, to a lesser extent, $CD4^+$ T cells to help IgA secretion in vitro. Both superinduction

of IL-6 secretion and increased capacity for cognate help by Mϕ appeared to facilitate VT-induced upregulation of IgA production in cultures from mucosal lymphoid tissues.

3.3 MATERIALS AND METHODS

3.3.1 Chemical and reagents

All chemicals were of reagent grade quality or better and obtained from Sigma Chemical (St Louis, MO) except where otherwise noted.

3.3.2 Animals and VT exposure regimen

Male B6C3F1 mice (8-9 weeks) were obtained from Charles River Laboratories (Wilmington, MA) and kept in a humidity and temperature controlled university animal care facility room with a 12 hr light and dark cycle. Mice were housed in cages equipped with filter bonnets (Nalgene, Rochester, NY) and fed powdered semi-purified AIN-76A diet (ICN Nutritional Biochemical, Cleveland, OH) on arrival. Animals were acclimated for at least one wk prior to usage. Food and water were withdrawn from cages 2 hr before toxin administration.

VT was purchased from Romer Labs (Washington, MO). Mice (4 per group) were gavaged orally with 25 mg/kg BW VT in 500 μl of 0.01M carbonate-bicarbonate buffer (pH 9.6) and control mice received 500 μl vehicle only (Azcona-Olivera et al., 1995b). Food and water were restored after gavaging. Mice were humanely euthanized 2 hr after gavage, and their spleens, PP and/or peritoneal Mϕ were removed for isolation and culture.

3.3.3 Lymphocyte preparation

Spleens were teased apart with sterile tissue forceps in harvest buffer consisting of 0.01M PBS (pH 7.4), containing 2% (v/v) heat inactivated FBS (Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell

suspensions were held on ice for 10 min to allow settling of tissue particles. Supernatant was removed following centrifugation at 450 x g for 10 min. Erythrocytes were lysed for 3 min at room temperature in 0.02 M Tris buffer (pH 7.65) containing 0.14M ammonium chloride. Cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 10% (v/v) FBS, 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acid and 5×10^{-5} M 2-mercaptoethanol, and then counted using a hemacytometer (American Optical, Buffalo, NY) (Strober, 1991). PP were teased apart in harvest buffer, passed through a sterile 85 -mesh stainless steel screen and resuspended in the same buffer. Cells were centrifuged at 450 x g for 10 min, resuspended in supplemented RPMI-1640 medium and counted.

For some cultures, Mϕ were depleted as described by Warner et al., (1994) by gently mixing and incubating the whole PP or spleen cells with Myloclear cell Reagent (Biotex Labs. Inc., Edmonton, Alberta, Canada) for 1 hr. The mixture was layered onto Histopaque-1.119, centrifuged for 10 min at 200 x g and the buffy layer containing lymphocytes was collected. Effectiveness of Mϕ depletion was verified by esterase staining (Yam et al., 1971).

3.3.4 Peritoneal Mϕ isolation

Mϕ were collected from the peritoneal cavity of euthanized mice without activation as described by Kruisbeek (1994). Briefly, Mϕ were obtained by injecting 10 ml of RPMI medium into the peritoneal cavity and then withdrawing the peritoneal fluids slowly. Pooled peritoneal lavage fluids were centrifuged at 450 x

g for 10 min. The cell pellet was washed once in RPMI medium by centrifugation at 450 x g for 10 min and then resuspended in RPMI with 10% FBS for culture. The purity of collected Mϕ was verified by esterase staining (Yam et al., 1971).

3.3.5 B cell isolation

B cells were prepared by depletion of T cells as described by Warner et al., (1994). Briefly, Mϕ-depleted PP lymphocytes were incubated with a mixture of 100 µl each of monoclonal anti-Thy 1.2, anti-Lyt 2, and anti-L3T4 Abs (PharMingen, San Diego, CA) suspended in 2 ml of 10% FBS-RPMI medium for 45 min on ice. The mixture was washed with cold PBS and centrifuged for 10 min at 450 x g. The pellet was resuspended in 3 ml solution composed of 2.5 ml of 10% FBS-RPMI medium and 0.5 ml of baby rabbit complement (Accurate Chemical & Scientific Corporation, Westbury, NY) and incubated for 45 min at 37^0 C in a CO_2 incubator. The mixture was then washed with FBS-RPMI and centrifuged for 10 min at 450 x g. The cell pellet was resuspended in RPMI with 10% FBS for culture.

3.3.6 $CD4^+$ T cell isolation

$CD4^+$ T cells were purified from splenic lymphocyte preparations after Mϕ depletion by using the mouse $CD4^+$ cell Kit (Biotex) as described by Warner et al., (1994). Briefly, B cells were removed by absorption on a column coated with goat anti-mouse and goat anti-rat Abs. Lymphocytes were incubated on ice for 45 min with Ab specific for mouse $CD8^+$ cells (rat anti-mouse) prior to passage through the column. Thus both B and $CD8^+$ cells should adhere to the column. After cells were collected, erythrocytes were lysed for 2 min at room temperature in a buffer

containing 0.14 M ammonium chloride in 0.02 M Tris buffer (pH 7.65). Cells were centrifuged and resuspended in RPMI with 10% FBS for culture.

3.3.7 Cell cultures

Complete (undepleted) and Mϕ-depleted PP and spleen cells (5×10^5) were cultured in 1 ml of supplemented RPMI-1640 medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific Co., Corning, NY) at 37^0 under 7% CO_2 in a humidified incubator. Cultures were unstimulated or stimulated with the T cell inducing agents, PMA (10 ng/ml) plus ION (500 ng/ml) (PMA + ION) or the Mϕ-inducing agent LPS (20 μg/ml) from Salmonella typhimurium. Supernatant was collected at 1, 4 and 7 days and stored in aliquots at -20^0 C until analysis.

3.3.8 Reconstitution studies with fractionated cell populations

Mϕ-depleted PP cells (5×10^5) and peritoneal Mϕ from treatment and control animals were reconstituted in 1 ml RPMI-1640 medium with 10% FBS in 24-well tissue culture plates. Previous studies have suggested that a critical ratio of Mϕ to immune competent cells was important for stimulating Ig synthesis (Argyris, 1968). The ratio of Mϕ-depleted PP cells vs peritoneal Mϕ was chosen at 20:1 because the physiological concentration of Mϕ is 2 to 5% of the whole cell population in spleen (Verstovsek et al., 1994). A (5:1) ratio was also used for comparative purposes. Mixed cultures were unstimulated or stimulated with PMA + ION or with LPS as described above. Supernatant from reconstituted cell cultures was collected at 1, 4 and 7 days and stored in aliquots at -20^0 C until analysis.

Fractionated splenic $CD4^+$ T cells and peritoneal Mϕ from treatment or

control animals were reconstituted with purified PP B cells (1 x 10^4) from treatment or control animals in 1 ml RPMI-1640 medium with 10% FBS in 24- well tissue culture plates. Supernatant from reconstituted cell cultures was collected at 7 days and stored in aliquots at -20^0 C until analysis.

3.3.9 Transwell culture studies

Fractionated Mϕ-depleted PP cells and peritoneal Mϕ from treatment and control animals were reconstituted in Transwell 24-well cell culture plates (Costar, Cambridge, MA) with a permeable membrane to separate peritoneal Mϕ and Mϕ-depleted PP cells. As a control, mixed cells were cultured together in identical plates without a membrane insert. The ratio of Mϕ-depleted PP cells vs peritoneal Mϕ was 20:1. Mixed cultures were unstimulated or stimulated with PMA + ION or with LPS. Supernatant from reconstituted cell cultures was collected at 1 and 7 days and stored in aliquots at -20^0 C until analysis.

3.3.10 IgA quantitation

IgA was measured in culture supernatants by ELISA (Bondy and Pestka, 1991). Immunolon 4 Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^0 C with 50 µl/well of heavy-chain specific goat anti-mouse IgA (Cappel Worthington, Malvern, PA) at a concentration of 10 µg/ml in 0.1M bicarbonate buffer (pH 9.6). Coated plates were washed 3 times with 0.01M PBS (pH 7.2) containing 0.2% Tween 20 (PBST) to remove excess capture Abs. Plates were incubated with 300 µl of 1% (w/v) BSA-PBST at 37^0 C for 30 min to block nonspecific protein binding and then washed 4 times with PBST. For IgA

determination, standard mouse reference serum (Bethyl Laboratories, Inc, Montgomery, TX) or samples were diluted in 10% (v/v) FBS RPMI-1640 medium and 50 µl was added to appropriate wells. Plates were incubated at 37° C for 60 min, washed 4 times with PBST, and then 50 µl of goat anti-mouse IgA horseradish peroxidase (α-chain specific, Cappel Worthington, Malvern, PA), diluted 1:1000 in 1% (w/v) BSA in PBS, was added to each well. Plates were incubated at 37° C for 30 min and washed 6 times with PBST. Bound peroxidase was determined with 2,2-azino-bis (3-ethylbenzthiazolin-6-sulfonate) (ABTS) substrate [0.4mM ABTS, 50mM citrate buffer (pH 4.0), and 1.2mM hydrogen peroxide] as described previously by Pestka et al., (1980). Absorbance was measured at 405 nm and IgA was quantitated by using Vmax Software (Molecular Devices).

3.3.11 Cytokine quantitation

Commercial mouse recombinant cytokine IL-6 (PharMingen, San Diego, CA) was used as standard for IL-6 quantitation. IL-6 production was monitored by ELISA using modification of the procedure of Dong et al. (1994). Briefly, Immunolon 4 Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4° C with 50 µl/well of 1.0 µg/ml purified rat anti-mouse IL-6 capture Ab (PharMingen) in 0.1M sodium bicarbonate buffer (pH 8.2). Plates were washed 3 times with PBST, blocked with 300 µl of 3% (w/v) BSA-PBST at 37° C for 30 min and washed 4 more times with PBST. Standard murine IL-6 or samples were diluted in RPMI-1640 medium containing 10% (v/v) FBS and 50 µl aliquots were added to appropriate wells. Plates were incubated at 37° C for 60 min, washed 4

times with PBST, and 50 μl of biotinylated rat anti-mouse IL-6 detection monoclonal Ab (1.5 μg/well; PharMingen) diluted in BSA-PBST were added to each well. After incubation at room temperature for 60 min, plates were washed 6 times with PBST, 50 μl of streptavidin-horseradish peroxidase conjugate (1.5 μg/well in BSA-PBST) were added to each well and plates were incubated at room temperature for 60 min. The plates were then washed 10 times with PBST, and 100 μl of substrate [10mM citric-phosphate buffer (pH 5.5), containing 0.4mM tetramethylbenzidine (TMB; Fluka Chemical Corp, Ronkonkoma, NY) and 1.2mM H_2O_2] were added to each well. The reaction was stopped by adding an equal volume of 6 N H_2SO_4. Absorbance was read at 450 nm and IL-6 concentration was quantitated by using Vmax Software (Molecular Devices).

3.3.12 Statistics

The data were analyzed by Dunnett's test or Student-Newman-Keuls (SNK) test following one way ANOVA using SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A *p* value of less than 0.05 was considered statistically significant.

3.4 RESULTS

Effects of prior oral VT exposure on in vitro IgA and IL-6 production in PP and spleen cell cultures. To assess the role of Mϕ on VT-induced elevation of IgA and IL-6 secretion, I : (1) exposed mice orally to 0 and 25 mg/kg BW VT, (2) isolated PP and spleen cells 2 hr later, (3) cultured the cells with and without Mϕ depletion, and (4) monitored IgA and IL-6 concentrations in supernatants over a 7 day period. Both complete PP and spleen cultures isolated from VT-exposed mice produced as much as 2- to 6-fold more IgA at 1, 4 and 7 days than corresponding control cells when cultured in the absence or presence of PMA + ION or LPS (Figure 3.1 and 3.2). The greatest elevation in IgA responses (2- to 10-fold over control) were found in the LPS-stimulated PP and spleen cell cultures. Since both Mϕ and B cells are responsive to LPS (Raetz et al., 1991; Liu and Janeway, 1991; Berberich and Schimpl, 1992; Verstovsek et al., 1994), it seemed likely that VT had its greatest effects on one or both of these populations. It was further notable that overall IgA production was higher in VT-treated PP cultures (about 10-40 fold) (Figure 3.1) than in VT-treated spleen cultures (Figure 3.2). This evidence suggests that, relative to IgA, VT may have a greater effect in the gut mucosal compartment as compared to the systemic compartment, and that PP Mϕ or B cells are primary targets for VT-induced IgA production in this model.

Mϕ depletion markedly reduced IgA production by PP cells (about 85 to 95%) from VT-treated and control animals cultured without stimulation or with stimulation by PMA + ION or with LPS as compared to undepleted whole cultures

Figure 3.1. IgA production by PP cell cultures isolated from mice following exposure to 0 and 25 mg/kg BW VT. Mice were sacrificed at 2 hr after gavage. Both complete and Mϕ-depleted PP cells ($5x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days. Supernatant IgA levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from corresponding complete PP cells; and **b**, different from corresponding control PP cells.

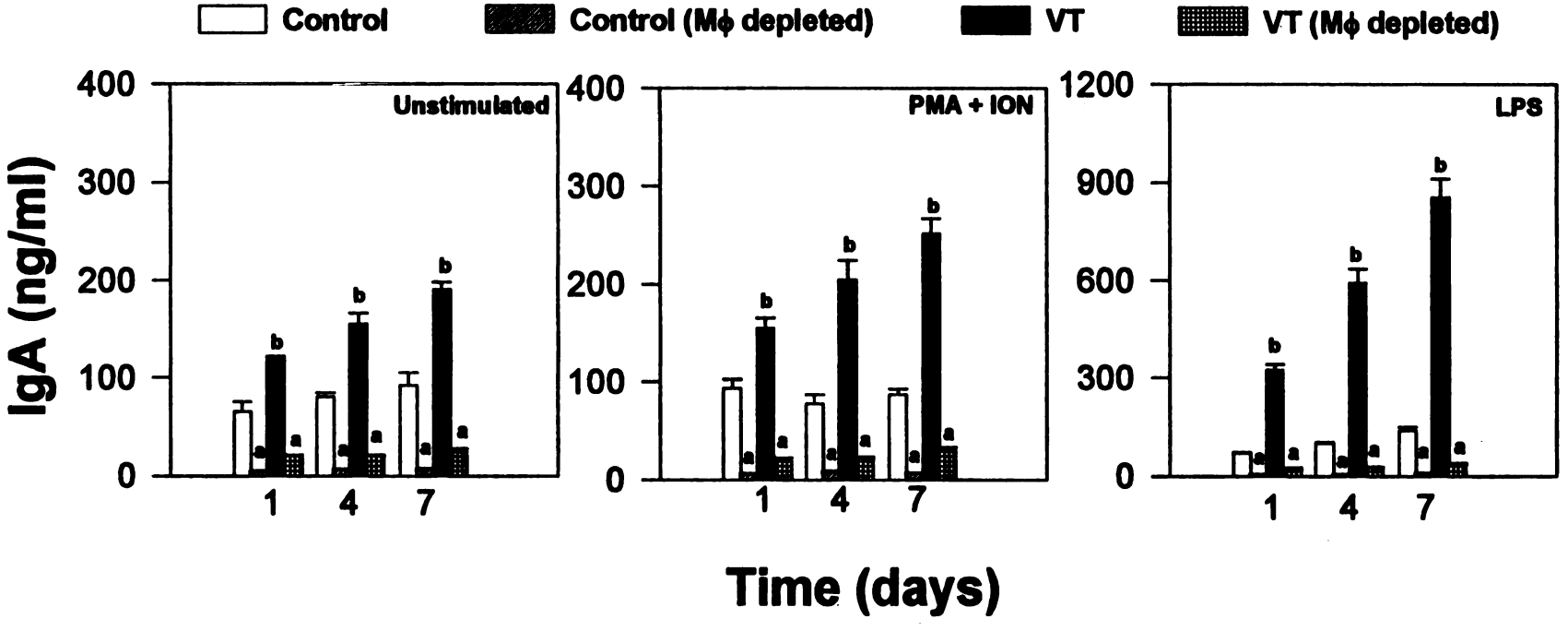
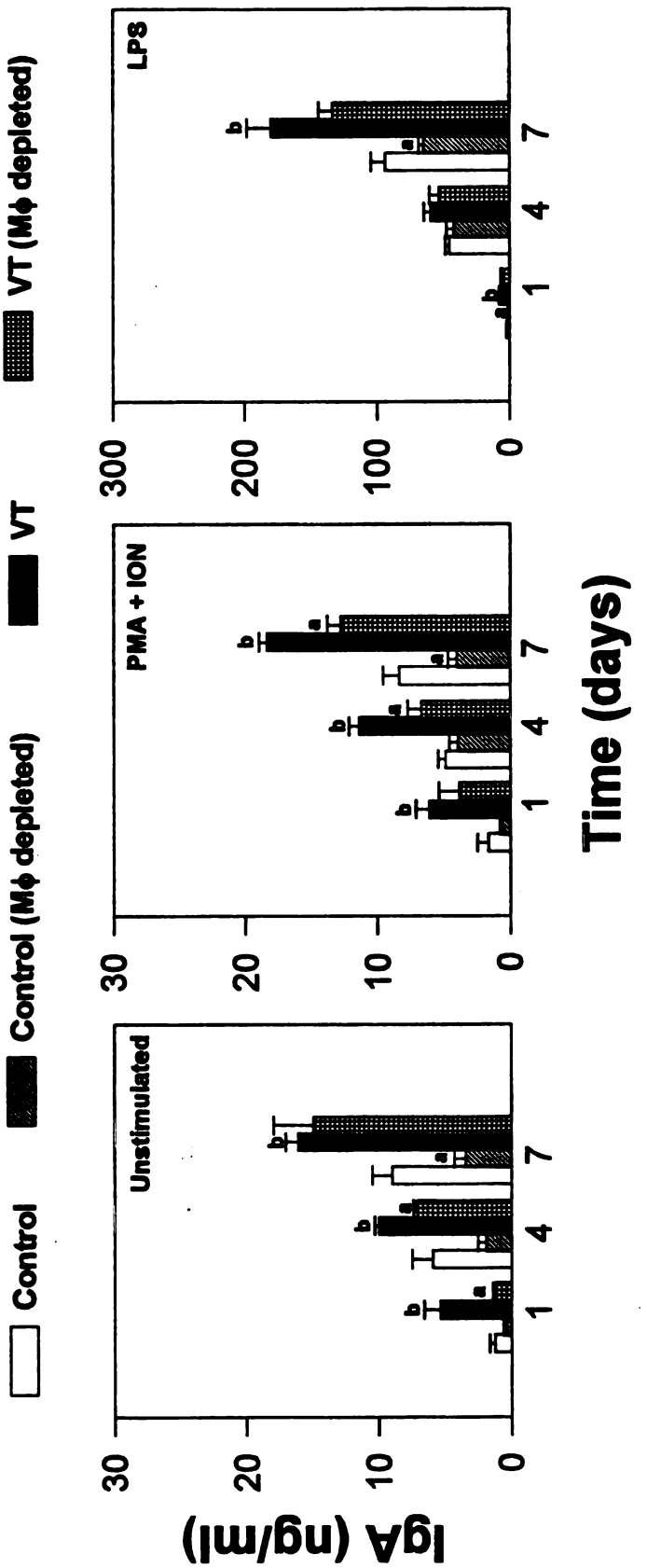

Control
Control (Mϕ depleted)
VT
VT (Mϕ depleted)
Unstimulated
PMA + ION
LPS
IgA (ng/ml)
400
300
200
100
0
1200
900
600
300
1
4
7
Time (days)
a
b

Figure 3.2. IgA production by spleen cell cultures isolated from mice following exposure to 0 and 25 mg/kg BW VT. Mice were sacrificed at 2 hr after gavage. Both complete and Mϕ-depleted spleen cells ($5x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days. Supernatant IgA levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from corresponding complete spleen cells; and **b**, different from corresponding control spleen cells.


Control
Control (Mϕ depleted)
VT
VT (Mϕ depleted)
Unstimulated
PMA + ION
LPS
IgA (ng/ml)
Time (days)
0
10
20
30
100
200
300
1
4
7
a
b

(Figure 3.1). In contrast to PP, VT-enhanced IgA production in spleen cultures was much less dependent on Mϕ (Figure 3.2). These results suggest that Mϕ was a key target of VT and was intimately involved with IgA hyperproduction.

Since IL-6 has been previously shown to promote B cell differentiation to IgA secretion in PP cells (Beagley et al., 1991; Beagley and Elson, 1992), the effect of acute VT exposure on production of this cytokine also was evaluated. As with IgA, IL-6 secretion was enhanced by VT pretreatment (Figure 3.3 and 3.4). IL-6 in LPS-stimulated complete cultures was 2- to 3-fold higher in the VT treatment groups as compared to controls at 7 days for PP cells (Figure 3.3) and 1, 4 and 7 days for spleen cells (Figure 3.4) whereas IL-6 was unaffected in unstimulated or PMA + ION-stimulated cultures. Since LPS is a potent stimulator of Mϕ (Raeta et al., 1991; Verstovsek et al., 1994) whereas PMA and ION have been used primarily for stimulating primary T cells (Gonzalez et al., 1994; Jeannin et al., 1995), the results suggested that Mϕ were the primary source for IL-6 in both PP and spleen. This possibility was supported by the observation that Mϕ depletion markedly depressed IL-6 levels in LPS-stimulated PP (60-95%) (Figure 3.3) and spleen (40-75%) (Figure 3.4) cell cultures from VT-treated and control animals as compared to undepleted whole cell cultures. In view of the key role of IL-6 in differentiation to IgA secretion and my previous finding that anti-IL-6 neutralizes VT's effects on IgA (see chapter 2), this cytokine is likely to play a mediatorial role in VT-enhanced IgA production.

Figure 3.3. IL-6 production by PP cell cultures isolated from mice following exposure to 0 and 25 mg/kg BW VT. Mice were sacrificed at 2 hr after gavage. Both complete and Mϕ-depleted PP cells ($5x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days. Supernatant IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). ND indicates non-detectable. Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from corresponding complete PP cells; and **b**, different from corresponding control PP cells.

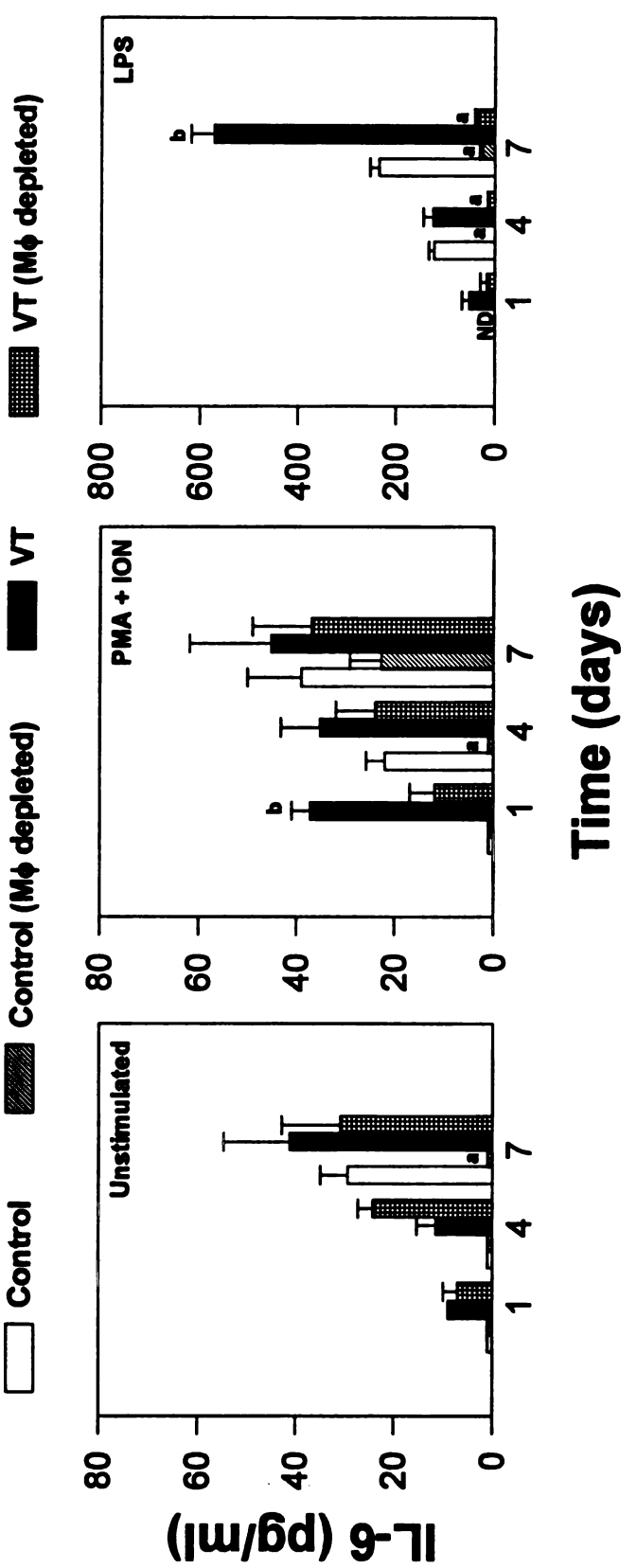
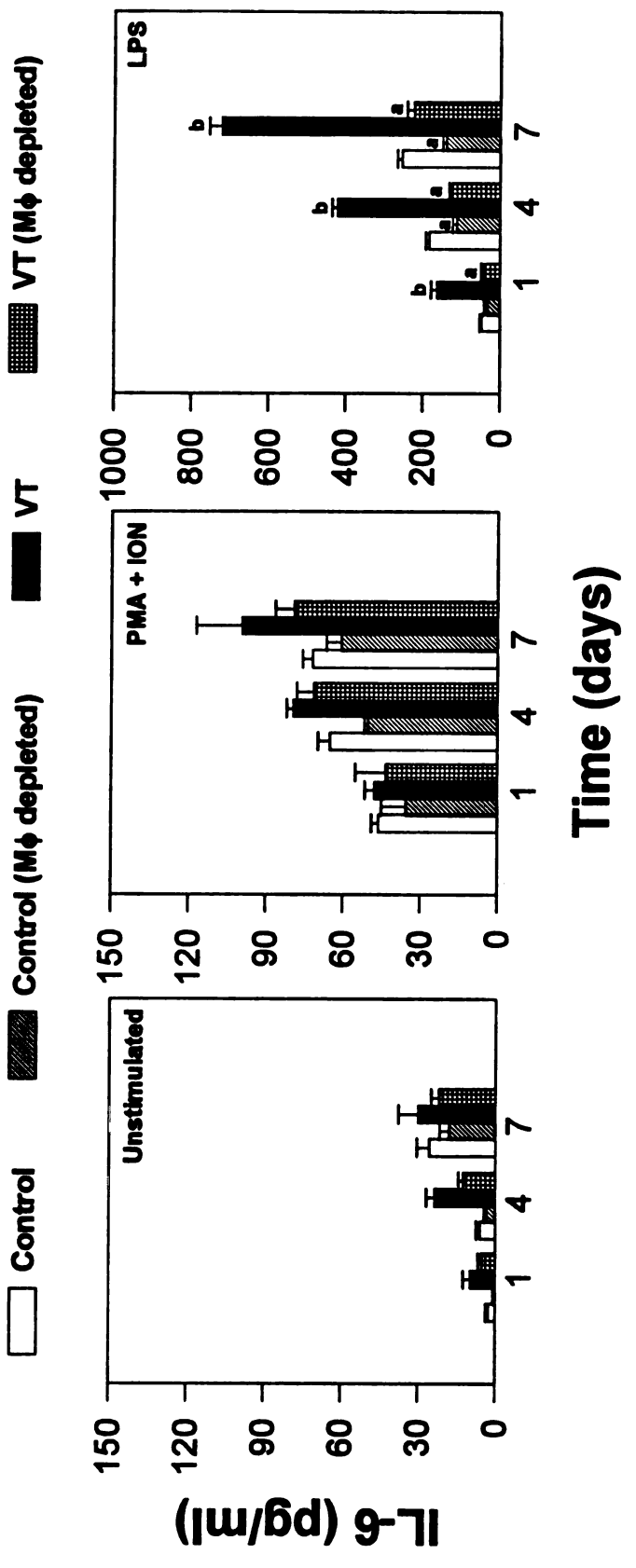

Control
Control (Mϕ depleted)
VT
VT (Mϕ depleted)
Unstimulated
PMA + ION
LPS
IL-6 (pg/ml)
Time (days)
ND
a
b

Figure 3.4. IL-6 production by spleen cell cultures isolated from mice following exposure to 0 and 25 mg/kg BW VT. Mice were sacrificed at 2 hr after gavage. Both complete Mϕ-depleted spleen cells ($5x10^5$/ml) were cultured with or without mitogens in 24-well plates for 1, 4 and 7 days. Supernatant IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from corresponding complete spleen cells; and **b**, different from corresponding control spleen cells.


Control
Control (Mϕ depleted)
VT
VT (Mϕ depleted)
Unstimulated
PMA + ION
LPS
IL-6 (pg/ml)
Time (days)
0
30
60
90
120
150
200
400
600
800
1000
1
4
7
a
b

Effects of prior oral VT exposure on in vitro IgA and IL-6 production in Mϕ-depleted PP cell cultures reconstituted with peritoneal Mϕ. The potential role of Mϕ in VT-induced IgA and IL-6 production was further assessed on Mϕ-depleted PP cultures reconstituted with Mϕ. Peritoneal exudate was used as an enriched source of Mϕ. Comparisons were made using cultures that contained: (1) control peritoneal Mϕ + control Mϕ-depleted PP cells; (2) control peritoneal Mϕ + VT-treated Mϕ-depleted PP cells; (3) VT-treated peritoneal Mϕ + control Mϕ-depleted PP cells; and (4) VT-treated peritoneal Mϕ + VT-treated Mϕ-depleted PP cells. IgA levels were consistently the highest in cultures where Mϕ-depleted PP cells from VT-treated animals were co-cultured with peritoneal Mϕ from VT-treated animals using a ratio of 20:1 as compared to the combinations of control Mϕ-depleted PP cells with control peritoneal Mϕ (Figure 3.5). These effects were most prominent when LPS was used as the mitogen. Notably, IgA production further increased when the ratio of VT-treated Mϕ-depleted PP cells to VT-treated peritoneal Mϕ was decreased to 5:1 (Figure 3.6). When compared to control Mϕ-depleted PP cells plus control peritoneal Mϕ, IgA levels were also increased to a lesser extent when VT-treated Mϕ-depleted PP cells were co-cultured with control peritoneal Mϕ or control Mϕ-depleted PP cells were co-cultured with VT-treated peritoneal Mϕ (Figure 3.5 and 3.6).

IL-6 levels were markedly increased in cultures where Mϕ-depleted PP cells from VT-treated animals were co-cultured with peritoneal Mϕ from VT-treated animals as compared to the combination of control Mϕ-depleted PP cells with

Figure 3.5. IgA and IL-6 production in reconstituted cultures containing Mϕ-depleted PP cells and peritoneal Mϕ at a 20:1 ratio. Mice were exposed to 0 and 25 mg/kg BW VT and sacrificed at 2 hr after gavage. Mϕ-depleted PP cells ($5x10^5$/ml) and peritoneal Mϕ from animals were reconstituted at ratio of 20:1 in 24-well tissue culture plates as follows: (1) control peritoneal Mϕ+ control Mϕ-depleted PP cells (CON Mϕ + CON $M\phi^-$ PP); (2) control peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (CON Mϕ + VT $M\phi^-$ PP); (3) VT-treated peritoneal Mϕ + control Mϕ-depleted PP cells (VT Mϕ + CON $M\phi^-$ PP); (4) VT-treated peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (VT Mϕ + VT $M\phi^-$ PP) . Mixed cultures were unstimulated or stimulated with the mitogen for 1, 4 and 7 days, respectively. Supernatant IgA and IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from CON Mϕ + CON $M\phi^-$ PP; and **b,** different from CON Mϕ + VT $M\phi^-$ PP.

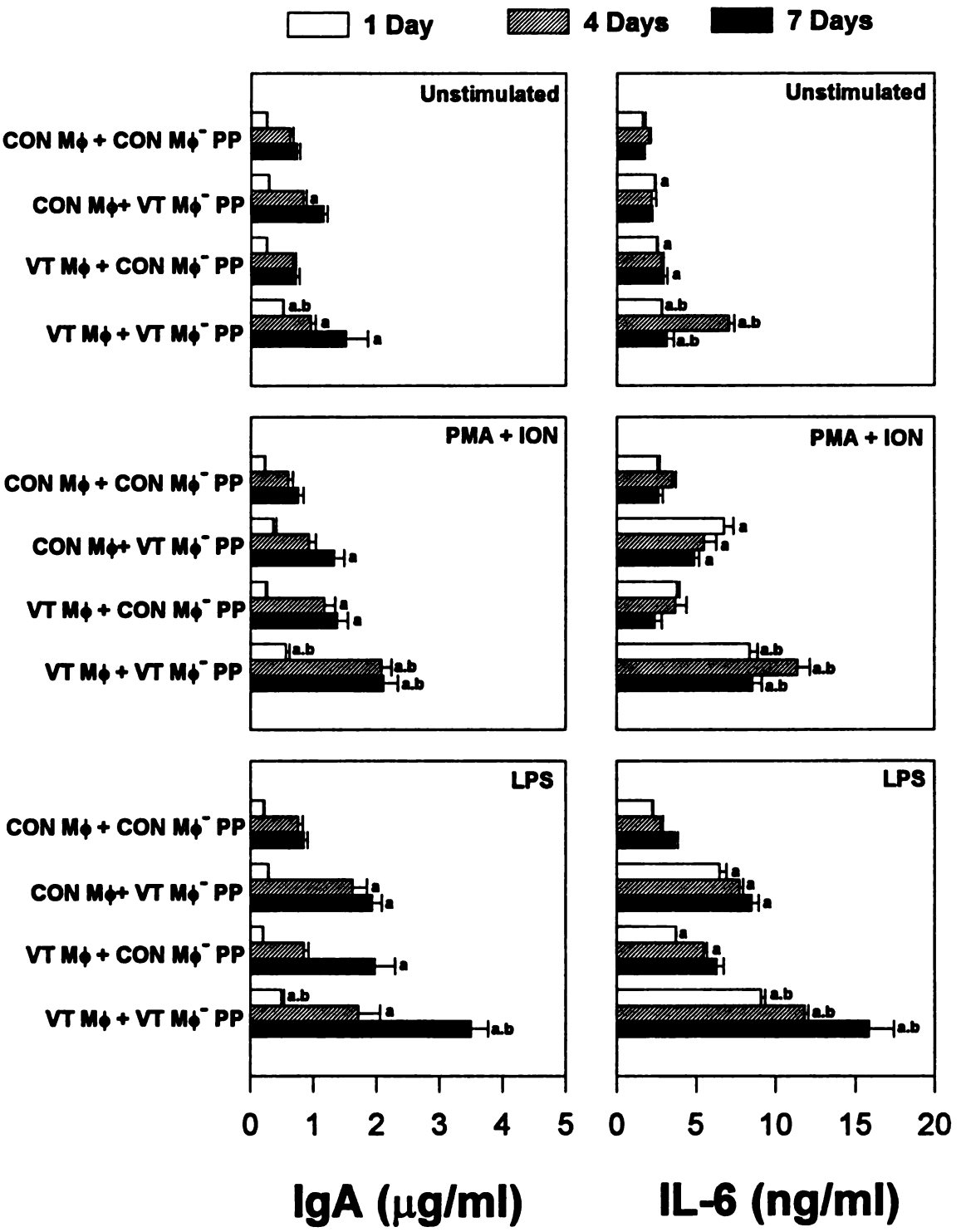
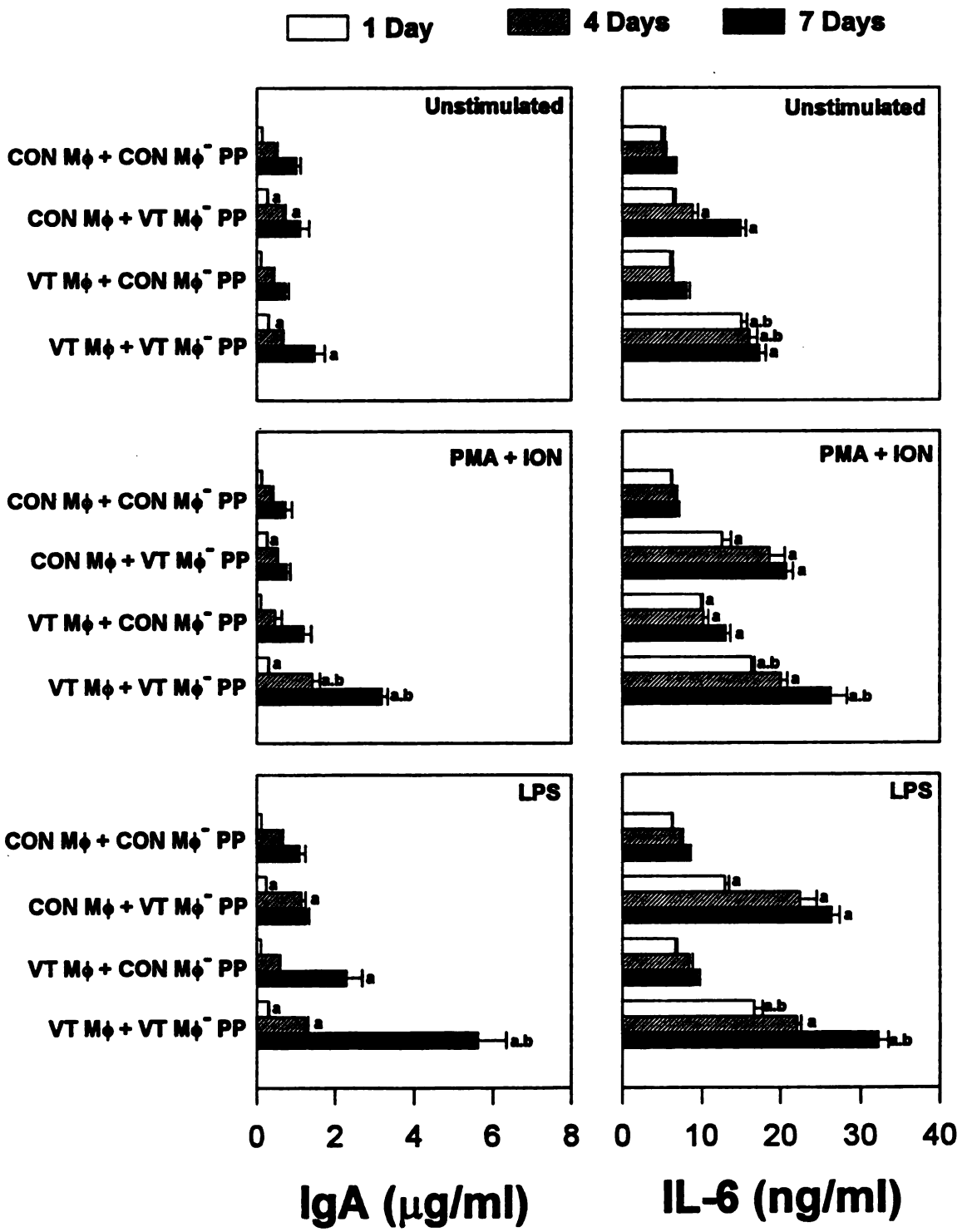

1 Day
4 Days
7 Days
Unstimulated
PMA + ION
LPS
CON Mϕ + CON Mϕ⁻ PP
CON Mϕ+ VT Mϕ⁻ PP
VT Mϕ + CON Mϕ⁻ PP
VT Mϕ + VT Mϕ⁻ PP
a
a.b
0 1 2 3 4 5
0 5 10 15 20
IgA (μg/ml)
IL-6 (ng/ml)

Figure 3.6. IgA and IL-6 production in reconstituted cultures containing Mϕ-depleted PP cells and peritoneal Mϕ at a 5:1 ratio. Mice were exposed to 0 and 25 mg/kg BW VT and sacrificed at 2 hr after gavage. Mϕ-depleted PP cells ($5x10^5$/ml) and peritoneal Mϕ from animals were reconstituted at ratio of 5:1 in 24-well tissue culture plates as follows: (1) control peritoneal Mϕ+ control Mϕ-depleted PP cells (CON Mϕ + CON Mϕ$^-$ PP); (2) control peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (CON Mϕ + VT Mϕ$^-$ PP); (3) VT-treated peritoneal Mϕ + control Mϕ-depleted PP cells (VT Mϕ + CON Mϕ$^-$ PP); (4) VT-treated peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (VT Mϕ + VT Mϕ$^-$ PP) . Mixed cultures were unstimulated or stimulated with the mitogens for 1, 4 and 7 days, respectively. Supernatant IgA and IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from CON Mϕ + CON Mϕ$^-$ PP; and **b**, different from CON Mϕ + VT Mϕ$^-$ PP.


1 Day
4 Days
7 Days
Unstimulated
PMA + ION
LPS
CON Mϕ + CON Mϕ⁻ PP
CON Mϕ + VT Mϕ⁻ PP
VT Mϕ + CON Mϕ⁻ PP
VT Mϕ + VT Mϕ⁻ PP
0 2 4 6 8
IgA (μg/ml)
0 10 20 30 40
IL-6 (ng/ml)

control peritoneal Mϕ (Figure 3.5 and 3.6). The effects were most pronounced in LPS-stimulated cell cultures thus suggesting that Mϕ were a primary source of IL-6. In further support of this contention, 2- to 4-fold more IL-6 was produced in cultures where VT-treated Mϕ-depleted PP cells were co-cultured with VT-treated peritoneal Mϕ at ratio of 5:1 (Figure 3.6) as compared to cultures employing a 20:1 ratio (Figure 3.5).

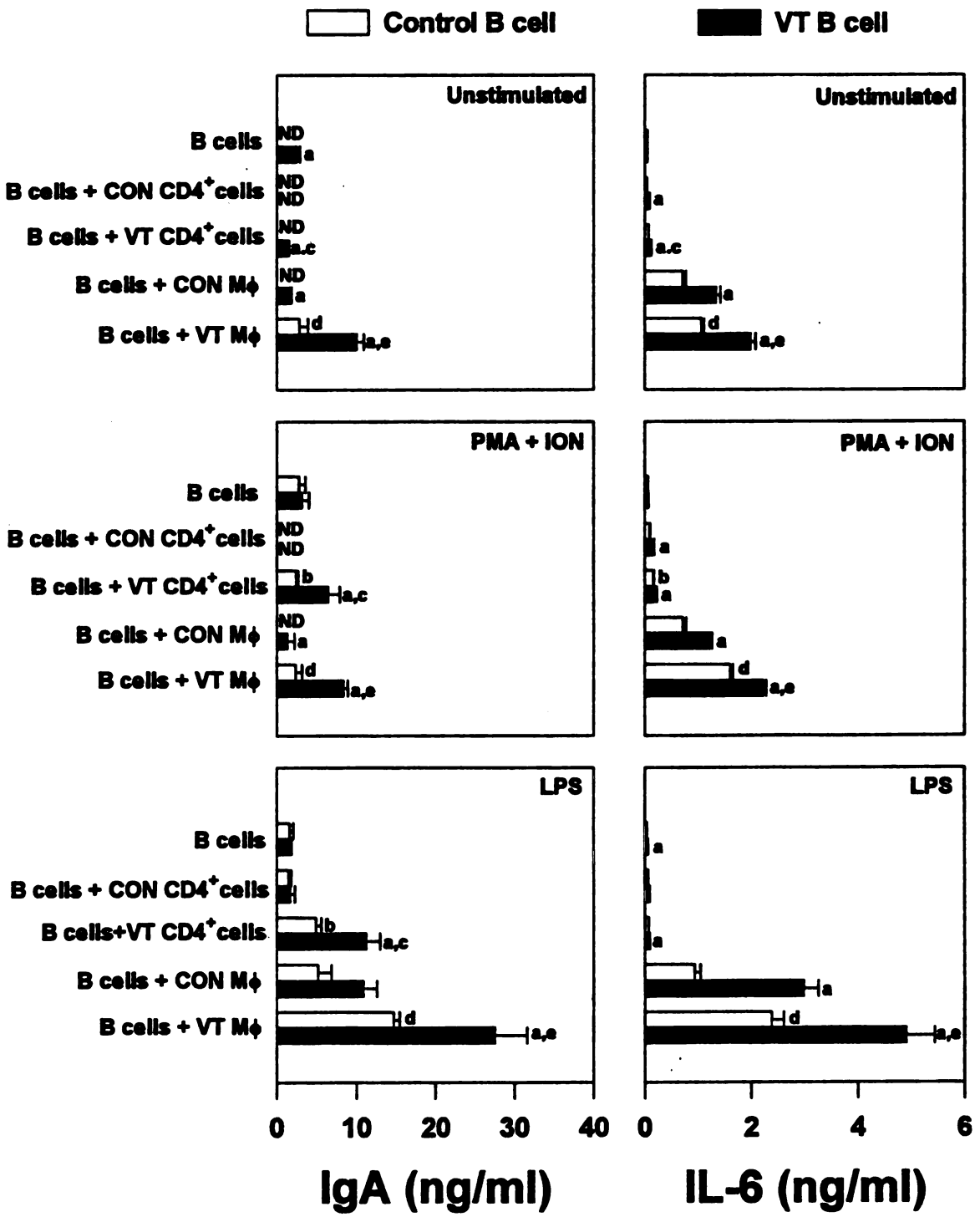
Higher IL-6 concentrations were observed in cultures where VT-treated Mϕ-depleted PP cells were co-cultured with VT-treated peritoneal Mϕ as compared to the combination of VT-treated Mϕ-depleted PP cells with control peritoneal Mϕ (Figure 3.5 and 3.6). However, IL-6 levels were also elevated in cultures when VT-treated Mϕ-depleted PP cells were combined with control peritoneal Mϕ as compared to the combination of control Mϕ-depleted PP cells with control peritoneal Mϕ (Figure 3.5 and 3.6). These results suggest that increased IgA production might be mediated by VT-induced enhancement of IL-6 production both in part by Mϕ and in part by a non-Mϕ populations such as Th cells.

Effects of prior oral VT exposure on <u>in vitro</u> IgA and IL-6 production by B cell cultures reconstituted with peritoneal Mϕ or splenic $CD4^+$ T cells. Previous results suggested the potential of both Mϕ and Th cells to promote IgA and IL-6 production. To further assess these possibilities, purified PP B cells were reconstituted with peritoneal Mϕ or splenic $CD4^+$ T cells. All three cell types were isolated from mice 2 hr after exposure to VT or vehicle and then cultured as

follows: (1) control B cells only; (2) VT-treated B cells only; (3) control B cells + control $CD4^+$ T cells; (4) VT-treated B cells + control $CD4^+$ T cells; (5) control B cells + VT-treated $CD4^+$ T cells; (6) VT-treated B cells + VT-treated $CD4^+$ T cells; (7) control B cells + control peritoneal Mϕ; (8) VT-treated B cells + control peritoneal Mϕ; (9) control B cells + VT-treated peritoneal Mϕ; and (10) VT-treated B cells + VT-treated peritoneal Mϕ. A ratio of 3:2 for PP B cells vs $CD4^+$ T cells was chosen based on the study of Bondy and Pestka (1991). A ratio of PP B cells vs peritoneal Mϕ was chosen at 20:1 for the reasons described above. As in previous experiments, cultures were unstimulated or stimulated with PMA + ION or with LPS. PP B cell cultures from control animals produced significantly more IgA when co-cultured with peritoneal Mϕ or $CD4^+$ T cells from VT-treated animals as compared to cultures containing control PP B cells and control peritoneal Mϕ or $CD4^+$ T cells (Figure 3.7). Similarly, IgA was markedly amplified in cultures when VT-treated PP B cells were co-cultured with VT-treated peritoneal Mϕ (about 14-fold) or $CD4^+$ T cells (about 5-fold) as compared to cultures containing VT-treated PP B cells and control Mϕ or $CD4^+$ T cells (Figure 3.7). As shown in previous experiments, the greatest IgA responses in Mϕ-containing cultures were observed in the LPS-stimulated cultures where VT-treated or control PP B cells were co-cultured with VT-treated peritoneal Mϕ. In general, these observations suggest that IgA production can be elevated by VT-treated Mϕ or, to a lesser extent, VT-treated $CD4^+$ T cells.

IL-6 levels were also markedly elevated in cultures where PP B cells from

Figure 3.7. IgA and IL-6 production by purified PP B cells reconstituted with purified peritoneal Mϕ or splenic $CD4^+$ T cells. Mice were exposed to 0 and 25 mg/kg BW VT and sacrificed at 2 hr after gavage. PP B cells ($1x10^4$/ml) and splenic $CD4^+$ T cells or peritoneal Mϕ from animals were reconstituted at ratios of 3:2 and 20:1, respectively, in 24-well tissue culture plates as follows: (1) control or VT-treated B cells only (B cells); (2) control or VT-treated B cells + control $CD4^+$T cells (B cells + CON $CD4^+$ cells); (3) control or VT-treated B cells + VT-treated CD^+ T cells (B cells + VT $CD4^+$ cells); (4) control or VT-treated B cells + control peritoneal Mϕ (B cells + CON Mϕ); (5) control or VT-treated B cells + VT-treated peritoneal Mϕ (B cells + VT Mϕ). Mixed cultures were unstimulated or stimulated with the mitogens for 7 days. Supernatant IgA and IL-6 levels were analyzed by ELISA. Data are mean ± SEM (n=4). ND indicates non-detectable. Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a**, control B cells vs VT-treated B cells; **b**, control T cells vs VT-treated T cells in control B cell groups; **c**, control T cells vs VT-treated T cells in VT-treated B cell groups; **d**, control Mϕ vs VT-treated Mϕ in control B cell groups; and **e**, control Mϕ vs VT-treated Mϕ in VT-treated B cell groups.

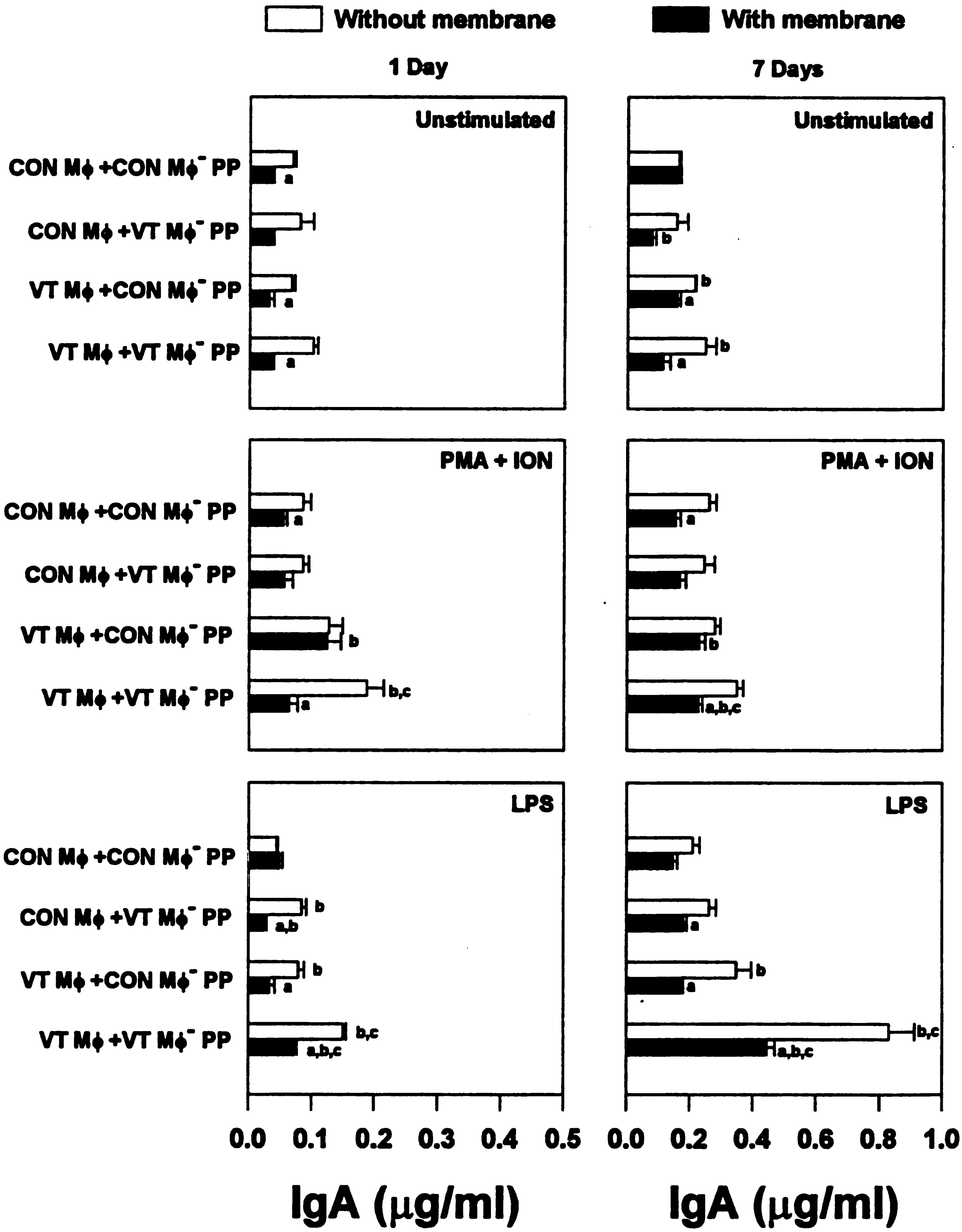

Control B cell
VT B cell
Unstimulated
PMA + ION
LPS
B cells
B cells + CON CD4+cells
B cells + VT CD4+cells
B cells + CON Mϕ
B cells + VT Mϕ
B cells+VT CD4+cells
ND
a
a,c
d
a,e
b
0
10
20
30
40
2
4
6
IgA (ng/ml)
IL-6 (ng/ml)

control animals were co-cultured with peritoneal Mϕ from VT-treated animals as compared to control PP B cells plus control peritoneal Mϕ (Figure 3.7). This effect was markedly amplified in cultures when VT-treated PP B cells were co-cultured with VT-treated peritoneal Mϕ as compared to cultures containing VT-treated PP B cells and control peritoneal Mϕ (Figure 3.7). The greatest response was observed in the LPS-stimulated cultures (35-60 fold increase) whereas PMA + ION appeared to have no effect when compared to unstimulated cultures. IL-6 levels were only significantly increased in PMA + ION-stimulated cultures where control PP B cells were co-cultured with VT-treated $CD4^+$ T cells as compared to the combinations of control PP B cells with control $CD4^+$ T cells (Figure 3.7). Notably, approximately 2-fold more IL-6 secretion was observed in cultures where VT-treated or control PP B cells were co-cultured with VT-treated peritoneal Mϕ as compared to co-cultures containing control peritoneal Mϕ. These data suggest that increased IgA production in mucosal tissues could be mediated by both Mϕ and to a lesser extent $CD4^+$ T cells but that the elevation of IL-6 levels was mediated mainly by Mϕ. Interestingly, prior exposure of B cells to VT in vivo consistently enhanced the capacity of Mϕ to increase IgA and IL-6 production in combination cultures.

Role of soluble mediators and cell-cell interactions on the capacity of Mϕ to stimulate in vitro IgA production. It was of further interest to verify whether Mϕ enhanced IgA production by providing cognate cell-cell interactions and/or soluble mediators (e.g. IL-6). Peritoneal Mϕ and Mϕ-depleted PP cells were obtained from

mice 2 hr after exposure to 0 and 25 mg/kg BW VT and were co-cultured in plates containing a permeable membrane (Transwell) to separate peritoneal Mϕ and Mϕ-depleted PP cells. As controls, mixed cells were cultured together in identical plates without a membrane. The cells were reconstituted as follows: (1) control peritoneal Mϕ + control Mϕ-depleted PP; (2) control peritoneal Mϕ + VT-treated Mϕ-depleted PP cells; (3) VT-treated peritoneal Mϕ + control Mϕ-depleted PP cells; and (4) VT-treated peritoneal Mϕ + VT-treated Mϕ-depleted PP cells. As expected, IgA levels were increased in cultures where VT-treated Mϕ-depleted PP cells were co-cultured with VT-treated peritoneal Mϕ in the absence of a membrane (Figure 3.8). IgA levels were significantly decreased (about 50%) in the reconstituted cell cultures with or without PMA + ION or LPS and employing a membrane as compared to those cell cultures without a membrane (Figure 3.8). These findings suggest that in addition to Mϕ-secreted mediators such as the cytokine IL-6, cell-cell interactions might be important for mediating VT-enhanced IgA production.

Figure 3.8. IgA production in reconstituted cultures containing Mϕ-depleted PP cells and peritoneal Mϕ at a 20:1 ratio. Mice were exposed to 0 and 25 mg/kg BW VT and sacrificed at 2 hr after gavage. Mϕ-depleted PP cells ($1x10^5$/ml) and peritoneal Mϕ from animals were reconstituted at ratio of 20:1 in 24 well Transwell cell culture plates which contain a permeable membrane to separate Mϕ and Mϕ-depleted PP cells. As controls, mixed cells were cultured together in identical plates without a membrane. The cells were reconstituted as follows: (1) control peritoneal Mϕ+ control Mϕ-depleted PP cells (CON Mϕ + CON $M\phi^-$ PP); (2) control peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (CON Mϕ + VT $M\phi^-$ PP); (3) VT-treated peritoneal Mϕ + control Mϕ-depleted PP cells (VT Mϕ + CON $M\phi^-$ PP); (4) VT-treated peritoneal Mϕ + VT-treated Mϕ-depleted PP cells (VT Mϕ + VT $M\phi^-$ PP) . Mixed cultures were unstimulated or stimulated with the mitogens for 1 and 7 days, respectively. Supernatant IgA levels were analyzed by sandwich ELISA. Data are mean ± SEM (n=3). Bars marked with letter indicate significantly different ($p < 0.05$) as follows: **a,** different from cell cultures without a membrane; **b**, different from CON Mϕ + CON $M\phi^-$ PP; and **c**, different from CON Mϕ + VT $M\phi^-$ PP.



Without membrane
With membrane
1 Day
7 Days
Unstimulated
PMA + ION
LPS
CON Mϕ +CON Mϕ⁻ PP
CON Mϕ +VT Mϕ⁻ PP
VT Mϕ +CON Mϕ⁻ PP
VT Mϕ +VT Mϕ⁻ PP
0.0 0.1 0.2 0.3 0.4 0.5
0.0 0.2 0.4 0.6 0.8 1.0
IgA (μg/ml)
IgA (μg/ml)

3.5 DISCUSSION

VT has been shown to be both immunostimulatory and immunosuppressive in a variety of animal and cell culture models (Pestka and Bondy, 1994). The stimulatory effects of prolonged dietary VT exposure previously observed in our laboratory include: (1) elevation of serum IgA production (Forsell et al., 1986; Pestka et al., 1989), (2) induction of IgAN (Pestka et al., 1989; Pestka and Bondy, 1990; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b); and (3) increase in the percentages of membrane IgA^+ cells, T cells, and $CD4^+$ T cells as well as $CD4^+/CD8^+$ T cell ratios in PP and spleen (Pestka et al., 1990a; Bondy and Pestka, 1991). Collectively, all these findings are indicative of dysregulation of IgA production by VT. Several lines of evidence suggest that VT can specifically dysregulate cytokine production. For example, in vitro superinduction of IL-2, IL-4, IL-5 and IL-6 mRNA expression by VT has been observed in murine splenic $CD4^+$ T cells (Ouyang et al., 1995; 1996a; Azcona-Olivera et al., 1995a; Warner et al., 1994) as well as EL-4 thymoma cultures (Dong et al., 1994). Additionally, in vivo enhancement of mRNA levels of the proinflammatory cytokines (IL-6, IL-1β, TNF-α and IFN-γ) that are suggestive of Mϕ activation is observed in PP and spleens of mice exposed acutely to VT (Azcona-Olivera et al., 1995b; Zhou et al., 1997). Thus, the potential exists for VT to alter Ig secretion via cytokines. The results presented herein revealed that both IgA and IL-6 secretion were markedly decreased in Mϕ-depleted PP and spleen cell cultures isolated from mice 2 hr after exposure to 25 mg/kg VT. This evidence

suggests that Mϕ produce IL-6 and that this cytokine may be related to VT-induced IgA production. In this study, I used a short term VT exposure (2 hr) model to identify potential leukocyte populations that mediate this novel isotype specific dysregulation. Several key findings were made. First, PP was a primary target for VT-induced IgA production as compared to spleen. Second, both IgA and IL-6 secretion by VT-treated and control PP cultures were diminished after depletion of Mϕ. Third, enhanced production of murine IL-6 occurred concurrently with increased IgA production in Mϕ-depleted PP cell cultures reconstituted with peritoneal Mϕ. Fourth, increased IgA production was evident in PP B cell cultures reconstituted with both VT-exposed Mϕ and $CD4^+$ T cells with Mϕ having the greatest effect. Finally, both soluble factors and cognate interactions between Mϕ and the lymphocyte populations appeared necessary for increased IgA production in PP cultures following VT exposure.

PP in the gut-associated lymphoid tissue is a major source of B cell precursors for IgA-secreting plasma cells (Craig and Cebra, 1971). In contrast to PP, spleen is not a major source of B cells for IgA production. Both Mϕ and B cells are responsive to LPS (Raetz et al., 1991; Liu and Janeway, 1991; Berberich and Schimpl, 1992; Verstovsek et al., 1994). Elevation of IL-6 secretion in LPS-stimulated spleen cultures coupled with a small relative increase in IgA production suggests that Mϕ were the primary source for IL-6 in spleen but that spleen was not a primary target for VT-induced IgA production in this model. This contention was supported by the observation that production of IgA was affected more by VT in PP

cultures than in spleen cultures. Thus, VT potentially has a greater effect in the gut mucosal compartment as compared to the systemic compartment.

It well-established that certain cytokines are involved in regulating IgA response in PP via enhancement of activation, switching and differentiation of B cells into IgA-secreting plasma cells (McGhee et al., 1989; Beagley and Elson, 1992; McGhee and Kiyono, 1993). Notably, IL-6 has been demonstrated to elevate IgA production when it is added to PP B cell cultures (Beagley et al., 1989; 1991). One of the primary sources of IL-6 is the Mϕ (Bauer et al. 1988; Bauer, 1989). In previous studies, I found that acute oral VT exposure in mice can increase the capacity of PP and spleen cell cultures to secrete IL-6 and, furthermore, specific neutralizing anti-IL-6 Ab inhibits in vitro IgA production by PP cultures obtained from mice exposed to single oral dose of VT (see chapter 2). Thus, there is a strong possibility that, by secreting cytokines such as IL-6, Mϕ may contribute co-stimulatory function for B cell production of IgA. Recently, VanCott et al., (1996) demonstrated that oral exposure to Salmonella expressing fragment C of tetanus toxin induces high levels of Mϕ-secreting IL-6 in murine PP and spleen cultures, and that this IL-6 elevated plasma cell production of IgA at mucosal effector sites. The production of IL-6 from local intestinal mucosa has also been reported (Bao et al., 1993). Mucosal production of IL-6 is important because this cytokine could regulate a number of local immune responses including IgA production (Akira et al., 1990). This conclusion was supported by recent observations that the numbers of mucosal IgA-producing cells were dramatically decreased in IL-6 knock-out mice

(Ramsay et al., 1994b). Thus, it is reasonable to suggest that IL-6 produced by Mϕ plays a central role in regulation of VT-induced IgA responses.

Recently, Okahashi et al., (1996) demonstrated that oral administration of IL-4 knock-out mice with tetanus toxin and CT induced CT-specific $CD4^+$ T cell-producing IL-6 and mucosal IgA anti-CT responses. This suggested that IL-6 from Th2-type cells plays an important compensatory role in the induction and regulation of mucosal IgA response. Thus, T cells may be another source of IL-6 that arises oral VT exposure in mice. This is supported by several lines of evidence. First, increased IL-6 and IgA production were also observed in cultures where VT-treated Mϕ-depleted PP cells were co-cultured with control peritoneal Mϕ. After depleting Mϕ from PP cells, T cells are still presumed to be present and therefore could provide an alternative signal for IL-6 secretion. Second, IgA production in control PP B cell cultures was enhanced when co-cultured with VT-treated $CD4^+$ T cells as compared to cultures containing control PP B cells and control $CD4^+$ T cells. IL-6 secretion was significantly increased only in PMA + ION-stimulated cultures where control PP B cells were co-cultured with VT-treated $CD4^+$ T cells as compared to co-cultured with control $CD4^+$ T cells. Third, previous studies have observed that in vitro exposure of murine splenic $CD4^+$ T cells to VT can significantly elevate IgA production by B cells (Warner et al., 1994). In these studies, IL-6 levels were enhanced significantly in Con A-stimulated $CD4^+$ T cells after exposure to VT when compared to control. Fourth, previous studies have demonstrated that significant increases in IgA production occur when control B cells are co-cultured with PP T

cells isolated from mice fed 25 ppm VT for 8 wks (Bondy and Pestka, 1991). Finally, elevated percentages of $CD4^+$ T cells and $CD4^+/CD8^+$ T cell ratios were also found in PP and spleens of VT-fed mice (Pestka et al., 1990a). Collectively, these results suggest that T cells might also play a role in regulating VT-induced IgA production.

Since the results indicated the potential of both Mϕ and T cells from VT-treated animals to promote IgA and IL-6 secretion, a key question relates to which cell type (Mϕ or T cells) is the major contributor. It is critical to note that IgA and IL-6 production were highest in cultures where VT-treated or control PP B cells were co-cultured with VT-treated Mϕ than co-cultured with VT-treated $CD4^+$ T cells. Furthermore, the greatest effects on IgA and IL-6 production were found in the LPS-stimulated cultures consisting of VT-treated or control PP B cells and VT-treated peritoneal Mϕ. These observations suggest in the model described herein that Mϕ may have a much greater effect on IgA and IL-6 production than $CD4^+$ T cells.

There were several reasons for selecting peritoneal Mϕ and splenic $CD4^+$ T cells in reconstitution studies. First, results of preliminary experiments indicated that IL-6 secretion was increased in peritoneal Mϕ cultures obtained from mice 2 hr after oral exposure to VT (data not shown). This evidence suggests that VT was capable of stimulating peritoneal Mϕ to release IL-6. Second, peritoneal exudates are a far richer source of Mϕ than PP and thus minimized the need for experimental animals. Third, murine spleen was used as an enriched source of $CD4^+$ T cells because previous studies have shown that exposure to VT <u>in vitro</u> superinduces IL-

2, IL-4 IL-5 and IL-6 mRNA expression in murine splenic $CD4^+$ T cells (Ouyang et al., 1995; 1996a; Azcona-Olivera et al., 1995a; Warner et al., 1994). Finally, in vitro exposure of murine splenic $CD4^+$ T cells to VT can significantly enhance IgA production by B cells (Warner et al., 1994). These observations suggest that splenic $CD4^+$ T cells may play a role in regulating IgA production by secretion of cytokines such as IL-6.

In addition to Mϕ and Th cells, it is worth noting that B cells can also produce IL-6 (Van-Snick, 1990). Yee et al., (1989) has proposed that B cells synthesize, secrete and utilize IL-6, and this autocrine pathway could promote both growth and Ig secretion. Clearly, the autocrine or paracrine actions of B cell-secreted IL-6 could lead to augmented differentiation of IgA-committed B cells and result in increasing IgA production. Thus, it was notable that control and VT-treated B cells cultured alone did not show a major difference of IgA and IL-6 production following oral exposure to VT whereas there was a marked increase in IgA and IL-6 production when VT-treated B cells were combined with untreated and treated Mϕ or treated $CD4^+$ T cells.

A number of studies have demonstrated that the regulation of the Ig response not only needs soluble cytokines but also requires cell-cell interactions. Vercelli et al., (1989) have observed that a physiological concentration of IL-4 induces purified B cells to synthesize IgE only after a cognate interaction with T cells. Cytokine combinations alone were unable to replace T cells and monocytes in inducing IgE synthesis in peripheral blood mononuclear cells, suggesting that

cell-cell contact is an essential requirement. It was notable that IgA levels were markedly less in the reconstituted cell cultures where VT-treated Mϕ were separated with a semi-permeable membrane as compared to those cell cultures without a membrane. One explanation for these findings is that although the soluble mediators were allowed to pass through the membrane, cell-cell interactions between Mϕ and the lymphocyte populations were prevented. Mϕ are required as accessory cells in the activation of T lymphocytes (Rosenwasser and Rosenthal, 1978). Mϕ can be stimulated to secrete cytokines by contact with Ag (Farr et al., 1977), mitogen activated lymphocytes (Mizel et al., 1978) and by cytokines secreted from activated lymphocytes (Meltzer and Oppenheim, 1977). This indicates that Mϕ-lymphocyte interactions are bidirectional and can be mediated either by cell-cell contact or by cytokines. Erb and Feldmann, (1975a) reported that Mϕ and T cell interactions are required for the induction of Th cell in the reaction to Ags in vitro. Some previous studies have demonstrated that activation of Mϕ effector function is required the cognate (cell:cell) interactions between Mϕ and Th cells (Sypek and Wyler, 1991; Sypek et al., 1991; Stout and Suttles, 1993). Subsequently, many recent studies also demonstrated that interactions between activated T cells and monocytes are necessary for the induction of IL-1β mRNA expression (McAllister and Ellis, 1996) as well as the production of IL-1β (See et al., 1992; Dayer et al., 1993; Wagner et al., 1994), TNF-α (See et al., 1992) and IL-12 (Shu et al., 1995) by monocytes. Therefore, I believe that in addition to soluble mediators, interactions between Mϕ and T cells are required in VT-induced enhancement of IgA

production. These interactions are likely to play an integral role in regulation of IgA response in mucosal tissues and susceptibility to VT-induced IgAN.

It is now also clear that induction of T-cell activation for regulating immune responses requires the costimulatory signals which involve the attachement of CD40 ligand (CD40L) on activated T cells to CD40 on B cells (Noellet et al., 1992; Van-den-Eertwegh et al., 1993) and the interactions of CD28 on the T cells with B7 on the Ag presenting cells (Mondino and Jenkins, 1994). The costimulatory signals involved in interactions between T and B cells, and T cell and Mϕ are shown in Figure 3.9. B7 is a cell surface molecule and is expressed by multiple cell types including Mϕ and B cells (Hathcock et al., 1994). Previous studies have demonstrated that interactions of CD28 with its natural ligand B7 expressed on the surface of activated Mϕ or B cells will induce proliferation and cytokines secretion in activated T cells (Freedman et al., 1991; Razi-Wolf et al., 1992; Linsley et al., 1991). Based on the observation that VT could induce secretion of IL-6 by Mϕ for regulating IgA production, it seems possible that induction of expression of cell surface molecule B7 also occurred on VT-treated Mϕ and resulted in providing a costimulatory signal for regulating IgA response. Furthermore, cognate interactions between Mϕ and T cells appeared necessary for VT-induced enhancement of IgA production. Further investigation of the potential of VT on inducing expression of B7 molecule on Mϕ is thus warranted.

Taken together, these results suggest that superinduction of IL-6 secretion

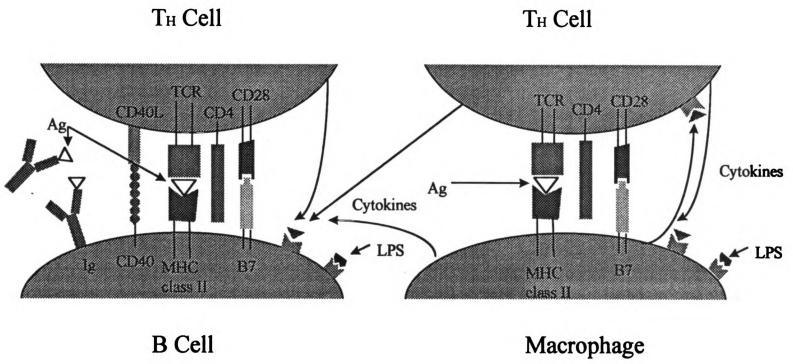


Figure 3.9 Costimulatory signals between T and B cells, and T cell and macrophage.

by both Mϕ and to a lesser extent $CD4^+$ T cells may be responsible for upregulation of IgA production in mice exposed orally to VT. For Mϕ, this is likely to involve both secretion of soluble mediators such as IL-6 and cognate interactions.

CHAPTER 4

INDUCTION OF NEPHROPATHY BY INJECTION WITH DIETARY VOMITOXIN-INDUCED IgA MONOCLONAL ANTIBODIES INTO MICE

4.1 ABSTRACT

Oral exposure to VT induces elevated levels of serum IgA, circulating IgA-IC and causes mesangial IgA deposition and hematuria in mice. These manifestations mimic the hallmark clinical signs of human IgAN. To further assess the role of VT-induced IgA in inducing this disease in the mouse, two strains of mice, B6C3F1 and BALB/C, were injected i.p. with various monoclonal IgA Abs previously obtained from hybridomas derived from PP of VT-exposed mice and several immunopathologic markers were subsequently monitored. In B6C3F1 mice, 2- to 5-fold serum IgA and IgA-IC levels were increased in treatment groups after 4 and 6 wks as compared to controls. Serum IgG and IgM levels as well as urinary erythrocyte counts were elevated in treatment groups after 2, 4 and 6 wks as compared to controls. Concurrent increases in IgA and IgG complexes containing casein, the dietary protein source, were found in treatment mice. Mesangial IgA, IgG, IgM and C3 deposition were significantly increased in all treatment mice after 6 wks. Furthermore, electron microscopy revealed that there was an elevation of electron-dense deposits in the glomeruli of IgA-injected mice after 6 wks. The above parameters were similarly affected in BALB/C mice. Injection of IgA-secreting hybridoma cells into BALB/C mice also increased serum IgA, IgA-IC and IgG levels as well as elevated mesangial IgA, IgG and C3 deposition and hematuria in treatment mice after 2 to 3 wks as compared to controls. These results indicated that injection of VT-induced monoclonal IgA Abs or IgA-secreting hybridoma cells into mice can induce elevation of serum IgA, IgA-IC, IgG and cause mesangial IgA,

IgG, C3 deposition and hematuria. Casein, an Ag found in the diet used for these mice, appeared to form IC with IgA or IgG and these IC may participate in pathogenesis leading to nephropathy.

4.2 INTRODUCTION

VT is a fungal secondary metabolite that belongs to a family of mycotoxins referred to as trichothecenes (Tanaka et al., 1988). This toxin is frequently found in cereal grains as well as other food and agricultural products (Abouzied et al., 1991; Rotter et al., 1996). VT and other trichothecenes are potent protein synthesis inhibitors (Ueno, 1985; Betina, 1989; Pestka and Casale, 1990) that can significantly alter cell-mediated immunity, humoral immunity and host resistance in animal models (Pestka et al., 1987; Pestka and Bondy, 1990). Trichothecenes can be both immunostimulatory and immunosuppressive in a variety of animal and cell culture models (Pestka and Bondy, 1994). In mice, dietary VT exposure induces extremely high levels of serum IgA (Forsell et al., 1986; Pestka et al., 1989), and increases circulating IgA-IC formation, glomerular IgA deposition and hematuria (Pestka et al., 1989; Pestka and Bondy, 1990; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b). These symptoms are clinically similar to human IgAN (Berger's Disease) which is the most common glomerulonephritis worldwide (D'Amico, 1987). These observations suggest that VT may be used as a probe to understand mechanism of IgAN and that it might be a possible etiological factor in IgAN.

Although the pathogenesis and etiology of IgAN are not fully understood, IgAN is likely to be caused by an aberrant mucosal immune response resulting in large increases in polyspecific serum IgA (especially polymeric IgA) (Monteiro et al., 1984). This apparently leads to a dramatic increase in glomerular IgA and IgA-IC

deposition within the kidney (Czerkinsky et al., 1986). IgG, IgM and C3 complement component deposition occurs concurrently and are also likely to contribute to glomerular dysfunction. It has been suggested that the mucosal stimulation following gastrointestinal /respiratory infection, genetic background or diet factors may contribute to the development of this disease (D'Amico, 1987).

There are several animal models for IgAN that mimic the clinical and morphologic features of the human syndrome (Rifai et al., 1979; Emancipator et al., 1983; Rifai and Millard, 1985; Emancipator et al., 1987). Notably, Rifai et al., (1979) and Rifai and Millard, (1985) have demonstrated that passive injection of IgA-IC results in deposition of IgA and C3 as well as elicit hematuria. These symptoms are frequently observed in human IgAN. It has also been noted that oral immunization with different proteins (bovine gamma globulin, ovalbumin and ferritin) can induce elevation of serum IgA and mesangial IgA deposits in mice (Emancipator et al., 1983). Recent studies in our laboratory have demonstrated that exposure to VT can dysregulate IgA production and induce glomerular IgA deposition and hematuria in both B6C3F1 and BALB/C mice (Pestka et al., 1989; Dong et al., 1991; Dong and Pestka, 1993; Rasooly and Pestka, 1994; Greene et al., 1994a; 1994b). Furthermore, polyspecific and autoreactive monoclonal IgA Abs have been produced by hybridomas derived from PP of VT-fed mice and these resemble IgAs encountered in sera of VT-exposed mice (Rasooly et al., 1994). In that study, elevation of mesangial IgG and C3 deposition and hematuria was also observed in BALB/C mice after injection of VT-induced monoclonal IgAs, thus suggesting that

autoreactive polyspecific IgAs might be pathogenic in this murine model (Rasooly et al., 1994). Further investigation of potential of VT-induced monoclonal IgAs in inducing nephropathy on both B6C3F1 and BALB/C mice is of considerable interest because it might provide an experimental model for understanding immunopathologic mechanism of IgA-induced nephropathy.

We hypothesize that the polyreactive IgA Abs induced by VT contribute to experimental IgAN induced by this trichothecene. The objective of this study was to induce experimental nephropathy by injection of VT-induced IgA Abs into mice. I specifically assessed serum IgA, circulating IgA-IC formation, mesangial IgA and C3 deposition and hematuria as indicators of IgAN. The results indicated that injection of IgA Abs into two strains of mice can induce the elevation of serum IgA, IgA-IC, IgG, IgM as well as cause accumulation of mesangial IgA, IgG and C3 and hematuria. One potential dietary Ag, casein, appeared to form IC with IgA or IgG and these IC may participate in pathogenesis of this disease.

4.3 MATERIALS AND METHODS

4.3.1 Chemical and reagents

All chemicals were of reagent grade quality or better and obtained from Sigma Chemical (St Louis, MO) except where otherwise noted.

4.3.2 Animal and IgA Abs injection

Two strains of mice were used because: (1) induction of experimental IgAN was previously observed in VT-fed B6C3F1 mice (Pestka et al., 1989; Pestka and Bondy, 1990; Dong et al., 1991; Dong and Pestka, 1993) and BALB/C mice (Rasooly and Pestka, 1994; Greene et al., 1994a), and (2) BALB/C mice have been previously used to produce IgA-secreting hybridomas derived from PP of VT-exposed mice (Rasooly et al, 1994). Female mice (B6C3F1 and BALB/C) were obtained from Charles River Laboratories (Wilmington, MA) and kept in a university animal care facility room with a humidity- and temperature-controlled and a 12 hr light and dark cycle. They were housed in cages equipped with filter bonnets (Nalgene, Rochester, NY) and fed powdered semi-purified AIN-76A diet (ICN Nutritional Biochemical, Cleveland, OH) upon arrival. Animals were acclimated for at least one wk prior to usage.

For Ab studies, mice (3 per group) were injected i.p. with purified IgA Abs (1 mg/mouse) in PBS twice per wk for 4 wks and 6 wks respectively. Control mice were injected with an equal volume of PBS. BALB/C mice (3 per group) were injected i.p. with IgA-secreting hybridoma cells (1×10^7/mouse) once for 3 wks. Control mice were injected with NS-1 cells (1×10^7/mouse) or with an equal volume of DMEM

medium.

4.3.3 IgA-secreting hybridoma cell cultures

Seven different monoclonal IgA-secreting hybridoma cell lines derived from PP of VT-fed mice, 3-1-G5, 7-1-E4, 8-2-F6, 11-1B5, 12-1-B5, 25-1-D5, and 42-2-B5 were kindly provided by Rasooly et al., (1994) and MOPC 315 is a murine IgA standard (American Type Culture Collection, Rockville, MD). These cells were cultured in DMEM medium supplemented with 20% (v/v) FBS (Gibco, Grand Island, NY), 1% NCTC (v/v), 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% (v/v) MCM in tissue culture plates (Fisher Scientific Co., Corning, NY) at 37^0 C under a 7% CO_2 in a humidified incubator. Monoclonal IgA supernatants were precipitated three times with 50% saturated ammonium sulfate (Harlow and Lane, 1988). Samples were dialyzed against 3 changes of 0.01M PBS (pH 7.4).

4.3.4 IgA purification by TNP-BSA immuno-affinity gel

The above described polyreactive monoclonal IgAs reacted strongly with TNP. This facilitated purification of these monoclonal IgAs using TNP-BSA immuno-affinity gel columns. TNP-BSA (trinitrophenylated BSA) was prepared by trinitrophenylation using picric acid (Good et al., 1980). Briefly, 20 mg of BSA was mixed with 20 mg potassium carbonate in 1 ml distilled water and then 20 mg of picrylsulfonic acid was added. The mixture was covered with aluminum foil and stirred at room temperature overnight. The conjugate was dialyzed in the dark at 4^0 C for 3 days against sodium bicarbonate (0.1 M, pH 9.0) with several buffer

changes. Following dialysis, the ratio of TNP bound to BSA was calculated by spectrophotometric analysis at 278 nm for protein and 348 nm for TNP (Good et al., 1980).

TNP-BSA conjugated at 51:1 mole ratio was coupled to immuno-affinity gel 15 (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's protocol. Briefly, TNP-BSA solution was concentrated using a Centriprep 10 concentrator (Amicon, Beverly, MA) and dissolved in 1 ml of MOPS coupling buffer (0.1 M, pH 7.5). TNP-BSA ligand in coupling solution (1 ml) was added to 1 ml of immuno-affinity gel 15 and agitated sufficiently to make a uniform suspension. After incubation at 4^0 C overnight, TNP-BSA-Gel was centrifuged for 10 min at 450 x g and used directly to absorb the Abs.

For affinity purification, 1 ml of IgA-containing solution was incubated with 1 ml of TNP-BSA-Gel at 4^0 C overnight for affinity purification. Following incubation the mixture was centrifuged for 10 min at 450 x g. After washing three times with PBS the bound IgA was eluted from the affinity gel with 0.1M glycine solution (pH 2.3). The eluent was collected by centrifuging for 10 min at 450 x g, and then dialyzed against PBS. IgA concentration was measured by ELISA. Samples were concentrated with a Centriprep 10 concentrator (Amicon) and filter sterilized prior to injection.

4.3.5 Hematuria analysis

Urine samples (approximately 2 ml/mouse/16 hr) were collected overnight in a metabolic cage at 2 wk intervals and centrifuged at 500 x g for 10 min.

Erythrocyte numbers in 10 random microscopic fields (x 45) were counted in the sediment and averaged as described by Dong et al., (1991).

4.3.6 ELISAs

Blood samples were collected orbitally from ether-anesthetized mice every 2 wks. Serum IgA, IgG and IgM were quantitated by ELISA (Bondy and Pestka, 1991). Briefly, Immunolon 4 Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated overnight at 4^0 C with 50 µl/well of heavy-chain specific goat anti-mouse IgA, IgG or IgM (Cappel Worthington, Malvern, PA) at a concentration of 10 µg/ml in 0.1M bicarbonate buffer (pH 9.6). Coated plates were washed 3 times with 0.01 M PBS (pH 7.2) containing 0.2% Tween 20 (PBST) to remove excess capture Abs. Plates were incubated with 300 µl of 1% (w/v) BSA in PBS (BSA-PBS) at 37^0 C for 30 min to block nonspecific protein binding, and then washed 4 times with PBST. For Ig determination, standard mouse reference serum (Bethyl Laboratories, Inc, Montgomery, TX) or serum samples were diluted in BSA-PBS and 50 µl was added to appropriate wells. Plates were incubated at 37^0 C for 60 min, washed 4 times with PBST, and then 50 µl of goat anti-mouse IgA, IgG or IgM horseradish peroxidase conjugates (Cappel Worthington, Malvern, PA) detection antibodies, diluted 1:1000 (for IgA) and 1: 500 (for IgG and IgM) in 1% (w/v) BSA-PBS, was added to each well. Plates were incubated at 37^0 C for 30 min and washed 6 times with PBST. Bound peroxidase was determined with 2,2-azino-bis (3-ethylbenzolin-6-sulfonate) (ABTS) substrate [0.4mM ABTS, 50mM citrate buffer (pH 4.0), and 1.2mM hydrogen peroxide) as

described previously by Pestka et al., (1980). Absorbance was measured at 405 nm and IgA, IgG and IgM were quantitated by using the Vmax Software (Molecular Devices).

For detection of circulating IgA-IC, sera were precipitated using 3.5% (w/v) polyethylene glycol (PEG 6000) as described by Dong et al., (1993) and then quantitated by IgA ELISA as described above.

For detection of casein Ig-IC, Immunolon 4 Removawell microtiter strip wells were coated overnight at 4^0 C with 50 µl/well of capture sheep anti-casein Ab (Cortecs Diagnostics, Deeside, U.K.) at a concentration of 10 µg/ml in 0.1M bicarbonate buffer (pH 9.6). Following BSA-PBS blocking step, standard mouse reference serum or serum sample was diluted in BSA-PBS and 50 µl was added to appropriate wells. Bound Ig was quantitated by ELISA as described above.

For casein inhibition studies, Immunol 4 Removawell microtiter strip wells were coated overnight at 4^0 C with 50 µl/well of capture sheep anti-casein Ab (Cortecs) at a concentration of 10 µg/ml in 0.1M bicarbonate buffer (pH 9.6). Coated plates were washed 3 times with PBST to remove excess capture Abs, and 100 µl/well of free casein (100 µg/ml) in PBS was added to each well. Plates were incubated at 37^0 C for 30 min, and washed 4 times with PBST to remove unbound casein. Following BSA-PBS blocking step, standard mouse reference serum or serum sample was diluted in BSA-PBS and 50 µl was added to appropriate wells, and then quantitated by ELISA as described above.

4.3.7 Quantitation of mesangial IgA, IgG, IgM and C3

BALB/C and B6C3F1 mice were humanely killed at 3, 4 and 6 wks following injection with IgA Abs or IgA-secreting hybridoma cells. Kidneys were removed and immediately frozen in liquid nitrogen then stored at -80^0 C for section. These kidneys were sectioned into 7 μm slices on a cryostat (Riechert-Jung, Cambridge Instruments, Buffalo, NY) and stained with fluorescein-labeled goat anti-mouse IgA, IgG, IgM and C3 Abs (Sigma) according to the procedure of Valenzuela and Deodhar, (1981). Sections from each mouse were analyzed under a Nikon epifluorescence microscope as described by Greene et al., (1994a; 1994b). Immunofluorescence staining intensities of the kidney mesangium (10 glomeruli/section/mouse) were measured using an ITM Densitometric Video Camera (Waltham, MA) and analyzed by JAVA image analysis system software (Jandel Scientific, San Rafael, CA).

4.3.8 Electron microscopy

Electron microscopy examination was done at department of pathology in MSU. B6C3F1 mice were humanely killed 6 wks after injection with IgA Abs. Kidneys were removed and fixed in 2.5% glutaraldehyde in 200 mOsm. 0.1M sodium phosphate buffer at a pH of 7.4 and rinsed with a 0.1M phosphate buffer (4 x 15 min per rinse). Samples were post-fixed in 1% Osmium tetroxide, rinsed with 0.1M phosphate buffer (2 x10 min per rinse). Dehydration of samples through a series of graded ethanols was followed by propylene oxide with infiltration and embedding in Polybed-Araldite resin. Samples were polymerized for two days at approximately 74^0 C. One micron sections were prepared 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 a diamond knives and placed on 300 mesh copper grids. Sections were contrasted with 2% aqueous Uranyl acetate and lead nitrate. All sections were examined at 60 KV on a Philips 301 electron microscope.

4.3.9 Statistics

The data were analyzed by Danaid's test or Student-Newman-Keuls (SNK) test following one way ANOVA using SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A p value of less than 0.05 was considered statistically significant.

4.4 RESULTS

Effects of monoclonal IgA Abs on B6C3F1 mice. Monoclonal IgA Abs had been previously produced from PP hybridomas of VT-fed mice and demonstrated to be polyreactive or autoreactive due to their ability for binding to various self and non-self Ags (Rasooly et al., 1994). To assess the potential of VT-induced IgA to induce experimental nephropathy in mice, several different polyreactive IgA Abs were used in this study. TNP was chosen for efficient IgA purification since all injected polyreactive IgAs have been previously demonstrated to react with TNP (Rasooly et al., 1994). Furthermore, MOPC 315 was used in this study as a murine IgA positive standard. Rasooly et al., (1994) also observed that MOPC 315 can strongly react with an Ag panel including DNA, PC, casein, inulin, TNP, thyroglobulin, collagen and cardiolipin. This suggested that MOPC 315 might also be polyspecific. The effects of injection of purified monoclonal IgA Abs on serum IgA, IgA-IC, IgG and IgM were assessed in B6C3F1 mice at 2 wks intervals. IgA levels were significantly increased 2- to 4-fold in treatment groups 12-1-B5 at 2 wks, in 3-1-G5, 8-2-F6, 12-1-B5, 25-1-D5 and MOPC 315 at 4 wks and in all treatment groups at 6 wks as compared to control groups (Table 4.1). IgA-IC levels were significantly increased by 5-fold in treatment groups 11-1-B5 and 12-1-B5 at 2 wks and by 2- to 5-fold in all treatment groups at 4 and 6 wks as compared to control groups (Table 4.2). Injection of IgA appeared to have the greatest effect on serum IgG levels which where enhanced significantly 2- to 4-fold at 2 wks, and 2- to 17-fold at 4 and 6 wks in treatment as compared to control groups (Table 4.3). IgM

Table 4.1. The effects of monoclonal IgA Abs administration on serum IgA accumulation in B6C3F1 mice[a,b]

	IgA (μg/ml)		
Material injected	wk 2	wk 4	wk 6
PBS (control)	323 ± 30	320 ± 23	334 ± 22
3-1-G5	515 ± 61	1311 ± 232[c]	1330 ± 325[c]
7-1-E4	399 ± 55	689 ± 140	966 ± 222[c]
8-2-F6	555 ± 61	900 ± 140[c]	822 ± 96[c]
11-1-B5	625 ± 141	579 ± 76	784 ± 75[c]
12-1-B5	832 ± 67[c]	759 ± 15[c]	850 ± 83[c]
25-1-D5	464 ± 122	1029 ± 221[c]	1369 ± 313[c]
42-2-B5	427 ± 48	723 ± 146	882 ± 224[c]
MOPC 315	425 ± 74	923 ± 218[c]	997 ± 69[c]

[a] Mice were injected i.P. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.2. The effects of monoclonal IgA Abs administration on serum IgA-IC formation in B6C3F1 mice[a,b]

	IgA-IC (μg/ml)		
Material injected	wk 2	wk 4	wk 6
PBS (control)	1.5 ± 0.2	1.7 ± 0.4	2.5 ± 0.8
3-1-G5	3.8 ± 0.6	6.4 ± 1.9[c]	7.3 ± 1.4[c]
7-1-E4	2.4 ± 0.9	5.8 ± 1.2[c]	6.3 ± 0.3[c]
8-2-F6	2.5 ± 0.4	6.9 ± 0.1[c]	8.0 ± 2.1[c]
11-1-B5	8.3 ± 1.5[c]	4.8 ± 0.4[c]	11 ± 0.9[c]
12-1-B5	8.2 ± 0.3[c]	9.8 ± 2.5[c]	9.3 ± 1.7[c]
25-1-D5	3.5 ± 1.7	4.2 ± 1.1[c]	5.2 ± 0.5[c]
42-2-B5	4.6 ± 2.3	8.9 ± 0.7[c]	9.1 ± 0.6[c]
MOPC 315	4.0 ± 0.5	6.8 ± 0.4[c]	13 ± 2.1[c]

[a] Mice were injected i.p. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.3. The effects of monoclonal IgA Abs administration on serum IgG accumulation in B6C3F1 mice[a,b]

	IgG (mg/ml)		
Material injected	wk 2	wk 4	wk 6
PBS (control)	3.7 ± 0.3	3.3 ± 0.4	3.6 ± 0.2
3-1-G5	8.3 ± 1.6[c]	16.5 ± 0.8[c]	20.9 ± 1.7[c]
7-1-E4	8.2 ± 0.3[c]	14.1 ± 2.5[c]	15.0 ± 4.0[c]
8-2-F6	8.1 ± 1.4[c]	8.4 ± 1.6[c]	10.3 ± 2.0[c]
11-1-B5	14.7 ± 3.2[c]	56.0 ± 25.5[c]	63.9 ± 30.8[c]
12-1-B5	13.3 ± 2.0[c]	36.7 ± 10.5[c]	42.0 ± 11.4[c]
25-1-D5	15.0 ± 1.8[c]	29.7 ± 1.6[c]	34.9 ± 3.0[c]
42-2-B5	9.1 ± 2.0[c]	12.6 ± 2.7[c]	13.4 ± 3.7[c]
MOPC 315	7.9 ± 1.2[c]	18.8 ± 8.6[c]	23.9 ± 11.1[c]

[a] Mice were injected i.p. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.

[b] Data reported as mean ± SEM (n=3).

[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

levels were significantly increased (2- to 5-fold) in most treatment groups at 2 to 6 wks as compared to control groups (Table 4.4). These observations demonstrated that VT-induced IgA Abs can induce elevation of serum IgA , IgG, IgM levels and IgA-IC formation.

Since casein is the primary dietary protein found in the AIN-76A diet, the effects of injection of IgA Abs on casein Ig-IC formation were also assessed in serum of mice. IgA-casein complexes (Table 4.5) and IgG-casein complexes (Table 4.6) levels were significantly elevated in groups treated with 3-1-G5, 11-1-B5, 12-1-B5, 25-1-D5 and MOPC 315 after 6 wks as compared to control groups. These complexes were inhibitable (90-95%) by free casein in the ELISA. The findings demonstrated that injection of IgAs can induce casein Ig-IC formation, and suggested that casein may play a role in formation of these IC.

Glomerular injury was determined at 2, 4 and 6 wks by enumeration of erythrocytes in the urine. All treatment groups showed significant elevation in the numbers of erythrocyte as compared to control groups with maximal effects being observed 6 wks after injection (Table 4.7). A significant increase in mesangial IgA, IgG, IgM and C3 deposition was observed in all treatment groups as compared to control groups (Table 4.8). Electron microscopy further revealed that there was marked electron-dense mesangial deposition in treatment animal after 6 wks (Fig. 4.1a), whereas electron-dense deposits were not observed in control animal (Fig. 4.1b). These results suggest that VT-induced IgA Abs could contribution to the pathological effects in this murine model.

Table 4.4. The effects of monoclonal IgA Abs administration on serum IgM accumulation in B6C3F1 mice[a,b]

	IgM (μg/ml)		
Material injected	wk 2	wk 4	wk 6
PBS (control)	128 ± 6.6	188 ± 21	139 ± 26
3-1-G5	512 ± 65[c]	325 ± 26	510 ± 256[c]
7-1-E4	334 ± 45[c]	424 ± 3.9[c]	417 ± 62[c]
8-2-F6	284 ± 26[c]	323 ± 57	393 ± 39[c]
11-1-B5	694 ± 33[c]	729 ± 96[c]	1136 ± 296[c]
12-1-B5	516 ± 19[c]	967 ± 242[c]	728 ± 98[c]
25-1-D5	353 ± 26[c]	347 ± 92	525 ± 77[c]
42-2-B5	237 ± 28[c]	388 ± 61	503 ± 226[c]
MOPC 315	300 ± 36[c]	612 ± 73[c]	537 ± 75[c]

[a] Mice were injected i.p. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.

[b] Data reported as mean ± SEM (n=3).

[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.5. The effects of monoclonal IgA Abs administration on serum IgA-casein complexes formation in B6C3F1 mice[a,b]

	IgA-casein complexes (ng/ml)	
Material injected	w/o casein blocking	w/ casein blocking[c]
PBS (control)	0.6 ± 0.3	< 0.1[e]
3-1-G5	44 ± 4.3[d]	2.8 ± 0.4[e]
11-1-B5	51 ± 9.1[d]	5.8 ± 1.1[e]
12-1-B5	73 ± 1.7[d]	6.0 ± 0.6[e]
25-1-D5	87 ± 9.2[d]	9.1 ± 1.4[e]
MOPC 315	54 ± 14.6[d]	2.9 ± 0.7[e]

[a] Mice were injected i.p. twice per wk for 6 wks with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Data were obtained by ELISA. Coated capture sheep anti-casein Ab was first blocked with free casein and then serum samples were added.
[d] Indicates significantly different ($p < 0.05$) from control group (injected PBS).
[e] Indicated significantly different ($p < 0.05$) from without casein blocking group.

Table 4.6. The effects of monoclonal IgA Abs administration on serum IgG-casein complexes formation in B6C3F1 mice[a,b]

	IgG-casein complexes (ng/ml)	
Material injected	w/o casein blocking	w/ casein blocking[c]
PBS (control)	0.5 ± 0.3	0.4 ± 0.2
3-1-G5	167 ± 17.1[d]	9.7 ± 1.2[e]
11-1-B5	512 ± 82.7[d]	19.3 ± 4.5[e]
12-1-B5	219 ± 57.3[d]	14.4 ± 2.1[e]
25-1-D5	347 ± 19.8[d]	14.8 ± 0.9[e]
MOPC 315	177 ± 34.7[d]	7.7 ± 1.3[e]

[a] Mice were injected i.p. twice per wk for 6 wks with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Data were obtained by ELISA. Coated capture sheep anti-casein Ab was first blocked with free casein and then serum samples were added.
[d] Indicates significantly different ($p < 0.05$) from control group (injected PBS).
[e] Indicated significantly different ($p < 0.05$) from without casein blocking group.

Table 4.7. Erythrocyte counts in urine of B6C3F1 mice injected with monoclonal IgA Abs[a,b]

	Erythrocyte/field		
Material injected	wk 2	wk 4	wk 6
PBS (control)	2.3 ± 0.4	4.1 ± 0.1	6.7 ± 0.2
3-1-G5	8.1 ± 2.7[c]	13.9 ± 1.5[c]	20.9 ± 0.4[c]
7-1-E4	13.0 ± 1.0[c]	18.0 ± 1.0[c]	28.0 ± 1.8[c]
8-2-F6	9.0 ± 1.7[c]	17.3 ± 1.5[c]	25.7 ± 0.7[c]
11-1-B5	16.1 ± 1.2[c]	21.7 ± 0.7[c]	26.6 ± 0.4[c]
12-1-B5	25.8 ± 1.1[c]	33.1 ± 0.4[c]	48.7 ± 1.7[c]
25-1-D5	17.6 ± 0.3[c]	22.3 ± 1.4[c]	29.6 ± 1.0[c]
42-2-B5	11.3 ± 0.7[c]	17.2 ± 0.9[c]	24.3 ± 0.3[c]
MOPC 315	19.6 ± 2.4[c]	25.1 ± 1.1[c]	28.4 ± 1.9[c]

[a] Mice were injected i.p. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.

[b] Data reported as mean ± SEM (n=3).

[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.8. The effects of monoclonal IgA Abs administration on IgA, IgG, IgM and C3 deposition in the kidney of B6C3F1 mice[a]

	Relative immunofluorescence[b]			
Material injected	IgA	IgG	IgM	C3
PBS (control)	30 ± 0.9	35 ± 1.4	20 ± 0.4	51 ± 0.5
3-1-G5	80 ± 1.9[c]	84 ± 0.5[c]	44 ± 1.5[c]	107 ± 2.5[c]
7-1-E4	56 ± 2.3[c]	68 ± 2.3[c]	35 ± 2.5[c]	109 ± 1.6[c]
8-2-F6	56 ± 2.2[c]	68 ± 2.8[c]	34 ± 4.0[c]	100 ± 1.7[c]
11-1-B5	62 ± 0.3[c]	87 ± 1.2[c]	53 ± 0.7[c]	130 ± 0.6[c]
12-1-B5	61 ± 3.0[c]	83 ± 3.9[c]	52 ± 3.3[c]	122 ± 0.9[c]
25-1-D5	64 ± 0.6[c]	83 ± 0.4[c]	39 ± 0.3[c]	119 ± 0.6[c]
42-2-B5	52 ± 0.5[c]	64 ± 0.9[c]	33 ± 0.4[c]	105 ± 0.6[c]
MOPC 315	56 ± 3.2[c]	74 ± 1.3[c]	36 ± 0.3[c]	118 ± 0.9[c]

[a] Mice were injected i.p. twice per wk for 6 wks with monoclonal IgA Abs or with an equal amount of PBS.

[b] Data reported as mean ± SEM of relative immunofluorescence on scale of 0-255 per pixel. Ten glomeruli measured per individual mouse/per group (3 mice/group).

[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

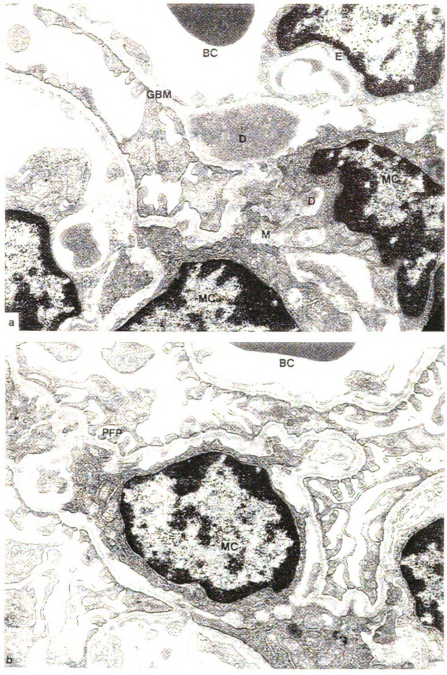


Figure 4.1. (**a**) Electron micrograph of a glomerulus from a B6C3F1 mouse injected with 1 mg VT-induced monoclonal IgA (12-1-B5) twice per wk for 6 wks. Electron-dense deposits (**D**) are seen under the glomerular basement membrane (**GBM**) and within the mesangial region (**M**). **MC**, mesangial cell; **BC**, blood capillary; **E**, endothelial cell; **PFP**, podocyte foot processes. (**b**) Electron micrograph of a glomerulus from a control mouse injected with PBS for 6 wks. Electron-dense deposits were not observed in mesangial region. (magnification x 11,000).

Effects of monoclonal IgA Abs on BALB/C mice. To assess whether the above immunopathological effects could be induced in the original strain from which IgA-secreting hybridomas were produced, BALB/C mice were also injected with purified monoclonal IgA Abs and serum IgA, IgA-IC, IgG and IgM were monitored at 2 wks intervals. IgA levels were increased 2-fold in treatment groups 12-1-B5 and MOPC 315 at 2 and 4 wks as compared to control groups (Table 4.9). Relatively, significant increases in IgA-IC (7- to 12-fold), IgG (10- to 44-fold) and IgM (2- to 4-fold) serum concentrations was also observed in all treatment after 2 and 4 wks as compared to control groups (Table 4.9). Consistent with B6C3F1 mice, all treatment groups had higher numbers of erythrocyte than control groups after 4 wks (Table 4.10). Mesangial IgA, IgG and C3 deposition were significantly increased in all treatment groups as compared to control groups (Table 4.11) but no differences were observed in IgM deposits. All these observations suggest that BALB/C mice exhibit similar immunopathological effects of nephropathy with B6C3F1 mice after injection of VT-induced IgA Abs and these IgAs also can induce the experimental nephropathy in this strain of mice. Thus the effect of IgG observed in B6C3F1 mice was not simply due to an anti-allotype response.

BALB/C mice response to IgA-secreting hybridoma cells. To further verify the effects of IgA Abs on inducing experimental nephropathy, BALB/C mice were also injected with IgA-secreting hybridoma cells. Serum IgA, IgA-IC, IgG and IgM were monitored at one wk intervals upto 3 wks. IgA levels were significantly increased in groups treated with 11-1-B5 and 12-1-B5 (37- to 190-fold) as well as

Table 4.9. The effects of monoclonal IgA Abs administration on serum Igs accumulation and IgA-IC formation in BALB/C mice[a,b]

Material injected	wk	IgA (μg/ml)	IgA-IC (μg/ml)	IgG (mg/ml)	IgM (μg/ml)
PBS (control)	0	743 ± 114	1.2 ± 0.3	1.2 ± 0.2	388 ± 32
	2	728 ± 128	2.1 ± 0.3	1.1 ± 0.2	320 ± 12
	4	631 ± 32	2.3 ± 0.5	1.1 ± 0.2	249 ± 14
12-1-B5	0	671 ± 52	0.9 ± 0.1	1.4 ± 0.2	334 ± 13
	2	1670 ± 308[c]	15 ± 1.2[c]	25.2 ± 5.6[c]	1104 ± 164[c]
	4	1296 ± 209[c]	15 ± 1.9[c]	44.7 ± 20.5[c]	994 ± 48[c]
25-1-D5	0	765 ± 126	2.0 ± 0.4	2.0 ± 0.7	324 ± 40
	2	1043 ± 97	14 ± 1.3[c]	22.2 ± 12.4[c]	890 ± 79[c]
	4	877 ± 96	15 ± 2.2[c]	27.9 ± 11.7[c]	1198 ± 177[c]
MOPC 315	0	998 ± 47	2.4 ± 0.4	2.2 ± 0.6	524 ± 40
	2	1467 ± 246[c]	15 ± 1.2[c]	10.2 ± 4.0[c]	805 ± 85[c]
	4	1067 ± 71[c]	24 ± 6.7[c]	26.0 ± 7.8[c]	1185 ± 297[c]

[a] Mice were injected i.p. twice per wk with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.10. Erythrocyte counts in urine of BALB/C mice injected with monoclonal IgA Abs[a,b]

Material injected	Erythrocyte/field
PBS (control)	7.5 ± 0.9
12-1-B5	40.7 ± 0.7[c]
25-1-D5	44.5 ± 2.5[c]
MOPC 315	26.7 ± 3.3[c]

[a] Mice were injected i.p. twice per wk for 4 wks with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM (n=3).
[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

Table 4.11. The effects of monoclonal IgA Abs administration on IgA, IgG, IgM and C3 deposition in the kidney of BALB/C mice[a]

	Relative immunofluorescence[b]			
Material injected	IgA	IgG	IgM	C3
PBS (control)	35 ± 0.2	41 ± 0.6	28 ± 0.5	60 ± 0.7
12-1-B5	71 ± 1.2[c]	100 ± 1.8[c]	29 ± 0.6	166 ± 3.6[c]
25-1-D5	77 ± 0.4[c]	101 ± 1.7[c]	33 ± 0.6	154 ± 2.6[c]
MOPC 315	67 ± 0.9[c]	91 ± o.9[c]	32 ± 0.4	142 ± 3.0[c]

[a] Mice were injected i.p. twice per wk for 4 wks with monoclonal IgA Abs or with an equal amount of PBS.
[b] Data reported as mean ± SEM of relative immunofluorescence on scale of 0-255 per pixel. Ten glomeruli measured per individual mouse/per group (3 mice/group).
[c] Indicates significantly different ($p < 0.05$) from control group (injected PBS).

11-1-B5 and MOPC 315 (27- to 44-fold) after 2 to 3 wks respectively (Table 4.12). IgA-IC levels were also significantly increased in groups treated with 12-1-B5 and 25-1-D5 (17- to 60-fold) and 11-1-B5 (20-fold) after 2 to 3 wks respectively (Table 4.13). Significant increases in IgG levels were observed in groups treated with 25-1-D5 or MOPC 315 but not 12-1-B5 or 11-1-B5 (Table 4.14). IgM levels were not affected in any of the treatment groups (data not shown). All treatments resulted in increased numbers of erythrocytes in the urine (Table 4.15) as well as mesangial IgA, IgG and C3 deposition (Table 4.16). These findings suggest that some of the immunopathological effects of nephropathy were also induced in BALB/C mice by injection of IgA-secreting hybridoma cells.

Table 4.12. The effects of IgA-secreting hybridoma cell injection on serum IgA accumulation in BALB/C mice[a,b]

	IgA (μg/ml)			
Cell line injected	wk 0	wk 1	wk 2	wk 3
Medium (control)	388 ± 27	498 ± 77	431 ± 40	543 ± 35
11-1-B5	343 ± 24	648 ± 125	15991± 7782[d]	24030± 3372[d]
12-1-B5	367 ± 114	1511± 405[d]	82257± 19644[d]	Nd[c]
25-1-D5	336 ± 144	560 ± 139	3459 ± 1540	2621 ± 637
NS-1	266 ± 94	194 ± 60	157 ± 20	297 ± 32
MOPC 315	408 ± 36	623 ± 113	521 ± 98	15180 ± 1276[d]

[a] Mice were injected i.p. once with IgA-secreting hybridoma cells (1×10^7) or with an equal amount of NS-1 cells.
[b] Data reported as mean ± SEM (n=3).
[c] Not determined (mice died before 3 wks).
[d] Indicates significantly different ($p < 0.05$) from control group (injected medium).

Table 4.13. The effects of IgA-secreting hybridoma cell injection on serum IgA-IC formation in BALB/C mice[a,b]

	IgA-IC (μg/ml)	
Cell line injected	wk 2	wk 3
Medium (control)	0.8 ± 0.3	1.2 ± 0.5
11-1-B5	3.0 ± 0.6	25 ± 5.3[d]
12-1-B5	48 ± 13[d]	Nd[c]
25-1-D5	14 ± 6.7[d]	4.6 ± 0.9
NS-1	0.8 ± 0.4	0.8 ± 0.2
MOPC 315	1.7 ± 0.7	2.5 ± 0.9

[a] Mice were injected i.p. once with IgA-secreting hybridoma cells (1×10^7) or with an equal amount of NS-1 cells.
[b] Data reported as mean ± SEM (n=3).
[c] Not determined (mice died before 3 wks).
[d] Indicates significantly different ($p < 0.05$) from control group (injected medium).

Table 4.14. The effects of IgA-secreting hybridoma cell injection on serum IgG accumulation in BALB/C mice[a,b]

	IgG (μg/ml)			
Cell line injected	wk 0	wk 1	wk 2	wk 3
Medium (control)	502 ± 66	493 ±122	451 ± 141	446 ± 202
11-1-B5	391 ± 50	366 ± 27	300 ± 84	77 ± 13
12-1-B5	486 ± 113	1542 ± 772	407 ± 80	Nd[c]
25-1-D5	781 ± 24	770 ± 230	711 ± 219	1083 ± 133[d]
NS-1	331 ± 18	666 ± 274	571 ± 256	365 ± 103
MOPC 315	437 ± 78	952 ± 262	1233 ± 204[d]	180 ± 88

[a] Mice were injected i.p. once with IgA-secreting hybridoma cells (1×10^7) or with an equal amount of NS-1 cells.
[b] Data reported as mean ± SEM (n=3).
[c] Not determined (mice died before 3 wks).
[d] Indicates significantly different ($p < 0.05$) from control group (injected medium).

Table 4.15. Erythrocyte counts in urine of BALB/C mice injected with IgA-secreting hybridoma cell[a,b]

Cell line injected	Erythrocyte/field
Medium (control)	4.5 ± 0.2
11-1-B5	14.2 ± 0.6[c]
12-1-B5	17.7 ± 1.3[c]
25-1-D5	20.5 ± 0.6[c]
NS-1	10.3 ± 1.3[c]
MOPC 315	24.1 ± 1.6[c]

[a] Mice were injected i.p. once for 2 wks with IgA-secreting hybridoma cells (1×10^7) or with an equal amount of NS-1 cells.
[b] Data reported as mean ± SEM (n=3).
[c] Indicates significantly different ($p < 0.05$) from control group (injected medium).

Table 4.16. The effect of IgA-secreting hybridoma cell injection on IgA, IgG, IgM and C3 deposition in the kidney of BALB/mice[a]

	Relative immunofluorescence[b]			
Cell line injected	IgA	IgG	IgM	C3
Medium (control)	34 ± 0.7	40 ± 0.5	31 ± 0.4	53 ± 0.2
11-1-B5	127 ± 0.3[c]	73 ± 0.5[c]	34 ± 0.6	80 ± 0.2[c]
12-1-B5	148 ± 2.4[c]	71 ± 0.7[c]	29 ± 0.5	86 ± 0.8[c]
25-1-D5	98 ± 0.4[c]	77 ± 0.5[c]	33 ± 1.2	92 ± 1.1[c]
NS-1	33 ± 0.4	66 ± 0.9[c]	55 ± 1.2	95 ± 0.8[c]
MOPC 315	90 ± 0.8[c]	80 ± 1.3[c]	31 ± 0.5	93 ± 0.1[c]

[a] Mice were injected i.p. once for 3 wks with IgA-secreting hybridoma cells (1×10^7) or with an equal amount of NS-1 cells.

[b] Data reported as mean ± SEM of relative immunofluorescence on scale of 0-255 per pixel. Ten glomeruli measured per individual mouse/per group (3 mice/group).

[c] Indicates significantly different ($p < 0.05$) from control group (injected medium).

4.5 DISCUSSION

The results presented in this study demonstrated that excessive IgA exposure can increase serum IgA and IgA-IC levels, glomerular IgA deposition and hematuria in both B6C3F1 and BALB/C mice. These observations are very similar to previous reports that exposure to dietary VT induces hyperelevated serum IgA and circulating IgA-IC concentrations, and increased glomerular IgA deposition and hematuria in B6C3F1 mice (Forsell et al., 1986; Pestka et al., 1989; Dong et al., 1991; Dong and Pestka, 1993; Greene et al., 1994a; 1994b) and in BALB/C mice (Rasooly and Pestka, 1994; Greene et al., 1994a). Dong and Pestka, (1993) suggested that persistent stimulation of IgA secretion caused by VT results in renal injury and consequently IgAN. The results presented herein suggest that elevation of serum IgA production, IgA-IC formation and mesangial IgA deposition may play critical role in nephropathy severity.

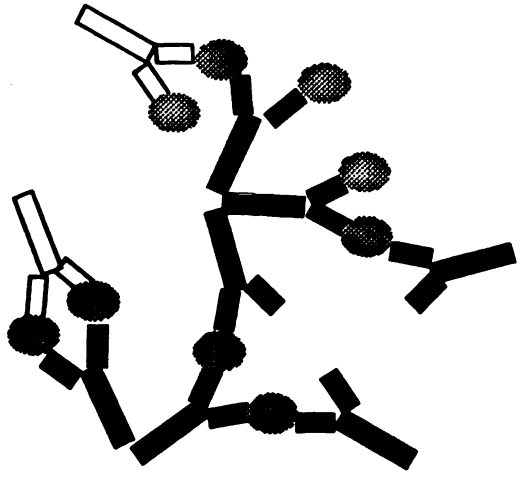
Since kidney damage is revealed as hematuria, it was notable that both IgA-injected B6C3F1 and BALB/C mice showed increases in the parameter as early as 2 wks. This result is consistent with previous studies (Rasooly et al., 1994) where hematuria was found in BALB/C mice at 4 wks after injection of VT-induced monoclonal IgA Abs. The presented results also indicated that 12-1-B5 IgA-injected B6C3F1 mice exhibited the highest numbers of erythrocyte in their urine as compared to other IgA-injected mice. This finding suggests that this monoclonal IgA Ab was particularly effective in inducing severe hematuria and may have predominant contribution to pathogenic effects in this experimental model.

Elevated IgA-containing IC have been well demonstrated in the circulation of patients with IgAN (Lesavre et al., 1982) and in several experiment animal studies of IgAN (Rifai et al., 1979; Emancipator et al., 1987; Montinaro et al., 1991; Dong et al., 1991; Dong and Pestka, 1993). Woodroffe et al., (1980) have reported that dysregulation of IgA production against dietary Ag or pathogens leads to form circulating IgA-IC and subsequent deposition in the kidney result in glomerular damage. Consistent with these findings, our laboratory has previously reported that serum IgA-IC levels were significantly increased in VT-fed B6C3F1 mice (Dong et al., 1991; Dong and Pestka, 1993). Since VT-induced IgA Abs have been previously demonstrated to be polyreactive and autoreactive Abs due to their ability to bind to various self or non-self Ags (Rasooly et al., 1994), it seems possible that the IgA Abs produced as a consequence of VT exposure might react with some foreign or self Ags to form IC and subsequently deposit in mesangial regions, and that IgA-IC may play a potential role in the pathogenesis of this experimental model.

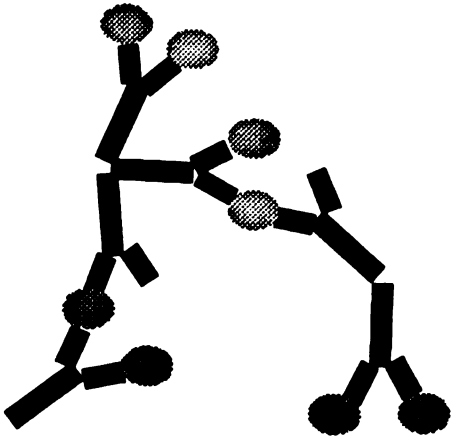
Casein is a dietary protein source that is present at a high levels (30%) in AIN-76A diet. Previous studies have demonstrated increased production of anti-casein IgA Abs as a consequence of exposure to VT in mice (Pestka et al., 1990), suggesting that casein is a candidate Ag for induction of IgA secretion and potential IC formation. Rasooly and Pestka, (1994) demonstrated that casein can inhibit VT-induced serum IgA from binding other Ags such as sphingomyelin and cardiolipin, and suggested that this IgA may be polyreactive. This contention was further demonstrated by studies that IgA eluted from kidney sections of VT-fed mice shows

a marked reactivity with several other Ags such as TNP, PC, inulin, DNA and casein (Rasooly and Pestka, 1994). Rasooly et al., (1994) also reported that approximately 30% of the over 120 VT-induced monoclonal IgA Abs can react with casein. Collectively, these observations strongly suggest that casein was capable of binding IgA. In this study, casein complexes containing IgG or IgA were significantly increased in B6C3F1 mice after injection of IgA Abs and specificity was verified by 90-95% inhibition when free casein was present prior to addition of the serum samples. These results suggest that injected polyreactive IgAs have an ability to directly bind the dietary Ag casein. These could form large pathogenic IC with longer persistence in the serum, which might lead to glomerular injury and hematuria. The IgA and IgG-casein complexes formation is shown in Figure 4.2. As dietary Ags gain access through mucosal membranes, it is possible that the presence of IgA at intestinal surfaces further promotes absorption of casein and thus deposition in the glomerular mesangium. In support of this contention, Russell et al., (1986) have observed that dietary protein Ags such as casein could be deposited in association with IgA in the glomerular mesangium of patients with IgAN. Woodroffe et al., (1980) also demonstrated similar IC formation to dietary Ag which resulted in glomerular damage. Notably, these IC also contain IgG isotype. Therefore, our observations suggest that casein as a dietary Ag may play a potential role in contribution to pathogenic effects by forming IC with IgA or IgG in this experiment model.

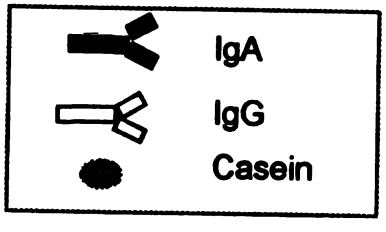
Although IgA is the predominant isotype deposited in the kidney mesangium

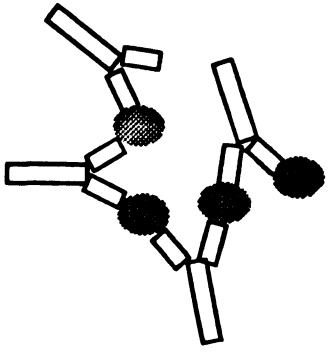


Mixed IgA and IgG-casein complexes



IgA-casein complexes





IgG-casein complexes

Figure 4.2 IgA and IgG-casein complex formation.

of IgA patients, codeposition of IgG and C3 is often detectable in patients with IgAN(D'Amico, 1983). Furthermore, mesangial codeposition of IgG or C3 is also reported in several experimental animal studies of IgAN (Isaacs et al., 1981; Isaacs and Miller, 1982; Rifai and Millard, 1985; Emancipator et al., 1987; Emancipator and Lamm, 1989; Montinaro et al., 1992). This suggests that the codeposition event may be responsible for the alterations in glomerular function in IgAN. Accumulation of C3 in glomeruli is important, since IgA-IC can activate the alternative complement pathway (Gotze and Muller-Eberhard, 1971). Stad et al., (1993) demonstrated that injection of polymeric IgA Abs into rats led to the mesangium deposits of C3 in association with IgA-IC. These findings suggest that alternative pathway activation may play a pathogenic role in glomerular injury and IgAN. My observation of mesangial C3 accumulation further supports previous reports that mesangial C3 deposition occurred in IgA-injected mice (Rasooly et al., 1994) and VT-fed mice (Greene et al., 1994a; 1994b), and suggests that this mediator might play a pathogenic role in VT-induced IgAN.

In this study, injection with IgA Abs into mice caused an extensive increase in serum IgG and IgM as well as mesangial IgG deposition. This result conflicts directly the previous observations (Pestka et al., 1989; Dong et al., 1991; Dong and Pestka, 1993; Greene et al., 1994a; 1994b) in which serum IgG and IgM concentrations as well as mesangial IgG deposition were decreased in VT-fed B6C3F1 mice but is in agreement with a previous report (Rasooly et al., 1994) that increased of mesangial IgG deposition in BALB/C mice was observed after injection

of VT-induced IgAs. One potential explanation is that persistent elevation of serum IgA-casein complex formation and inadequate clearance might be immunogenic to IgG production. The observation of increased serum IgG-casein complex formation supports this possibility. Alternatively, it is possible that IgA might function as the Ag in the immune response. Berger, (1979) has suggested that IgA may aggregate and form deposits in the glomerular mesangial regions, serving as an Ag. Thus another potential possibility is that injected IgA may be an Ag to induce an IgG anti-IgA idiotype immune response. Stall, (1996) reported that anti-idiotype Abs are often induced when monoclonal Abs are used as an immunogen. For example, induction of anti-idiotype Ab synthesis occurs following injection of anti-CEA (carcinoembryonic antigen) monoclonal Ab into mice (de-Moraes et al., 1992) or anti-LPS monoclonal Ab into hamster (Field et al., 1993). Another possibility is that the extensive IgG response is caused by different Ig allotypic specificities (Stall, 1996). However, since elevation of serum IgG was observed in both B6C3F1 and BALB/C mice, it seems likely that this is not an anti-allotype response. Further clarification of the existence of IgG anti-IgA idiotype immune response by injection of IgA Abs into mice is warranted.

Rasooly et al., (1994) have demonstrated that approximately 80% of the monoclonal IgAs from PP hybridomas of VT-fed mice were reactive with more than one of self and non-self Ags that included casein, TNP, sphingomyelin, thyroglobulin, PC, DNA, inulin, collagen, and cardiolipin. Furthermore, one Ag can inhibit binding of some monoclonal IgAs to another Ag. These observations suggest

that these monoclonal IgA Abs were polyspecific and autoreactive. The cross-reactivity of injected monoclonal IgA Abs with an Ag panel is shown in Table 4.17. In this investigation, IgA Abs 3-1-G5, 11-1-B5 and 12-1-B5 showed more effects than other IgA Abs on immunopathologic parameters of IgAN including the elevation of serum IgA, IgA-IC, IgG, IgM levels and mesangial IgA, IgG, C3 deposition with hematuria. These three IgA Abs had been previously demonstrated to react with most Ags (Table 4. 17). Such IgAs might easily form IC with self or non-self Ags which are nephritogenic. Collectively, these findings suggest that polyreactive IgAs 3-1-G5, 11-1B5 and 12-1B5, especially 12-1-B5, are particularly effective in inducing the experimental nephropathy, and that a particular IgA Ab specificity may contribute the pathogenic effects in this experimental animal study of nephropathy.

In this study, injection of VT-induced monoclonal IgAs into BALB/C mice resulted in increasing mesangial IgG and C3 deposition and hematuria. This observation is consistent with a previous report (Rasooly et al., 1994) that increase of mesangial IgG and C3 deposition and hematuria was found in BALB/C mice after injection of VT-induced IgAs. In addition to these same observations, the results presented herein also showed that injection of VT-induced IgAs can elevate serum IgA, IgA-IC, IgG and IgM levels as well as glomerular IgA deposition in BALB/C mice. Thus, presented results further demonstrate that VT-induced polyspecific IgAs might be pathogenic in this experimental murine model.

It is notable that increases in serum IgA, IgA-IC and IgG levels as well as

mesangial IgA, IgG and C3 deposition with hematuria also occurred in BALB/C mice after injection of IgA-secreting hybridoma cells. Higher levels of serum IgA in treatment mice are probably due to IgA-secreting hybridoma cells can grow and continually produce IgA in vivo, which would lead to augmenting serum IgA and IgA-IC levels as well as accumulation of IgA in mesangium. These results further support the evidence that IgA-mediated nephropathy in mice was induced by injection of IgA Abs.

In conclusion, the results presented herein demonstrated that injection of VT-induced monoclonal IgA Abs into both B6C3F1 and BALB/C mice can induce the elevation of serum IgA, IgA-IC, IgG, IgM levels and cause mesangial IgA, IgG, C3 deposition and hematuria. One potential dietary Ag, casein, appeared to form IC with IgA or IgG and these IC may participate in the pathogenesis of nephropathy. These studies showed that, with the exception of IgG effects, IgA-induced murine nephropathy model to be mechanistically similar to VT-induced IgAN.

Table 4. 17. ELISA reactivity of representative monoclonal IgA supernatants (10 μg/ml) with Ag panel (from Rasooly et al., 1994)

Clone	DNA	PC	Casein	Inulin	TNP	Thyro	Collag	Cardio
3-1-G5	+	+	+	+	+++	++	nd	nd
7-1-E4	++	+	++	+	+++	+++	+	++
8-2-F6	+	+	+	+	++	++	nd	+
11-1-B5	nd	+++	nd	nd	++	++	nd	+
12-1-B5	++	nd	++	++	++	++	+	++
25-1-D5	nd	nd	nd	nd	+++	nd	nd	nd
42-2-B5	nd	nd	nd	++	+++	+	nd	nd
MOPC 315	+++	++	++	+++	+++	++	+++	+

PC=phosphorylcholine Thyro= thyroglobuli Collag=collagen Cardio=cardiolipin

Values are reactivity of each clone to each Ag as expressed by absorbance: + = low binding (OD<0.1); ++ = medium binding (OD = 0.1-0.5); +++ = high binding (OD> 0.5); nd = no detectable reactivity.

CHAPTER 5

SUMMARY AND FUTURE STUDIES

In this study, I evaluated possible early and late mechanisms for VT-induced IgAN. This investigation suggests that the superinduction of cytokines IL-5 and IL-6 expression may, in part, be responsible for upregulation of IgA production in mice exposed orally to VT. I further demonstrated that both Mϕ and to a lesser extent $CD4^+$ T cells may be responsible for upregulation of IgA production in mice exposed orally to VT. For Mϕ, this is likely to involve both secretion of soluble mediators such as IL-6 and cognate interactions. Furthermore I also demonstrated that injection of VT-induced monoclonal polyreactive IgA Abs into mice can induce the elevation of serum IgA, IgA-IC, IgG and IgM concentrations as well as cause mesangial IgA, IgG, C3 deposition and hematuria. One potential dietary Ag, casein, appeared to form IC with IgA or IgG and these IC may participate in the pathogenesis of nephropathy. This study showed that, with the exception of IgG effects, IgA-induced murine nephropathy model to be mechanistically similar to VT-induced IgAN.

It has been known that the costimulatory signals such as interactions between CD28 on the T cells and B7 on the Mϕ are required for induction of T-cell activation and for regulating immune responses. Based on the observation that VT could induce secretion of IL-6 by Mϕ for regulating IgA production, it is possible that induction of expression of cell surface molecule B7 also occurred on VT-treated Mϕ and that the provided a costimulatory signal for regulating IgA response. Furthermore, cognate interactions between Mϕ and T cells appeared necessary for VT-induced enhancement of IgA production, Therefore, further investigation of the

potential for VT on inducing expression of B7 molecule on Mϕ is warranted. Flow cytometry in conjunction with phenotypic immunostaining can be used to detect the expression of cell surface molecule B7 on Mϕ. Furthermore, based on the findings that injection of VT-induced monoclonal IgA Abs into both B6C3F1 and BALB/C mice resulted in elevation of serum IgG and mesangial IgG deposition, it is possible that injected IgA may be an Ag to induce an IgG anti-IgA idiotypic immune response. Therefore, further clarification of the existence of IgG anti-IgA idiotypic immune response by injection of IgA Abs into mice is warranted. Both B6C3F1 and BALB/C mice can serve as the murine model for future studies because IgA-mediated experimental nephropathy was induced in these two strains of mice.

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