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**DETECTION AND IMMUNOTOXICOLOGIC MECHANISMS OF TRICHOTHECENE MYCOTOXINS**

presented by

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has been accepted towards fulfillment
of the requirements for

Doctoral degree in Food Science and Human Nutrition and Institute for Environmental Toxicology

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Date 9-26-02

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# DETECTION AND IMMUNOTOXICOLOGIC MECHANISMS OF TRICHOTHECENE MYCOTOXINS

By

Yongjoo Chung

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition
and
Institute for Environmental Toxicology

2002

**ABSTRACT**

DETECTION AND IMMUNOTOXICOLOGIC MECHANISMS OF TRICHOTHECENE MYCOTOXINS

By

Yongjoo Chung

Antibodies for macrocyclic trichothecene, satratoxin G (SG), were produced upon immunization of rabbits with SG bis-hemisuccinate conjugated to BSA. The antibodies could detect free SG using a competitive direct-ELISA with a range of detection from 0.1 to 100 ng/ml. The observed antibody cross-reactivity may facilitate simultaneous detection of other satratoxins and to a lesser extent, other macrocyclic trichothecenes. Methanol content up to 20% in samples did not affect the immunoassay and this stability expands applicability of the antibodies. The capacity of representative macrocyclic trichothecenes to alter TNF-α and IL-6 production and viability was assessed in a murine macrophage model. Macrocyclic trichothecenes can superinduce the proinflammatory cytokine, TNF-α, at low cytotoxic concentrations, whereas these compounds are cytotoxic and reduce cytokine production at higher concentrations. These immunomodulatory effects were observed at relatively low (ng/ml) concentrations, suggesting that macrocyclic mycotoxins may pose a hazard to humans exposed to *Stachybotrys*. In order to expand understanding of how trichothecenes affect gene regulation, we have isolated vomitoxin (VT)- and SG-responsive genes, macrophage inflammatory protein-2 (MIP-2) and complement 3a receptor (C3aR), from a murine macrophage cell line using differential display-PCR. Both VT and SG up-regulated expression of MIP-2, which is a chemokine responsible for chemotaxis of neutrophils to inflammation site, whereas VT selectively up-regulated C3aR, which is a receptor involved

in activation by complement. VT up-regulated splenic MIP-2 mRNA and its protein in serum in a mouse model. To investigate roles of mitogen-activated protein kinase (MAPK) in cytokine superinduction by VT, the effects of VT on MAPK activation, as well as the relationship of MAPKs to VT-induced mRNA expression, TNF-α promoter activity, TNF-α mRNA stability, and TNF-α protein production were assessed in RAW 264.7 murine macrophage cells. The results indicate that VT may superinduce TNF-α expression by increasing its transcripts and mRNA stability through activation of MAPK. Trichothecene-induced activation of MAPK may play a critical role in superinduction of proinflammatory cytokine, TNF-α, and succeeding pathologic events.

To my heavenly Father, my wife, parents, and three children

## ACKNOWLEDGMENTS

I am greatly grateful to Dr. James J. Pestka, my major advisor, for his guidance, assistance, encouragement, and understanding throughout this study. I wish to express my gratitude to Dr. John E. Linz, Dr. L. Patrick Hart, and Dr. Kate Claycombe for their accessibility and advise and for being members of my graduate guidance committee.

I wish to thank Dr. Joseph J. Schroeder, Dr. Hui-Ren Zhou, Dr. Zahid Islam, Dr. Rebecca Uzarski, Mr. Matthew Rarick, and Mr. Michael Miller for their suggestions, practical help, and friendship.

I would like to thank my wife, Cindy Y. Chung and my children, Ashley, Joshua, and Eunice for their love, patience, understanding, and support during this study in my life. I am also grateful to my father, Woohong Chung, my mother, Kyungsoon Song, and mother-in law, Junghee Lim, and my twin brother Dr. Yongsoo Chung for their unconditional love and support in my life.

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# INTRODUCTION

Trichothecene mycotoxins are a group of secondary metabolites produced by *Fusarium*, *Stachybotrys*, and other molds growing on food or in the environment. Over 180 trichothecenes are identified and categorized into four groups (type A-D) based on the functional groups attached to the trichothecene ring structure. From a public health standpoint, type D (e.g. satratoxins, verrucarin A, and roridin A), type A (e.g. T-2 toxin and diacetoxyscirpenol), and type B (e.g. vomitoxin [VT or deoxynivalenol] and nivalenol) are of particular interest since these toxins are present in the environment and in food. These mycotoxins bind eukaryotic ribosomes and inhibit protein synthesis. Exposures of farm animals and humans to trichothecenes have been reported worldwide and have been related to outbreaks of alimentary toxic aleukia, vomiting, gastroenteritis, leukocytosis, and circulatory shock.

Satratoxins, a group of macrocyclic trichothecenes, are mainly produced by *Stachybotrys atra*, which grows on cellulose materials in water-damaged buildings. Satratoxins are 10 to 100 times more toxic than are other groups of trichothecenes in mouse $LD_{50}$ studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays. These toxins have been etiologically associated with stachybotryotoxicosis in animals in Central Europe. Clinical signs of this disease include leukopenia, thrombocytopenia, hemorrhage, arrhythmic heartbeat, and death. Outbreaks of *Stachybotrys*-associated diseases in water-damaged homes have been reported (Etzel *et al.*, 1998; Johanning *et al.*, 1996). Recently, the toxigenic *S. atra* was reported to be involved in pulmonary hemorrhage and hemosiderosis in infants living in *S. atra*-contaminated homes

(Etzel *et al.*, 1998).

Given the potential harmful effects of macrocyclic trichothecenes on human health, it is important to monitor their presence in the environment. Current methods for detection and quantitation of the toxins include thin-layer chromatography, high performance liquid chromatography, and mass spectrometry, which require time-consuming extraction, sample clean-up, and/or expensive instrumentation. Enzyme-linked immunosorbent assay (ELISA) has been widely applied for the detection of mycotoxins because of its high specificity and sensitivity. It is desirable and feasible to develop a specific ELISA for the satratoxins.

Leukocytes are particularly sensitive to trichothecenes. Depending on dose and duration of exposure, these toxins can be immunostimulatory as well as immunosuppressive. Trichothecene-induced immunostimulatory effects include increased cytokine production and upregulation of immunoglobulin (Ig) production. Immunosuppressive effects of trichothecenes include apoptosis and impairment of humoral immunity (Bondy and Pestka, 2000). To date, most work on the immunostimulatory effects of trichothecenes has focused on the type A and type B groups. It is important to better understand effects of macrocyclic trichothecenes on immune function for their potential effects on human health.

The trichothecene VT causes a wide range of toxicological and immunological effects in mice. Experimentally, high dose oral exposure to VT causes impairment to immune cells whereas low dose can result in vomiting, diarrhea, and gastroenteritis. Dietary VT in mice affects mitogen-induced proliferation of lymphocytes, host resistance, Ig production, especially IgA, and cytokine expression. One of the most prominent effects of dietary VT is dysregulation of IgA production, which is highly analogous to human IgA nephropathy.

Relevant effects of VT include increases in serum IgA, circulating IgA immune complexes, kidney mesangial IgA, and hematuria as well as polyclonal activation of IgA secreting cells. Overproduction of IgA by VT is mediated through alteration of cytokine production by macrophages and helper T cells.

Cytokine superinduction by VT is well documented in vivo and in vitro. Acute, oral exposure of mice to VT increased Th1 cytokine, IL-2 and IFN-γ mRNA expression, Th2 cytokines IL-4, -5, -6 and their mRNAs, and proinflammatory cytokines IL-6, IL-1β, and tumor necrosis factor (TNF)-α. Superinduction of IL-2 and TNF-α and IL-6 expression by VT were observed in a murine T-cell culture (EL-4 cell line) model and in RAW 264.7 macrophage cells, respectively. In order to expand understanding of how trichothecenes affect gene regulation, murine RAW 264.7 macrophage cells were treated with VT or satratoxin G (SG) and expressed genes were assessed by differential display PCR (DD-PCR). The results indicate that both toxins up-regulated expression of macrophage inflammatory protein-2 (MIP-2), which is a chemokine responsible for chemotaxis of neutrophils to inflammation sites, whereas VT selectively up-regulated complement 3 a receptor (C3aR), which is a receptor involved in activation through complement. Induction of these two genes might contribute to trichothecene-induced immunotoxicity.

Mitogen-activated protein kinase (MAPK) cascades are major signaling systems in which cells transduce extracellular stimuli into intracellular responses in leukocytes and other cells. MAPKs have been associated with a wide array of cellular responses including cytokine production, cell growth, differentiation, and apoptosis. Three distinct MAPKs have been characterized in the mammalian system; the extracellular signal-regulated kinase

(ERK1/2 or p42/p44), the c-Jun amino-terminal kinases (JNK1/2, also known as stress-activated protein kinases, SAPK), and p38 MAPK. Activation of these MAPKs by stimuli turns to signal-specific transcriptional and posttranscriptional activation, and determine the cellular responses.

MAPKs may contribute to gene expression at a transcriptional level by phosphorylating transcription factors and at post-transcriptional level by mediating phosphorylation of RNA binding proteins. Activated transcription factors bind their binding sites in DNA which they subsequently transcribe to mRNA. Phosphorylated RNA binding protein may regulate binding affinity to its target site and result in stabilization of mRNA.

MAPKs may be critical mediators of VT-induced cytokine production. VT is known to superinduce TNF-α and IL-6 in RAW 264.7 cells and IL-2 in EL-4 cells. This superinduction is found to be due to both increased transcription and increased mRNA stability. The increased transcripts for TNF-α and IL-6 were also explained by an increase of binding activity of NF-κB, AP-1 and NF-IL6 transcription factors to their promoter regions. The increased TNF-α and IL-6 mRNA stability was explained by the observation that LPS-induced TNF-α and IL-6 mRNAs were stabilized by VT in the presence of transcription inhibitor, 5,6-dichloro-beta-D-ribofuranosyl-benzimidazole (DRB). Recently, VT was reported to induce p38 kinase, ERK1/2, and JNK1/2. The involvement of MAPKs in increased TNF-α mRNA expression by VT has yet to be explained.

The aforementioned issues were addressed in this dissertation, which is comprised of five parts. Chapter I reviews the literature on trichothecenes, effect of trichothecenes on immune modulation, proinflammatory mediators and trichothecenes, and MAPKs and their

effect on cytokine mRNA regulation. Chapter II describes development of antibodies to satratoxins and application to direct competitive enzyme-linked immunosorbent assay. Chapter III describes modulation of lipopolysaccharide-induced proinflammatory cytokine production by satratoxins and other macrocyclic trichothecenes in the murine macrophage. Chapter IV describes up-regulation of macrophage inflammatory protein-2 and complement 3a receptor by the trichothecenes deoxynivalenol and satratoxin G. Chapter V describes MAPK effects on TNF-α mRNA expression by deoxynivalenol. Chapter VI includes summary and conclusion of these studies.

# CHAPTER I

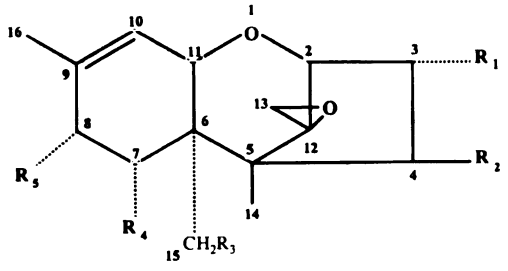
# LITERATURE REVIEW

## A. Trichothecenes

### *A.1. Introduction*

Trichothecenes are a group of mycotoxins, which are secondary metabolites produced by various strains of fungi including *Fusarium* and *Stachybotrys*. Fungal infection, colonization, and toxin production on grain crops can occur in the field, at harvest, and during storage. Favorable environmental conditions such as high moisture and cool temperature support mold colonization with subsequent trichothecene biosynthesis (Ishii, 1983). More than 180 trichothecenes have been identified and are categorized into four types (A-D) depending on the functional groups attached to the trichothecene ring structure (Figure 1.1) (Grove, 1988; Grove, 1993; Grove, 1996). Deoxynivalenol (vomitoxin, VT) and nivalenol (type B trichothecenes) and T-2 toxin and diacetoxyscirpenol (type A trichothecenes), are among the most common contaminants in cereal grains (Agriculture, 1989). Satratoxins, the macrocyclic trichothecenes, are type D trichothecenes and are often found in feeds or water-damaged structures (Bata *et al.*, 1985; Harrach *et al.*, 1983; Johanning *et al.*, 1996). Because these food-borne mycotoxins are resistant to processing (Jackson and Bullerman, 1999; Scott *et al.*, 1984; Wolf-Hall *et al.*, 1999), they are potential contaminants of human and animal food supplies. Consequently, trichothecene-contaminated grains have been a concern with food industries and animal feed manufacturers due to their possible health risks (Biabani and Dowling, 1996).

Trichothecenes have been associated with acute toxicoses of farm animals and humans (Bhat *et al.*, 1989; Ueno, 1983a; Yagen and Joffe, 1976). With humans, exposure to trichothecenes has led to outbreaks of alimentary toxic aleukia, vomiting, and



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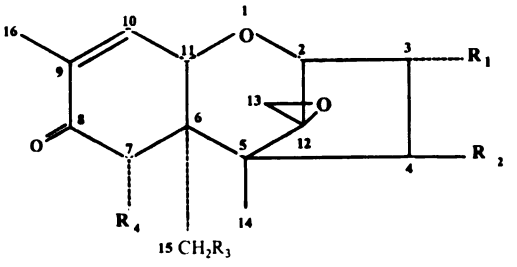

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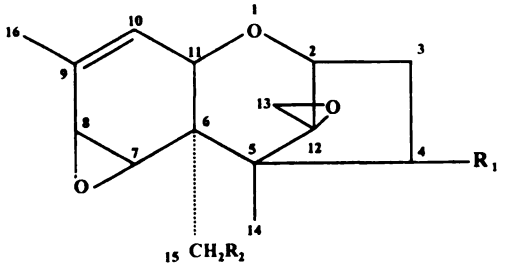

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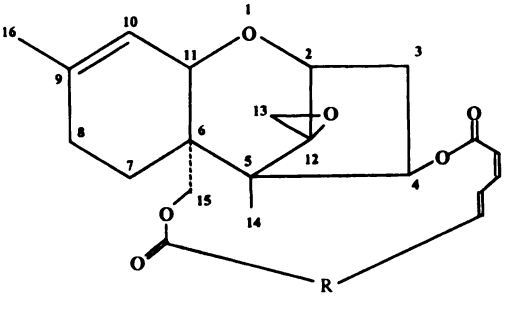

**Type D**

Figure 1.1 Major groups of trichothecenes

gastroenteritis (Bhat *et al.*, 1989; Li *et al.*, 1999; Luo *et al.*, 1990). Acute effects of VT in animals are diarrhea, vomiting, leukocytosis, circulatory shock, and death while chronic effects are feed refusal, reduced rate of weight gain, and immunotoxicity (Arnold *et al.*, 1986; Bondy and Pestka, 2000; Forsyth *et al.*, 1977; Friend *et al.*, 1982).

In addition to health concerns, contamination of grains by trichothecenes has been a major economic concern. A VT outbreak occurred across many Mid-Western states including Michigan in 1996 (Biabani and Dowling, 1996). Kellogg's, one of the biggest cereal producing companies in the world refused to purchase wheat from producers in the state of Michigan due to the possible human health risks from VT. Since 1996, American barley has become less competitive, partly due to contamination with VT (Service, 2000). Grains contaminated with VT also has reduced value as animal feed due to feed refusal and reduced weight gain in the animals (Biabani and Dowling, 1996; Kalish, 1995). A current FDA guideline in U.S. is 1 ppm for bran, flour, and germ intended for human consumption while 5 and 10 ppm are the levels for feed ingredients intended for swine diet and cattle diet, respectively (Trucksess, 1995).

### *A.2. Vomitoxin (Deoxynivalenol)*

VT is one of the major trichothecenes produced by *Fusarium graminearum* and *F. culmarum*, which is common contaminant of several grains including corn, wheat, and barley (Perkowski, 1998; Trigo-Stockli *et al.*, 1998; Wetter *et al.*, 1999; Yoshizawa and Jin, 1995). Wheat and barley from the U.S. Midwest were widely contaminated with VT following the heavy rain and cool weather of 1993-1996 (Trigo-Stockli *et al.*, 1998; Trucksess *et al.*,

1995). A recent report indicates that 71% of the 237 commercially available cereal-based foods including infant foods were positive for VT (Schollenberger *et al.*, 1999).

VT induces a wide range of toxicological and immunological effects. A high and acute dose (≥100 mg/kg) of VT causes necrosis of the gastrointestinal tract, bone marrow, and lymphoid tissues as well as lesions in heart and kidney (Forsell *et al.*, 1987). Subchronic dietary exposure to VT impairs the functions of the intestinal tracks in mice (Hunder *et al.*, 1991). VT induces anorexia at low doses and emesis at higher acute doses in animals (Forsyth *et al.*, 1977; Pestka *et al.*, 1987; Prelusky and Trenholm, 1993). Dietary VT affects mitogen-induced proliferation of lymphocytes, host resistance, immunoglobulin (Ig) production, especially IgA production, and cytokine expression (Rotter *et al.*, 1996).

The capacity of VT to alter immune function has been extensively studied (Bondy and Pestka, 2000). Depending on dose and frequency of exposure, VT can be both immunosuppressive and immunostimulatory. Immunosuppression by VT is manifested by rapid onset of leukocyte apoptosis (Islam *et al.*, 2002; Pestka *et al.*, 1994; Yang *et al.*, 2000; Zhou *et al.*, 2000; Zhou *et al.*, 1999). On the other hand, low doses of VT exposure enhance expression of various cytokines in vitro and in vivo (Azcona-Olivera *et al.*, 1995a; Li *et al.*, 1997; Wong *et al.*, 1998; Yan *et al.*, 1997, 1998b; Zhou *et al.*, 1997). These VT-induced immunomodulatory effects are further related to activation of mitogen-activated protein kinases (MAPKs) (Moon and Pestka, 2002; Yang *et al.*, 2000).

### *A.3. Macrocyclic Trichothecenes*

Macrocyclic trichothecenes are characterized by a cyclic diester or triester ring which

connects C-4 to C-15 of the trichothecene structure (Figure 1.1). These trichothecenes are 10 to 100 times more toxic than Type A and Type B trichothecenes in mouse $LD_{50}$ studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays (Ueno, 1983b). Several potentially important macrocyclic trichothecenes are produced by *Stachybotrys atra* (also known as *S. chartarum* ). This mold grows on cellulose substrates with a high moisture content such as wet hay and straw (Bata *et al.*, 1985; Harrach *et al.*, 1981). Macrocyclic trichothecenes have been etiologically associated with stachybotryotoxicosis in horse and sheep in Central Europe (Bata *et al.*, 1985; Harrach *et al.*, 1983; Harrach *et al.*, 1981; Hintikka, 1978). Clinical signs of this disease include leukopenia, thrombocytopenia, hemorrhage, arrhythmic heartbeat, and death (Forgacs, 1972).

Humans can also be exposed to *Stachybotrys* and macrocyclic trichothecenes via the environment. Outbreaks of *Stachybotrys*-associated diseases in water-damaged structures have been reported (Etzel *et al.*, 1998; Johanning *et al.*, 1996). The characteristics of the diseases include recurrent cough, irritation of the eyes and skin, mucous membrane disorder, headache, and fatigue. Recently, a toxigenic strain of *S. atra* was reported to be involved in pulmonary hemorrhage and hemosiderosis in infants living in *S. atra*-contaminated homes (Etzel *et al.*, 1998). Furthermore, this fungus was isolated from the lungs of a child with pulmonary hemorrhage (Elidemir *et al.*, 1999). This was the first report of the organism in human tissue, supporting the concept that *Stachybotrys* is involved in the lung disease. The involvement of macrocyclic trichothecenes, however, was not determined.

The ability of macrocyclic trichothecenes to modulate immune effect has partially been characterized. For example, macrocyclic trichothecenes have been shown to

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superinduce IL-2 production in EL-4 thymoma cells as well as induce and impair blastogenesis of lymphocytes in the presence of mitogen (Hughes *et al.*, 1990; Pestka and Forsell, 1988). These toxins have been observed to induce apoptosis through activation of MAPK in Jurkat T cells or RAW 264.7 murine macrophage cells (Shifrin and Anderson, 1999; Yang *et al.*, 2000). Spores from *S. atra* induce production of proinflammatory cytokines, TNF-α and IL-6, in RAW 264.7 cells (Ruotsalainen *et al.*, 1998). However, the capacity of macrocyclic trichothecenes to induce those cytokines has not been studied.

### *A.4 Detection of macrocyclic trichothecenes*

Given the potential harmful effects of macrocyclic trichothecenes on human health, it is important to monitor their presence in the environment. Current methods for detection and quantitation of the toxins include thin-layer chromatography, high performance liquid chromatography, and mass spectrometry (Krishnamurthy *et al.*, 1989; Stack and Eppley, 1980; Tuomi *et al.*, 1998). The methods used for macrocyclic trichothecenes, however, necessitate time-consuming extraction, sample clean-up and/or expensive instrumentation (Hinkley and Jarvis, 2000). A protein translation assay employing luciferase mRNA was developed for the purpose of detection of trichothecenes in the spores of toxigenic fungi (Yike *et al.*, 1999). This assay is sensitive due to the effect of protein synthesis inhibition by all trichothecenes present in spores and this method can not distinguish the effects of a single trichothecene from other trichothecenes or other chemicals in a sample. A further limitation of this assay is its expense and requirement for special training and equipment.

Enzyme-linked immunosorbent assays (ELISA) have been widely applied to

detection of mycotoxins because of their high specificity and sensitivity (Abouzied *et al.*, 1993; Azcona-Olivera *et al.*, 1992; Monti *et al.*, 1999; Thirumala-Devi *et al.*, 2000; Yuan *et al.*, 1999). Although the development of ELISA for a variety of food-borne non-macrocyclic trichothecenes has been accomplished (Abouzied *et al.*, 1993; Barna-Vetro *et al.*, 1994; Lee *et al.*, 1989; Park and Chu, 1996; Yuan *et al.*, 1999), relatively little effort to date has been placed on the environmentally important macrocyclic trichothecenes. In one exception, an ELISA for the macrocyclic trichothecene, roridin A, was developed using rabbit polyclonal antibodies (Martlbauer *et al.*, 1988). The antibodies were highly specific for roridin A and showed low cross-reactivity against satratoxin H (15%) and SG (6.5%), which are two of the major toxins in *Stachybotrys* spores(Jarvis *et al.*, 1998). Therefore, it was desirable and feasible to develop a specific ELISA for the satratoxins.

## B. Effect of trichothecenes on immune modulation

### *B.1 Introduction*

VT and other trichothecenes act in a number of cellular systems as protein synthesis inhibitors by binding to eukaryotic ribosomes (Feinberg and MacLaughlin, 1989; Liao *et al.*, 1976; Middlebrook and Leatherman, 1989; Ueno *et al.*, 1968). VT targets actively dividing cells in immune organs and intestinal mucosa (Azcona-Olivera *et al.*, 1995a; Yan *et al.*, 1998a; Zhou *et al.*, 2000) and consequently modulates immune function. One of the most prominent effects of dietary VT in a mouse model is dysregulation of IgA production, which is highly analogous to human IgA nephropathy (D'Amico, 1987; Dong *et al.*, 1991; Emancipator and Lamm, 1989).

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### *B.2 IgA Nephropathy*

IgA nephropathy is recognized worldwide as the most common form of glomerulonephritis (D'Amico, 1987; Emancipator and Lamm, 1989). It is characterized by the predominant deposition of IgA immune complexes (IgA-IC) in the glomerular mesangium. Common features in IgAN patients are an increased circulating IgA-IC and raised serum IgA concentration (Czerkinsky *et al.*, 1986; Lesavre *et al.*, 1982). Cytokines produced by infiltrating monocyte/macrophages, resident glomerular cells, and neutrophils are suspected as mediators for inflammatory damage in human glomerulonephritis including IgAN (Kincaid-Smith *et al.*, 1989; Lai *et al.*, 1994b; Yoshioka *et al.*, 1993).

### *B.3 Role of cytokines in IgA Nephropathy*

Cytokines are key mediators of proliferation and differentiation in lymphoid cells (de Vries *et al.*, 1993; Esser and Radbruch, 1990). They are also involved in immunoglobulin class-switching to different isotypes (Lebman and Edmiston, 1999). Changes in cytokine profile may influence the increased IgA production observed in IgAN. An increase of circulating "type 1" T helper (Th1) and "type 2" T helper (Th2) lymphocytes subset has been observed in IgAN patients (Lai *et al.*, 1994a). Cytokines IL-4, IL-5 and IL-6 produced by Th2 cells are of particular importance in differentiation of B cells to IgA production (McGhee *et al.*, 1990; McGhee *et al.*, 1989; Murray *et al.*, 1990). Other cytokines such as IL-2 and IFN-γ, which are produced by Th1 cells, can stimulate other cytokine secretion and activate macrophage cells, respectively. Transforming growth factor-β (TGF-β) produced by Th1 cells can induce IgA class switch recombination (Defrance *et al.*, 1992). IL-2, -4, and

IFN-γ levels were higher in mitogen-stimulated peripheral blood mononuclear cells (PBMC) from IgAN patients compared with controls (Lai *et al.*, 1994b; Lai *et al.*, 1991; Schena *et al.*, 1989). TGF-β expression was increased in $CD4^+$ cells in IgAN patients (Lai *et al.*, 1994c). Overall, different cytokine profiles are found in IgAN patients compared to healthy controls.

### *B.4 VT-induced IgA Nephropathy*

VT can induce IgAN in a mouse model in which alteration of cytokine production may influence induction of the disease. The level of serum IgA increased while IgG and IgM levels decreased when dietary VT was fed to mice (Forsell *et al.*, 1986). Chronic dietary exposure of mice to VT resulted in an elevation of serum IgA and led to IgA nephropathy (Bondy and Pestka, 1991; Dong and Pestka, 1993; Pestka *et al.*, 1989). Accumulation of mesangial IgA and increased IgA immune complex in serum were observed in VT-fed mice (Dong *et al.*, 1991). Dysregulation of IgA production is suspected to result from an alteration of cytokine profile caused by VT.

VT modulates T helper cytokine profile as well as proinflammatory cytokine profile *in vivo* and *in vitro*. Acute, oral exposure of mice to VT increased Th1 cytokine, IL-2 and IFN-γ mRNA expression, Th2 cytokines, IL-4, -5, -6, and their mRNAs (Azcona-Olivera *et al.*, 1995b; Ouyang *et al.*, 1996), and proinflammatory cytokines, IL-6, IL-1β, and tumor necrosis factor (TNF)-α (Azcona-Olivera *et al.*, 1995a). With in vitro studies, superinduction of IL-2 mRNA and its protein by VT was observed in a murine T-cell culture (EL-4 cell line) model (Li *et al.*, 1997). TNF-α and IL-6 mRNA and their proteins were superinduced in RAW 264.7 macrophage cells when VT and lipopolysaccharide (LPS) were

cotreated (Wong *et al.*, 1998). The changes in these cytokine profile are generally suspected for upregulation of IgA production, leading to IgAN (Dong *et al.*, 1994; Warner *et al.*, 1994; Yan *et al.*, 1997). From a study with IL-6-"knockout" mice, IL-6 was shown to be an essential cytokine in VT-induced IgA dysregulation (Pestka and Zhou, 2000). However, from a study with TNF-α receptor "knockout" mice, TNF-α was not involved in VT-mediated dysregulation of IgA production (Pestka and Zhou, 2002).

## C. Proinflammatory mediators and trichothecenes

### *C.1 Introduction*

Inflammatory mediators can be categorized into four groups: cytokines, chemokines, plasma enzyme mediators including complement, and lipid inflammatory mediators. The major proinflammatory cytokines are TNF-α, IL-6, and IL-1. These cytokines generally function in increasing fever, synthesis of acute phase protein by liver, increased vascular permeability, and activation of B- and T-cells. Chemokines such as IL-8 and macrophage inflammatory protein-2 (MIP-2), are small polypeptides that attract other leukocytes and regulate the expression of adhesion molecules in leukocyte membranes. Plasma enzyme mediators include the kinin system, clotting system, fibrinolytic system, and complement system. Binding of complement split products to receptors on cell membranes induces various immune responses. Lipid inflammatory mediators are prostaglandins, leukotrienes, and platelet-activating factors, which function in increased vascular permeability, neutrophil chemotaxis, and platelet aggregation (Kuby, 1997).

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### *C.2 Effect of VT on production of proinflammatory cytokines*

Proinflammatory cytokines such as TNF-α and IL-6 are mainly produced by macrophage upon stimulation. TNF-α is known to mediate biological responses such as cell death, cytokine and chemokine induction, antiviral activity, and sepsis (Beutler and van Huffel, 1994; Sedgwick *et al.*, 2000; Smith *et al.*, 1990; Van Damme *et al.*, 1987). Exposure to VT increases TNF-α mRNA and protein level *in vivo* and *in vitro* (Azcona-Olivera *et al.*, 1995a; Wong *et al.*, 1998). IL-6 is involved in the inflammatory response by increasing fever, synthesis of acute-phase proteins by liver, increased vascular permeability, and T- and B-cell activation(Kuby, 1997). In addition, IL-6 promotes B cell differentiation to IgA secreting cells in the Peyer's patch (Beagley *et al.*, 1991; Beagley and Elson, 1992). Acute, oral exposure to VT increases IL-6 production as well as IgA production and the elevation of IL-6 is mediated mainly by macrophage cells (Yan *et al.*, 1998b).

### *C.3 Macrophage inflammatory protein-2*

MIPs are chemokines, a member of structurally related proteins that can induce migration of a specific subset of leucocytes. Three MIPs (MIP-1a, MIP-1b and MIP-2), that are structurally and functionally similar, have been identified (Sherry *et al.*, 1988; Wolpe *et al.*, 1988; Wolpe *et al.*, 1989). MIPs are produced in response to various stimuli such as LPS, proinflammatory cytokines, and oxidative stress in a wide range of cells including alveolar macrophages, mast cells, peritoneal macrophages, epithelial cells, and fibroblasts (Burd *et al.*, 1989; Driscoll *et al.*, 1993; Kopydlowski *et al.*, 1999; Shi *et al.*, 1999). MIP-1α and MIP-1β possess proinflammatory properties and can activate both neutrophils and

mononuclear cells (Driscoll, 1994). MIP-2 is a potent neutrophil chemoattractant and epithelial cell mitogen and it is involved in acute pulmonary inflammation (Driscoll *et al.*, 1995; Huang *et al.*, 1992; Schmal *et al.*, 1996; Yoshidome *et al.*, 1999).

Several *in vivo* studies demonstrate that MIP-2 is involved in tissue injury and inflammation. Expression of MIP-2 mRNA and accumulation of neutrophils in lung tissue were associated with development of lung edema during hepatic ischemia/reperfusion in mice (Yoshidome *et al.*, 1999). In that study, passive immunization against MIP-2 with neutralizing antibody reduced lung edema indicating that MIP-2 is involved in tissue injury. The role of MIP-2 in LPS-induced lung injury in rats was studied demonstrating that LPS instillation into rat lung up-regulates MIP-2 mRNA in a time dependent manner and MIP-2 is responsible for neutrophil accumulation in lungs (Schmal *et al.*, 1996). These studies demonstrate that increased MIP-2 expression followed by neutrophil accumulation results in tissue damage and inflammation.

MIP-2 has been considered a functional analog of human IL-8 (Sherry *et al.*, 1992). IL-8 mediated neutrophil recruitment to the site of inflammation can occur in patients with adult respiratory stress syndrome, idiopathic pulmonary fibrosis, rheumatoid arthritis, and hepatic ischemia-reperfusion injury (Colletti *et al.*, 1995; Donnelly *et al.*, 1994; Koch *et al.*, 1994; Lynch *et al.*, 1992). A significant increase of IL-8 in sera of IgAN patients was detected compared to healthy controls. The free IL-8 autoantibodies of the IgA were more frequently detected in IgAN patients (Lai *et al.*, 1996). Recently, VT induced IL-8 production in human macrophage cells (Sugita-Konishi and Pestka, 2001).

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### *C.4 Complement 3a Receptor*

Complement 3a receptor (C3aR) is a seven-transmembrane receptor that is functionally coupled to G proteins (Roglic *et al.*, 1996). C3aR is distributed in both myeloid cells (monocytes/macrophage, neutrophils and eosinophils) and nonmyeloid cells (mast cells, glial cells, astrocytes, and smooth muscle cells) throughout various tissues including lung, heart, kidney, liver, testis, and brain (Davoust *et al.*, 1999; Gasque *et al.*, 1998; Legler *et al.*, 1996; Martin *et al.*, 1997; Sayah *et al.*, 1999). C3aR is activated on binding C3a ligand, which is one of the anaphylatoxins produced by proteolytic cleavage during complement activation (Hugli, 1990). C3a ligand stimulates numerous biological responses including lysosomal enzyme secretion from neutrophils, histamine release from mast cells, smooth muscle contraction, and modulation of the humoral and cellular immune responses (Glovsky *et al.*, 1979; Morgan, 1986; Sayah *et al.*, 1999; Showell *et al.*, 1982; Stimler *et al.*, 1983).

C3a can modulate the immune response by stimulating or suppressing cytokine production. The effects of C3a on IL-6 gene expression and protein production were studied in the presence of LPS in human PBMC (Fischer *et al.*, 1999). In this study, both IL-6 mRNA and protein were increased with a co-treatment of C3a and LPS by PBMC while IL-6 was not enhanced with C3a alone. Pretreatment of PBMCs with pertussis toxin inhibited the production of IL-6. In another study with human astrocytomas, stimulation by C3a enhanced IL-6 mRNA level and protein level while IL-1β, TNF-α and TGF-β mRNA expression remained unchanged (Sayah *et al.*, 1999). IL-6 mRNA expression was blocked by treatment of pertussis toxin or C3aR antibody, which indicates that the IL-6 response was specific to stimulation of C3a in astrocytomas. Regulation of B cell functions by C3a was studied by

measuring IgG, TNF-α, and IL-6 production in *Staphylococcus aureus* Cowan strain I (SAC)/IL-2-activated B cells (Fischer and Hugli, 1997). Incubation with C3a in SAC/IL-2-activated B cells suppressed polyclonal immune response, IL-6, and TNF-α production. Taken together, C3a has immunomodulatory effects upon binding C3aR in various cell types.

## D. MAPK and their effect on mRNA regulation of cytokines

### *D.1 Introduction*

MAPK cascades are major signaling systems in which cells transduce extracellular stimuli into intracellular responses. The essential cascades in MAPK pathways consist of three protein kinases (Figure 1.2). The stimulated MAPK kinase kinase (MAPKKK or MEKK), which is the most upstream of the MAPK cascades, phosphorylates and activates the MAPK kinase (MAPKK or MEK). The activated MAPKK, in turn, phosphorylates closely conserved threonine and tyrosine residues in MAP kinase (MAPK). These highly sequenced phosphorylation events turn the extracellular stimuli to signal-specific transcriptional and posttranscriptional activation, and determine the cellular responses (Tibbles and Woodgett, 1999).

Three distinct, but partially "cross-talking", MAPKs have been characterized in the mammalian system; the extracellular signal-regulated kinase (ERKs or p42/p44), the c-Jun amino-terminal kinase (JNKs, also known as stress-activated protein kinases, SAPK), and p38 MAPK. The ERK pathway is stimulated at the cellular membrane by either a mitogen or growth factor. ERK causes cell proliferation, differentiation, and mitosis. JNK and p38 cascades are activated by multiple environmental insults such as LPS, heat stress, UV

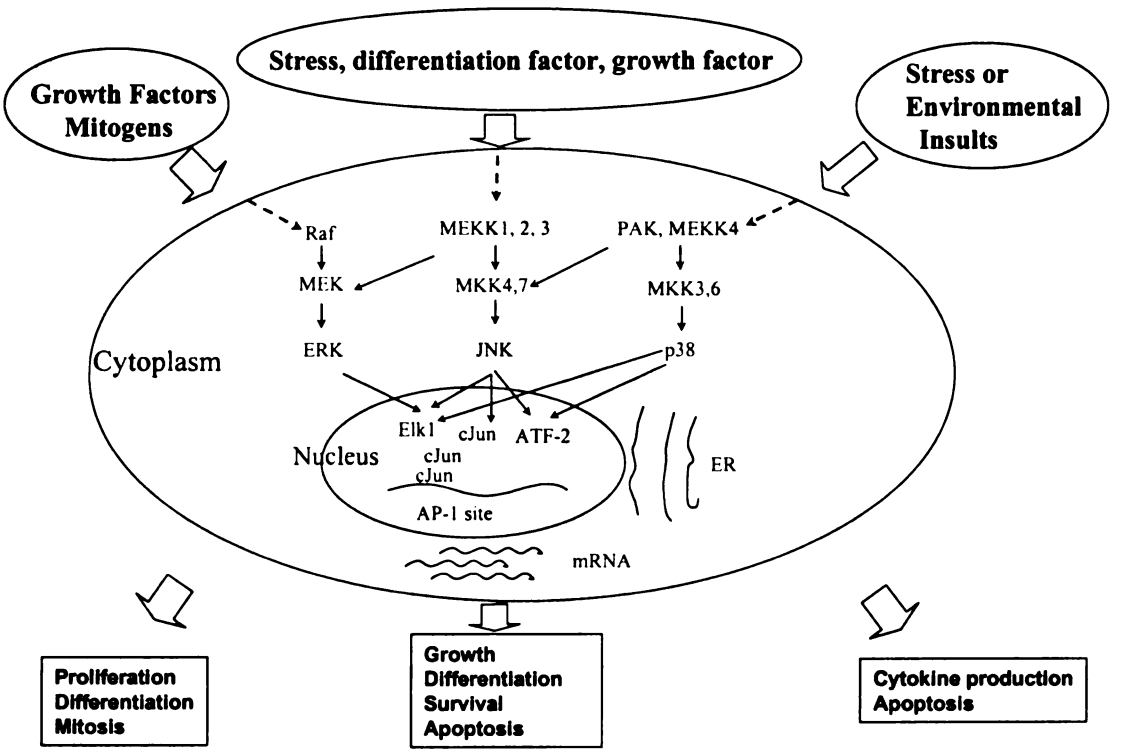


Figure 1.2 Activation of MAPKs and their cellular response

radiation, and osmotic stress. Activation of JNK and/or p38 mediates cellular processes such as apoptosis, immune activation, inflammation and adaptation to stress (Tibbles and Woodgett, 1999).

### *D.2 Transcriptional control*

MAPKs contribute to gene expression at a transcriptional level by phosphorylating transcription factors. Activation of p38 leads to phosphorylation of activating transcription factor-2 (ATF-2). The phosphorylated ATF-2 functions as a transcriptional activator via binding to the cAMP-response element (CRE) (Brinkman *et al.*, 1999). Activated JNK1/2 can also phosphorylate c-Jun and ATF-2 (Hazzalin *et al.*, 1996). Phosphorylated c-Jun binds to the activating protein-1 (AP-1) binding site resulting in transcriptional activation. Activation of ERK1/2 can phosphorylate Elk-1, which is a c-fos regulatory transcription factor (Babu *et al.*, 2000). In addition, all three MAPK pathways are involved in activation of nuclear factor-kB (NF-kB) by phosphorylating IkBa (Lee *et al.*, 1997; Schwenger *et al.*, 1998; Zhao and Lee, 1999). CRE, AP-1, and NF-kB cis-acting sites are known to play key roles in proinflammatory cytokine gene expression, including TNF-α.

### *D.3 Post-transcriptional control*

MAPKs also contribute to gene expression at a posttranscriptional level. Stabilization of cytokine mRNAs can be achieved by different signaling events in all three MAPK pathways. For example, IL-6, IL-8, and TNF-α mRNAs are stabilized by activation of the p38 kinase pathway by stimuli such as lipopolysaccharide (LPS) and IL-1 (Brook *et*

*al.*, 2000; Miyazawa *et al.*, 1998; Winzen *et al.*, 1999). The JNK pathway also participates in stabilization of IL-2 and IL-3 mRNAs by stimulation of ionomycin (Chen *et al.*, 1998; Ming *et al.*, 1998). The ERK pathway is responsible for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 mRNA stabilization induced by TNF-α (Esnault and Malter, 2002; Jijon *et al.*, 2002).

The mechanisms by which MAPK contributes to cytokine mRNA stabilization are not clearly understood. However, phosphorylation of RNA binding proteins by MAPK might be one of the important factors in regulating RNA stability (Sirenko *et al.*, 1997). AU rich element (ARE) in 3'-untranslated region (UTR) of mRNA is found in many short-lived cytokine and proto-oncogene mRNAs and determines stabilization of their transcripts (Chen and Shyu, 1995; Gillis and Malter, 1991; Sachs, 1993; Winstall *et al.*, 1995). Several distinct proteins such as AUF1 (Sirenko *et al.*, 1997), HuR (Myer *et al.*, 1997), and tristetraproline (TTP) (Lai *et al.*, 1999), are known to bind to AREs and regulate mRNA turnover.

Depending on types of protein and state of phosphorylation, binding of these proteins on ARE contributes to either stabilization or destabilization of mRNA. For example, AUF1 selectively recognizes ARE and facilitates mRNA degradation (DeMaria and Brewer, 1996; Pende *et al.*, 1996; Sirenko *et al.*, 1997). Adhesion-dependent stabilization of IL-1β transcripts in monocytes was abolished when SK&F 86002, p38 kinase inhibitor, was treated (Sirenko *et al.*, 1997), which indicates that phosphorylation events are necessary for AUF1 to bind to ARE of the transcripts. HuR, a member of the ELAV-like protein family, is important in translocation and protection of ARE-containing RNA (Fan and Steitz, 1998). It is a TNF ARE binding protein which stabilizes mRNA containing TNF-α mRNA (Dean

*et al.*, 2001). In the study of Dean et al (2001), HuR-mediated stabilization was found to be independent on activation of p38 kinase. TTP is a zinc finger RNA binding protein that is responsible for destabilization of TNF-α mRNA (Lai *et al.*, 1999). Unphosphorylated form of TTP has high binding activity to ARE RNA probe and facilitates mRNA degradation while phosphorylated TTP has low binding activity and consequently increases mRNA stability (Carballo *et al.*, 2001). This protein is inducible by LPS in macrophages and phosphorylated by MAPK-activated protein kinase-2 (MAPKAPK2 or MK-2) through activated p38 kinase (Mahtani *et al.*, 2001).

### *D.4 Superinduction and involvement of MAPKs*

Contribution of MAPKs to gene expression at the transcriptional and posttranscriptional levels may be associated with the superinduction of cytokines by VT because both cytokine gene transcription and mRNA half life are increased by VT in cell line models (Li *et al.*, 1997; Wong *et al.*, 2001; Wong *et al.*, 1998). Superinduction is defined as the capacity of protein synthesis inhibitors such as VT to prolong stimulation of transiently induced cytokine genes in response to stimulants such as growth factors, cytokines, hormones, and interferons (Mahadevan and Edwards, 1991). Superinduction is observed with an increase of specific transcripts and /or their mRNA stabilization.

The causes of superinduction due to protein synthesis inhibitors are unknown, but there are several theories to explain it. For example, c-Fos is a transcription factor that activates expression of other genes. A labile molecule on the c-*fos* gene in an inactivated state may be depleted by protein synthesis inhibitors, causing superinduction (Morello *et al.*,

1990; Subramaniam *et al.*, 1989). Alternatively, protein synthesis inhibitors such as anisomycin can act as second-messenger agonists on intracellular kinases leading to activation of signal transduction cascades (Cano *et al.*, 1994; Mahadevan and Edwards, 1991). Activated kinases are strongly involved in signaling to c-*fos* and c-*jun* (Cano *et al.*, 1994). Further, superinduction can be modulated by controlling the repressive system that mediates degradation of mRNA (Roger *et al.*, 1998). AREs are widely accepted as mRNA unstability determinants (Chen and Shyu, 1995; Gillis and Malter, 1991). Specific binding proteins to ARE bind to various early response gene mRNAs and regulate degradation of those mRNAs (Nakamaki *et al.*, 1995; Sirenko *et al.*, 1997; Zhang *et al.*, 1993). Protein synthesis inhibitors may contribute to repress mRNA degradation in two ways. First, protein synthesis inhibitors may deplete the specific 3'-UTR binding proteins leading to increased mRNA half life and superinduction. Second, activation of MAPK by protein synthesis inhibitors may lead to phosphorylation of RNA binding protein, resulting in low binding activity to ARE and consequently increase mRNA half life (Carballo *et al.*, 2001; Mahtani *et al.*, 2001). VT may contribute to activation of MAPK following phosphorylation of RNA binding protein, especially, TTP and lead to increased mRNA half life and superinduction of TNF-α and IL-6.

VT can superinduce TNF-α and IL-6 in RAW 264.7 cells and IL-2 in EL-4 cells as indicated by both mRNA and protein levels (Li *et al.*, 1997; Wong *et al.*, 1998). This superinduction is due to both increased corresponding transcripts and increased mRNA stability (Li *et al.*, 1997; Wong *et al.*, 2001). The increased transcripts for TNF-α and IL-6 were explained by an increase of binding activity of NF-κB, AP-1 and NF-IL6 transcription

factors to their promoter regions (Wong *et al.*, 2002). The increased TNF-α and IL-6 mRNA stability was explained that LPS-induced TNF-α and IL-6 mRNAs were stabilized by VT in the presence of transcription inhibitor, DRB (Wong *et al.*, 2001). The increased binding activity of those transcription factors and increased mRNA stability of TNF-α and IL-6 imply activation of MAPKs (Brook *et al.*, 2000; De Cesaris *et al.*, 1998; Diaz and Lopez-Berestein, 2000; Liu *et al.*, 2000; Miyazawa *et al.*, 1998; Swantek *et al.*, 1997). Recently, VT was reported to induce p38 kinase, ERK1/2, and JNK1/2 (Moon and Pestka, 2002; Yang *et al.*, 2000). The involvement of MAPKs in increased mRNA expression during superinduction of TNF-α has yet to be explained.

**E. Rationale for this research**

VT and SG, protein synthesis inhibitors, are often found in foods and in the environment, respectively. They have immunemodulatory effects *in vivo* and *in vitro*. Furthermore, VT has been suspected as an etiological factor in human IgAN based on a mouse experimental model. From experiments of dietary exposure to VT, proinflammatory cytokines, especially IL-6 and TNF-α, are found to be important to the progression of IgAN. The ability of SG or other macrocyclic trichothecenes to induce cytokine expression has been incompletely studied. Study on cytokine production by macrocyclic trichothecenes in RAW 264.7 macrophage cells will provide information about how the toxins can modulate immune function. Recently, from differential display experiments, two genes, MIP-2 and C3aR, were found to be up-regulated by VT and/or SG in RAW 264.7 cells. Expression of these gene products might play an important role in the progression of IgAN since autoimmune

complexes against IL-8 (a functional analog of MIP-2 in human) is higher in mesangium in IgAN patients than healthy controls. Kinetic studies on the expression of MIP-2 will provide information about their possible involvement in the autoimmune disease. Finally, the induction of proinflammatory cytokines by VT in RAW 264.7 cells is due both to increased transcription and to increased mRNA half life. Study of MAPK involvement in mRNA expression will provide information about the mechanism by which mRNA expression is increased by VT. Overall, these studies will contribute to understanding how trichothecenes can modulate immune function. In the long run, results of these studies will be used to reduce health risks due to exposure to trichothecenes.

# CHAPTER II

# DEVELOPMENT OF ANTIBODIES TO SATRATOXINS AND APPLICATION TO DIRECT COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY[1]

[1] This work has been submitted for publication in a similar form with authors Chung, Y.J., Jarvis, B.B., Tak, H., and Pestka, J.J. in Mycopathologia.

## ABSTRACT

Although macrocyclic trichothecenes produced by *Stachybotrys chartarum* have been associated with numerous outbreaks of indoor illness, no simple method exists for the detection of these toxins in environmental samples. The goal of this study was to develop an enzyme-linked immunosorbent assay (ELISA) to the major *S. chartarum* macrocyclic trichothecene mycotoxin, satratoxin G (SG). SG was derivatized to its hemisuccinate and coupled to bovine serum albumin for use as an immunogen to produce antibodies in rabbits. A direct competitive ELISA employing SG antibodies and SG-hemisuccinate coupled to horseradish peroxidase (HRP-SG) was used to detect between 0.1 to 100 ng/ml of SG. The concentrations of SG, satratoxin H, isosatratoxin F, roridin A, and verrucarin A causing 50% inhibition of HRP-SG conjugate binding were 1.3, 5.4, 3.2, 25.1, and 52.5 ng/ml, respectively, indicating that the antibodies were most specific for SG and closely related satratoxins. Sensitivity was largely unaffected by methanol content up to 20% in assay diluents. The ELISA was applicable to detection of satratoxins in *S. chartarum* spore extracts and cultures. SG antibodies should be useful for identifying toxigenic *S. chartarum* isolates as well as the rapid detection of satratoxins in environmental samples from buildings contaminated with this fungus.


## INTRODUCTION

Macrocyclic trichothecenes are a group of mycotoxins produced by *Stachybotrys chartarum* (*S. atra* ) and other fungi (Bata *et al.*, 1985; Harrach *et al.*, 1981; Jarvis *et al.*, 1988; Jarvis and Wang, 1999; Namikoshi *et al.*, 2001) that have potential human health

implications. These trichothecenes are 10 to 100 times more toxic than non-macrocyclic trichothecenes in mouse $LD_{50}$ studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays (Ueno, 1983). *Stachybotrys* grows well on cellulose substrates that have a high moisture content such as wet hay and straw and this fungus's metabolites contribute etiologically to stachybotryotoxicosis in horse and sheep in Central Europe (Bata *et al.*, 1985; Habermehl *et al.*, 1985; Harrach *et al.*, 1983; Harrach *et al.*, 1981; Hintikka, 1978). Outbreaks of *Stachybotrys*-associated human disease have been epidemiologically associated with water-damaged buildings (Dearborn, 1997; Etzel *et al.*, 1998; Johanning *et al.*, 1996). Pulmonary hemorrhage and hemosiderosis in infants have been associated with toxigenic *S. chartarum* contaminated homes (Etzel *et al.*, 1998) and notably, *S. chartarum* strains isolated from these homes produce several macrocyclic trichothecenes including satratoxin G (SG), satratoxin H (SH), isosatratoxin F (iSF), roridin L-2, and trichoverrol B (Jarvis *et al.*, 1998). Recently, spores of this fungus were isolated from the lungs of a child with pulmonary hemorrhage (Elidemir *et al.*, 1999). Although this was the first report of the organism in human tissue, the specific involvement of macrocyclic trichothecenes in the disease was not determined.

Given the potential harmful effects of macrocyclic trichothecenes on human health, it is important to monitor their presence in the environment. Current methods for detection and quantitation of the toxins include thin-layer chromatography, high performance liquid chromatography, and mass spectrometry (Krishnamurthy *et al.*, 1989; Stack and Eppley, 1980; Tuomi *et al.*, 1998). These methods used for macrocyclic trichothecenes, however, necessitate time-consuming extraction, sample clean-up, and/or expensive instrumentation.

A protein translation assay employing luciferase mRNA has been developed to detect trichothecenes in the spores of toxigenic fungi (Yike *et al.*, 1999). This assay is quite sensitive as all trichothecenes in spores inhibit protein synthesis, but this method is not specific for any single trichothecene, and may give false positives in response to other metabolites in a sample. A further limitation of this assay is its expense and requirement for special training and equipment.

Enzyme-linked immunosorbent assay (ELISA) has been widely applied for the detection of mycotoxins because of its high specificity and sensitivity (Abouzied *et al.*, 1993; Azcona-Olivera *et al.*, 1992; Thirumala-Devi *et al.*, 2000). ELISAs have been developed for a variety of food-borne non-macrocyclic trichothecenes (Abouzied *et al.*, 1993; Barna-Vetro *et al.*, 1994; Park and Chu, 1996), however, relatively little effort to date has been placed on the environmentally important macrocyclic trichothecenes. In one exception, an ELISA for the macrocyclic trichothecene, roridin A, was developed using rabbit polyclonal antibodies (Martlbauer *et al.*, 1988). The antibodies were highly specific for roridin A but showed low cross-reactivity against SH (15%) and SG (6.5%), which are two of the major toxins in *Stachybotrys* spores. Therefore, it was desirable and seemed feasible to develop a specific ELISA for the satratoxins. The purpose of this study was (i) to device a chemical strategy for linking SG to the protein carrier bovine serum albumin (BSA), (ii) to produce specific antibodies against the BSA-SG in rabbits, and (iii) to apply these antibodies to the development of a competitive direct enzyme-linked immunosorbent assay (CD-ELISA) for SG as well as other satratoxins. The results suggested that high titer SG antisera could be produced and applied to a CD-ELISA for the rapid detection of SG and other satratoxins.

## MATERIALS AND METHODS

**Materials and Chemicals**. Bovine serum albumin (BSA, fatty acid free grade), 1,3-dicyclohexylcarbodiimide (DCCD), N-hydroxysuccinimide, N,N-dimethylformamide (DMF), complete and incomplete Freund's adjuvants, Tween-20, deoxynivalenol (DON), roridin A (RA), verrucarin A (VA), and verrucarol were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP, ImmunoPure® grade) was obtained from Pierce (Rockford, IL). SG, satratoxin H (SH), and isosatratoxin F (iSF) (Figure 2.1) were prepared from cultures as described by Jarvis *et al.* (Jarvis *et al.*, 1995).

**Synthesis of the bis-hemisuccinate of satratoxin G.** A solution of 4.0 mg (7.3 mmol) of SG, 3.7 mg (36.5 mmol) of succinic anhydride, and 1 mg of N,N-dimethylaminopyridine (DMAP) in 5 mL of chloroform (that had first been passed through a short column of alumina) was refluxed for 1.5 hr. The reaction mixture was cooled, diluted with 5 mL of dichloromethane, and the solution washed successively with 10 mL of 5%(v/v) HCl and 5%(w/v) $NaHCO_3$ to yield, after removal of organic solvent, 5 mg of an amorphous solid that resulted in a single spot on thin layer chromatography (TLC, 1:9 dichloromethane-ethyl acetate) analysis. The product was analyzed by NMR at the University of Maryland. Department of Chemistry and Biochemistry.

**Preparation of satratoxin G conjugates.** SG hemisuccinate was coupled to BSA and HRP. Briefly, 1 mg of SG hemisuccinate was dissolved in 50 µl of DMF and was activated with 1.14 mg of N-hydroxysuccinimide and 4.08 mg of DCCD in 100 µl of dry DMF for 18 h at room temperature. The reaction mixture (150 µl) was added dropwise to 5 mg of BSA or 5 mg of HRP dissolved in 1.0 ml of $NaHCO_3$ (130 mM) and slowly stirred

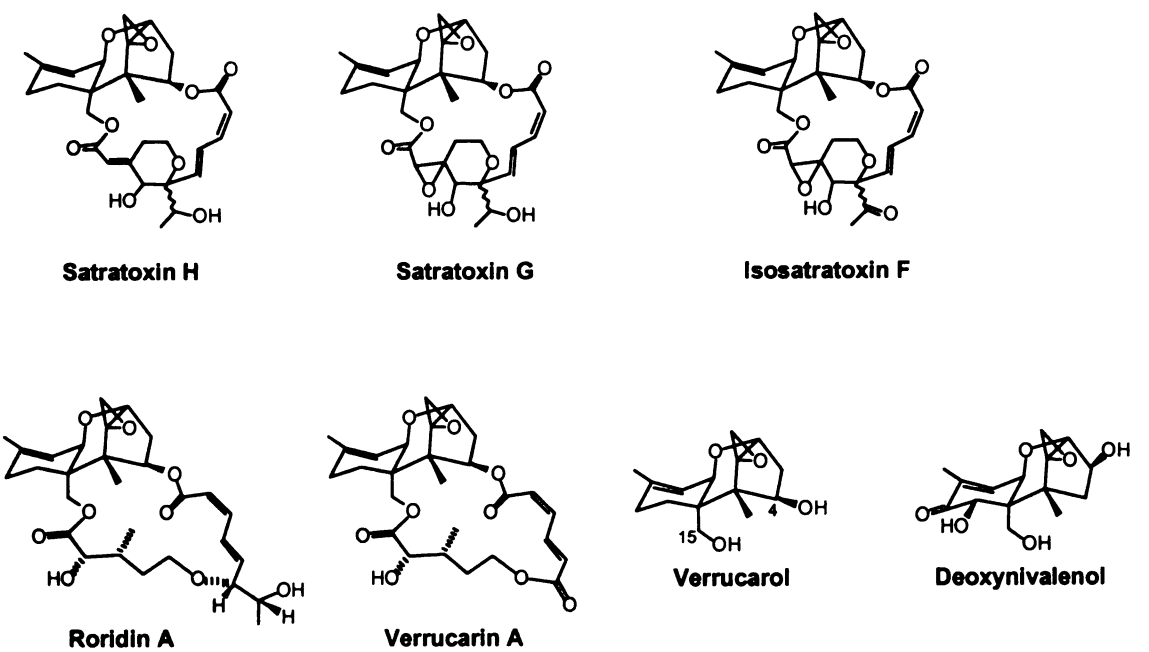


Figure. 2.1. Structures of macrocyclic and selected other trichothecenes

for 2 h at room temperature. The conjugates were dialyzed against 4 liters of phosphate buffered (pH 7.2, 10 mM) saline (PBS) at 4°C with four changes for 3 days. BSA-SG conjugate was stored frozen at - 20°C. HRP-SG conjugate was diluted in an equal volume of glycerol and stored at - 20°C.

**Rabbit immunization.** Three rabbits (Harlan, 6 weeks, female) were injected intradermally with BSA-SG conjugate (375 μg) in 1 ml of PBS-Freund's complete adjuvant (1:1) at 20 to 30 sites on a shaved back area. A booster consisting of BSA-SG conjugate (250 μg) in 1 ml of PBS-Freund's incomplete adjuvant (1:1) was injected subcutaneously at week 18. Blood samples were drawn via marginal ear vein at intervals. Sera were obtained after overnight incubation of blood at 4°C and centrifugation at 3,000 x g for 30 min. For long term storage, immunoglobulins were purified by 50% saturation with ammonium sulfate, lyophilized and stored at - 20°C.

**Antibody titration by direct ELISA.** Wells of polystyrene microtiter plates (Immulon 4 Removawells, Dynex Technologies Inc. Chantilly, VA) were coated with 100 μl of serially diluted serum in PBS (pH 7.2, 10 mM) overnight in a forced air drying oven at 42°C. Plates were washed three times with PBS-Tween 20 (0.02%, v/v). Wells were blocked with 300 μl of 3% (w/v) non-fat dried milk in PBS (NFDM-PBS), covered with parafilm, and incubated for 30-60 min at 37°C. Plates were washed four times with PBS-Tween. Then, 100 μl of HRP-SG conjugate (1:4,000 in NFDM-PBS) was added to each well and incubated at 37°C for 60 min. After seven washes with PBS-Tween, bound peroxidase was determined with 3,3',5,5'-tetramethylbenzidine (TMB) (Fluka Chemika, Buchs, Switzerland) substrate as described previously (Wong *et al.*, 1998). The reaction was

terminated after 10 min incubation at room temperature by adding 100 µl of 6 N sulfuric acid ($H_2SO_4$). The absorbance at 450 nm was measured using $V_{max}$ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

**Competitive direct (CD) ELISA.** Microtiter plates were coated with antiserum diluted 15,000-fold in PBS at 4°C overnight and blocked as described above. Then, 50 µl of standard SG or sample in PBS was simultaneously incubated with 50 µl of HRP-SG (1:2000 in NFDM-PBS) for 1 h at 37°C. Bound peroxidase was determined by adding TMB substrate as described above. The reaction was stopped after 30 min incubation at room temperature and absorbance read.

Cross-reactivity was measured by CD-ELISA as described above. Briefly, microtiter plates were coated with antiserum diluted 15,000-fold in PBS at 4°C overnight. After blocking with NFDM-PBS, 50 µl of SH, iSF, RA, VA, DON, or verrucarol serially diluted in PBS, was simultaneously incubated with HRP-SG conjugate (1:2000 in NFDM-PBS) and analyzed as described above.

Methanol sensitivity was measured by CD-ELISA as described above with some modification. Fifty microliters of standard SG dissolved in PBS containing 0 to 80% methanol was simultaneously incubated with 50 µl of HRP-SG conjugate in antibody coated wells for 1 h at 37°C and analyzed as described above.

Analysis of spore extracts to detect satratoxins in *Stachybotrys* spore, spore extracts from a toxigenic and non-toxigenic isolate, prepared as previously described (Yike *et al.*, 1999) were kindly provided by Drs. I Yike and D. Dearborn (Case Western Reserve University, Cleveland, OH) were dissolved in PBS. Briefly, 50 µl of serially diluted spore

extracts in PBS were simultaneously incubated with 50 µl of HRP-SG conjugate in antibody coated wells for 1 h at 37°C and analyzed as described above.

**Comparative analysis of *S. chartarum* cultures by HPLC and ELISA.**

S. chartarum cultures for this study were kindly provided by Ms. Janet Simpson (NIOSH) DRDS, Morgantown, WV). These cultures were previously collected in Cleveland, OH (Jarvis *et al.*, 1998). The general procedure for growth, extraction and HPLC analysis of rice cultures of *S. chartarum* was altered slightly from a recently published method (Hinkley *et al.*, 2000). Briefly, after 3 wk of growth, 25 ml of 85% (w/v) methanol in water was added to 5g of rice culture and the mixture sonicated for 30 min. The extraction was repeated and the extracts combined and filtered. This procedure tends to preferentially extract the trichothecenes from the culture (Hinkley *et al.*, 2000) and reduce problems with interfering analytes during HPLC analyses. Efficiency of recovery by this extraction protocol (approximately 50%) was assessed using standards and used to correct final estimates in HPLC analyses. For ELISA, 1.5 ml of 80% (w/v) methanol in water was added to 0.5 g of *S. chartarum* rice culture and sonicated for 30 min. Samples were filterd through a 0.45 µm filter; the extraction repeated and the filtrates combined. Extracts were diluted to attain 20% (w/v) methanol in water. Extracts were further diluted serially in 20% (w/v) methanol and subjected to ELISA in which standards were in the same diluent.

## RESULTS AND DISCUSSION

**Derivatization of SG to its bis-hemisuccinate.** Succinic anyhydride was used to convert SG its bis-hemisuccinate. NMR was used to verify the identity of the product as

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satratoxin G hemisuccinate {$^1$H NMR (400 MHz) ($CDCl_3$) d: 0.86 (1 H, s, H-14), 1.00 (3 H, d, J = 7 Hz, H-14'), 1.71 (3 H, s, H-16), 2.0 (4 H, m, H-7 and H-8), 2.0 (1 H, m, H-3b), 2.5 (1 H, m, H-3a), 2.5 (2 H, m, H-4'), 2.97 (2 H, center of AB system, J = 4 Hz, H-13), 3.58 (1 H, d, J = 5 Hz, H-11), 3.48 (1 H, s, H-2'), 3.84 (1 H, d, J = 5 Hz, H-2), 3.9 (2 H, m, H-5'), 4.21 (2 H, center of AB system, J = 12 Hz, H-15), 4.83 (1 H, s, H-12'), 5.32 (1 H, q, J = 7 Hz, H-13'), 5.42 (1 H, br d, J = 5 Hz, H-10), 5.84 (1 H, d, J= 16.5 Hz, H-7'), 5.96 (1 H, d, J = 10.5 Hz, H-10'), 6.0 (1 H, m, H-4), 6.68 (1 H, dd, J = 8.0 and 10.5 Hz, H-9'), and 6.97 (1 H, dd, J = 8.0 and 16.5 Hz, H-8'), $CH_2CH_2$'s of succinate: 2-7 (8 H, m); HREI-MS *m/z* 774.2640 $M^+$ ($C_{37}H_{36}O_{16}$ req. 774.2629)}.

**Production of antibodies against satratoxin G.** SG bis-hemisuccinate was conjugated to BSA for use as an immunogen and to HRP as an ELISA marker ligand. A direct ELISA was applied to monitor titers of SG antiserum whereby sera from three SG-BSA immunized rabbits was coated onto a microtiter plate and bound antibodies subsequently detected with HRP-SG conjugate. The titer was arbitrarily designated as the serum dilution that showed visually distinct color from pre-immune serum control at the same dilution. Titration curves for the antisera at week 6 are shown in Figure 2.2. Titers of the antiserum was 31250 whereas pre-immune serum control had negligible absorbance.

The titers from the three rabbits over 42 weeks are measured: the highest titer was 31250 at week 6 through week 10 and the titers dropped to 6250 at week 17. A booster injection to two rabbits at week 18 increased the titer of SG antiserum to 31250 from 27 to 31 weeks and the titers decreased slightly to 6250 for the remaining weeks. One rabbit (#2) was not given a booster injection due to granulomas on its back. The granuloma was self-

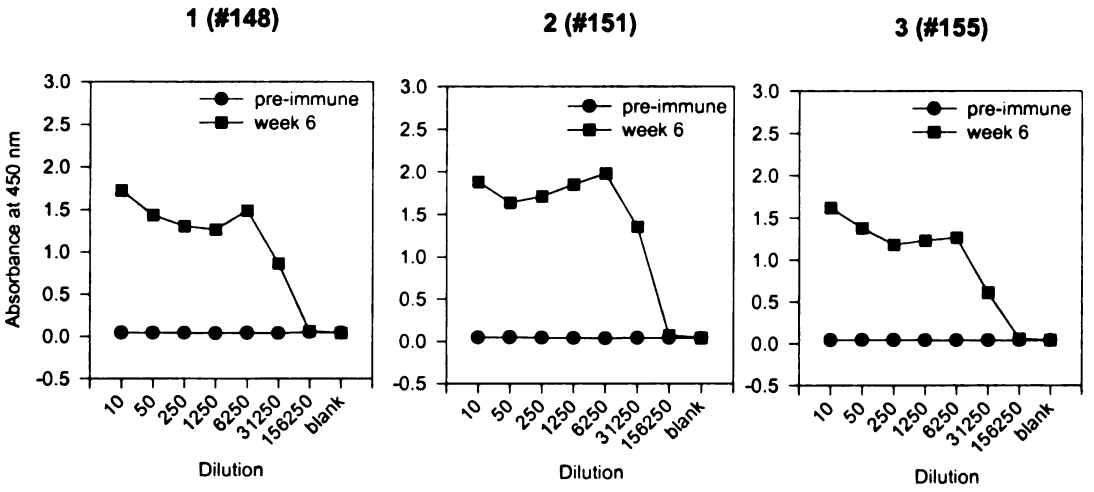


Figure 2.2. Direct ELISA titer determination of rabbit SG antibodies. Serially diluted antisera were coated onto microtiter wells and specific binding determined by incubation with HRG-SG. Three rabbits (#148, #151, and #155) were immunized with SG-BSA. Collected sera were subjected to direct ELISA for titer determination.

resolving but titers remained at 6250.

**Competitive direct ELISA.** A CD-ELISA was performed by simultaneously incubating standard SG and HRP-SG conjugate over antibodies coated on microtiter plates. Binding of HRP-SG conjugate could be inhibited by free SG and the resultant ELISA was highly sensitive for SG (Figure 2.3). The detection limit for the curve employing rabbit 2 serum was 0.1 ng/ml whereas it was 0.5 ng/ml for the other two rabbits. Ammonium sulfate precipitated immunoglobulin from rabbit 2 was therefore chosen to further characterize the properties of the antibodies.

**Specificity of satratoxin G antibody.** The specificity of the rabbit SG antiserum was tested by using other macrocyclic trichothecenes, verrucarol, and DON, a non-macrocyclic type B trichothecene, as competitors in the CD-ELISA (Figure 2.4). Different macrocyclic trichothecenes exhibited cross-reactivity while verrucarol and DON did not cross react with the antiserum. Concentrations at 50% inhibition ($ID_{50}$) of HRP-SG conjugate binding to antibodies on microtiter plates were 1.3, 5.4, 3.2, 25.1, and 52.5 ng/ml for SG, SH, iSF, RA, and VA, respectively. More than 1 μg/ml of verrucarol were required to inhibit 50% of HRP-SG conjugate binding. DON at 1 μg/ml did not inhibit binding of the SG-HRP.

The side chains in macrocyclic trichothecenes might play an important role in determining specificity of antibodies produced. Macrocyclic trichothecenes are characterized by a cyclic diester or triester ring connecting C-4 to C-15 of trichothecene structure (Jarvis *et al.*, 1995). The bis-hemisuccinate of SG facilitated conjugation of SG through the hydroxyls associated with the six membered ring component (cyclopentanone) in the cyclic structure (Figure 2.1). Thus, the resultant immunodominant epitopes for SG would be those

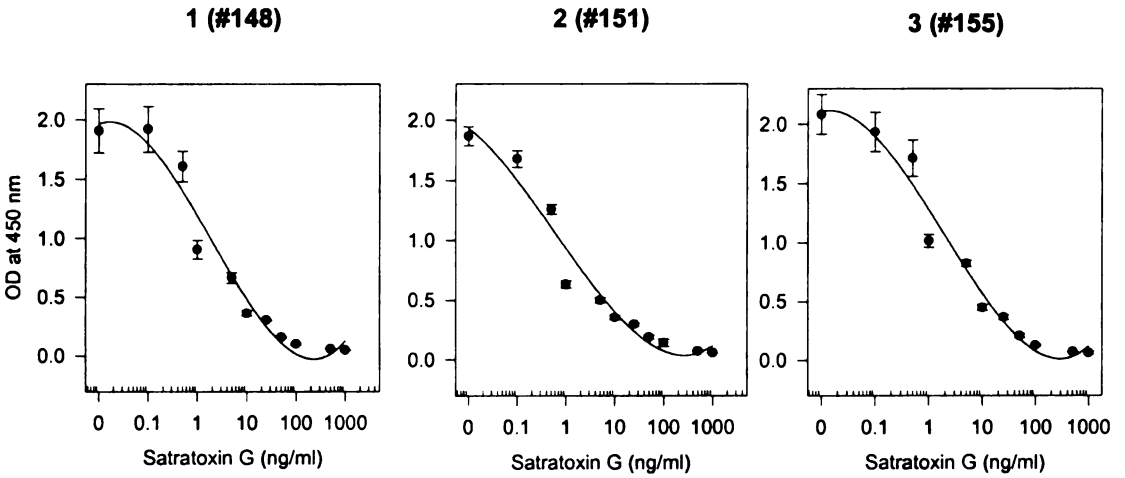


Figure 2.3. Standard curve of SG CD ELISA. Microtiter were coated with SG antibodies from rabbits (#148, #151, and #155) and then simultaneously incubated with Standard SG and HRP-SG. Each point represents the mean ± SD for three replicates.

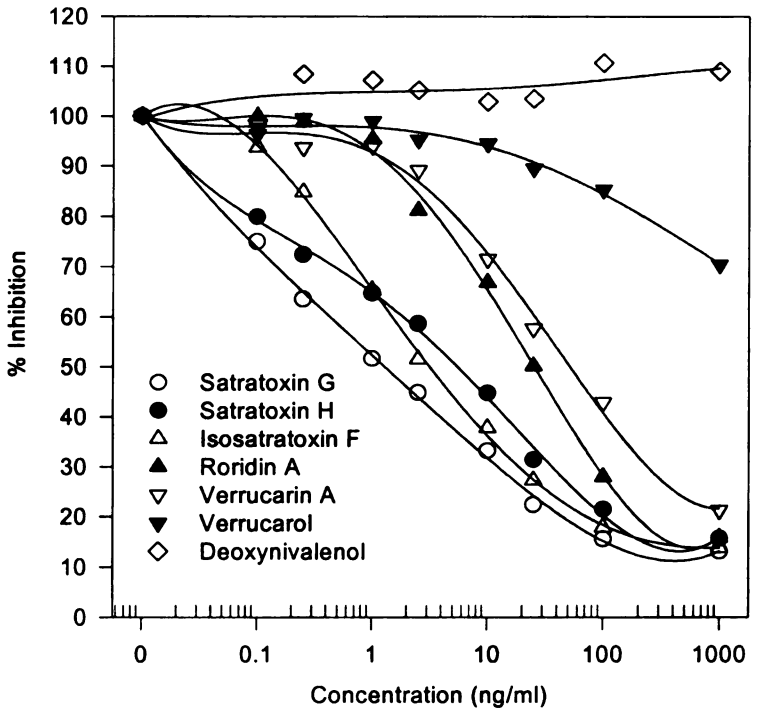


Figure 2.4. Cross-reactivity of SG Rabbit 2 antiserum toward macrocyclic trichothecenes, verrucarol, and deoxynivalenol. Wells were coated with SG antibody and incubated with HRP-SG in the presence of different toxins.

moieties projecting distally from these conjugation sites. The presence of the cyclopentanone in satratoxin H and isosatratoxin F may account for the relatively high cross-reactivity, approximately 24 and 41 %, respectively (based on the $ID_{50}$s), relative to SG. Roridin A and verrucarin A, which do not possess the six member ring structure, exhibited much less cross-reactivity, 5.2 and 2.5 %, respectively, compared to SG. The inability of verrucarol, a trichothecene that can be generated by base hydrolysis of macrocyclic trichothecenes (Figure 2.1), or deoxynivalenol, a non macrocyclic trichothecene, to compete in the ELISA indicated that there is an absolute requirement for a macrocyclic ring structure for antibody binding.

The observed cross reactivity raises the issue that absolute quantification of SG from samples might not be achievable. Nevertheless, the capacity of these antibodies to bind to other satratoxins or other macrocyclic trichothecenes could be beneficial in screening environmental samples for satratoxins and other macrocyclic trichothecenes as well as in toxigenicity determinations for *Stachybotrys* or *Myrothecium* isolates which potentially produce different macrocyclic trichothecenes (Bata *et al.*, 1985; Harrach *et al.*, 1981; Jarvis *et al.*, 1988; Jarvis and Wang, 1999; Namikoshi *et al.*, 2001). In such determinations, macrocyclic trichothecenes can be estimated semiquantitatively as "SG equivalents" when SG is used as the standard. When desired, the antibodies can be used to identify specific macrocyclic trichothecenes profiles using a chromatographic step such as immunoaffinity chromatography in conjunction with HPLC or using the HPTLC ELISAGRAM described previously by our laboratory (Pestka, 1991).

**Effect of methanol in CD-ELISA.** Methanol extraction of toxic components from

*S. chartarum* has been applied for the detection of macrocyclic trichothecenes (Sorenson *et al.*, 1987; Stack and Eppley, 1980) and for the removal of toxic components to study their effects on pulmonary inflammation (Rao *et al.*, 2000). Methanol might also be used in procedures for extracting environmental particulate or swab samples for satratoxins. Therefore, the effects of using different methanol contents in the assay diluent on the CD-ELISA was assessed. The presence of 20% methanol in the final CD-ELISA slightly affect the sensitivity of the method (Figure 2.5); however, higher methanol concentrations either depressed assay sensitivity (30%) or completely suppressed the ELISA. The stability of these antibodies in 20% methanol should facilitate the detection in extracts of fungal isolates and in environmental samples

**Application of CD ELISA to detect satratoxins from *Stachybotrys* spore extracts.** Two *S. chartarum* spore extracts from isolates obtained in a case control study (Yike *et al.*, 1999) were tested for satratoxin content. Previously, these extracts were estimated by protein translation inhibition assay to contain 670 fg SG equivalents/spore (Strain 58-17) and 80 fg SG equivalents/spore (strain 58-06) (I.Yike, personal comunication). Using ELISA, the 58-17 extract was found to contain 980 fg SG equivalent per spore. The other extract, 58-06, had 0.05 fg SG equivalent per spore. The reason for the differences between the two assays may relate to the presence of other trichothecenes in the extract that interfere in the translational assay of SG or enhance it. Nevertheless, these qualitative results suggest that the ELISA might be readily used to discriminate toxigenic from weakly toxigenic or non-toxigenic strains of *S. chartarum*.

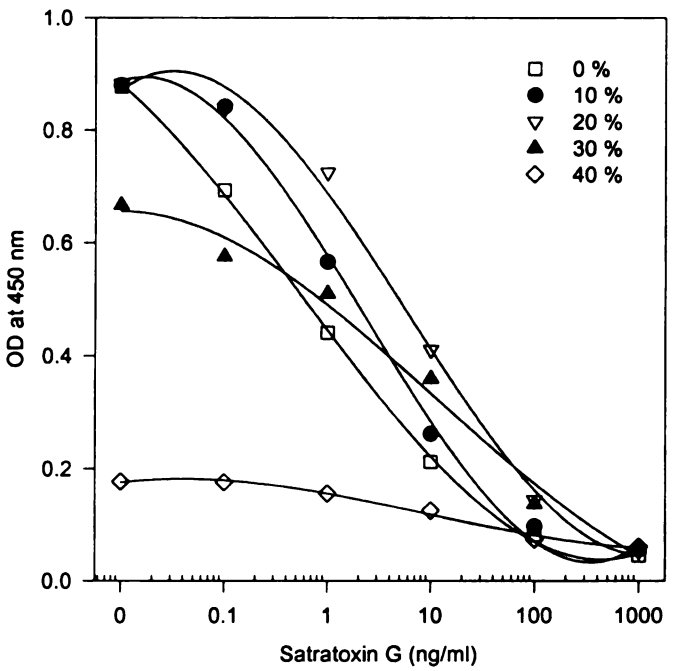


Figure 2.5. Effect of methanol on binding of HRP-SG to SG antiserum. SG antiserum was coated onto microwells and incubated with HRP-SG in the presence of methanol at concentrations ranging from 0 to 40%.

**Comparison of HPLC to CD ELISA in analysis of satratoxins in *S. chartarum* rice cultures.** Three toxigenic and three atoxigenic *S. chartarum* cultures isolated from an epidemiologic study (Jarvis *et al.*, 1998) were grown of rice for 3 wk and subjected to analysis by HPLC and CD ELISA (Table 2.1). The CD-ELISA correlated qualitatively with HPLC recovery and identified the three highly toxigenic cultures from the atoxigenic cultures. ELISA estimates compared to HPLC estimates were approximately identical for one sample (22-11), one half for a second sample (22-03) and two times higher for a third sample (22-08). The reason for these differences might be attributed to subsample variability arising from non-uniformity of culture growth and toxin production. Differences might also be associated with the fact the ELISA measured satratoxin equivalents which are affected by differential antibody cross reactivity for various macrocyclics cover, whereas HPLC measured total macrocylic trichothecenes. The reason for the slight positive ELISA responses (1-9 μg/g) in the three cultures that do not produce macrocyclics as detectable by HPLC may relate to the cultures capacity to produce the non-macrocyclic verrucarol which reacts weakly in the ELISA. Taken together, these data suggest that culture or environmental samples can be analyzed for satratoxins by ELISA after only a methanol-water extraction without any of the laborious clean-up steps or instrumental requirements associated with HPLC.

## CONCLUSIONS

In summary, high antibody titers to SG were readily achieved upon immunization of rabbits with SG bis-hemisuccinate conjugated to BSA. The antibodies produced could detect

**Table 2.1. Comparison of HPLC and CD-ELISA analyses of *S. chartanum* rice cultures[a].**

| Sample | HPLC[b] Total macrocyclic trichothecenes (μg/g) | CD-ELISA[c] Satratoxin equivalents (μg/g) |
|---|---|---|
| 22-03 | 680 | 303 ±140 |
| 22-04 | 0 | 0.8 ± 0.3 |
| 22-07 | 0 | 9.0 ± 0.2 |
| 22-08 | 180 | 374 ± 91 |
| 22-10 | 0 | 1.20 ± 0.1 |
| 22-11 | 174 | 177 ± 26 |

a. Cultures were isolated from homes in Cleveland during an epidemiologic study of *S. chartarum*-associated pulmonary hemorrhage.
b. Standard error for this method was typically 10 to 20% (12).
c. Mean of two separate 0.5 g subsamples.

free SG using a CD-ELISA with a range of detection from 0.1 to 100 ng/ml. The observed antibody cross-reactivity may facilitate simultaneous detection of other satratoxins and to a lesser extent, other macrocyclic trichothecenes. Methanol content up to 20% in samples did not affect the immunoassay and this stability expands applicability of the antibodies. As has been implemented on a widescale for foodborne mycotoxins (Abouzied *et al.*, 1993; Barna-Vetro *et al.*, 1994; Park and Chu, 1996), potential applications of these CD-ELISA would be on-site rapid detection of satratoxins in culture, wallboard, floor and air samples from *Stachybotrys*-contaminated buildings. Additional analytical studies on *Stachybotrys* isolates and environmental samples in which assessment of satratoxins by ELISA are compared with existing chemical methods such as HPLC would be helpful in this regard. Finally, since *Stachybotrys* spores can be inhaled in lungs, these antibodies might also be applied to the localization of toxigenic spores and released toxin in lung tissue.

## ACKNOWLEDGMENT

This work was supported by EPA Contract 00-5674 NAEX and NIH grant ES-03358. We thank Drs. I. Yike and D. Dearborn (Case Western Reserve University, Cleveland, OH) for providing spore extracts and related analytical data.

# CHAPTER III

# MODULATION OF LIPOPOLYSACCHARIDE-INDUCED PROINFLAMMATORY CYTOKINE PRODUCTION BY SATRATOXINS AND OTHER MACROCYCLIC TRICHOTHECENES IN THE MURINE MACROPHAGE [1]

[1] This work has been accepted for publication in a similar form with authors Chung, Y.J., Jarvis, B.B., and Pestka, J.J. in Journal of Toxicology and Environmental Health.

## ABSTRACT

The satratoxins and other macrocyclic trichothecene mycotoxins, are produced by *Stachybotrys*, a mold that is often found in water-damaged dwellings and office buildings. To test the potential immunomodulatory effects of these mycotoxins, RAW 264.7 murine macrophage cells were treated with various concentrations of satratoxin G (SG), isosatratoxin F (iSF), satratoxin H (SH), roridin A (RA), and verrucarin A (VA) for 48 hr in the presence or absence of suboptimal concentration of lipopolysaccharide (LPS, 50 ng/ml). Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production were assayed by ELISA. In LPS-stimulated cultures, TNF-α supernatant concentrations were significantly increased in the presence of 2.5, 2.5, and 1 ng/ml of SG, SH, and RA, respectively, whereas IL-6 concentrations were not affected by these macrocyclic trichothecenes. When cells treated with LPS and SG were evaluated by real time PCR, SG at 2.5 ng/ml increased TNF-α mRNA at 24, 36, and 48 hr compared to control cells. At higher concentrations, cytokine production and cell viability were markedly impaired in LPS-stimulated cells. Without LPS stimulation, neither TNF-α nor IL-6 was induced. These results indicate that low concentrations of macrocyclic trichothecenes superinduce expression of TNF-α whereas higher concentrations of these toxins are cytotoxic and concurrently reduce cytokine production. The capacity of satratoxins and other macrocyclic trichothecenes to alter cytokine production may play an etiologic role in outbreaks of *Stachybotrys*-associated human illnesses.

## INTRODUCTION

The trichothecenes are a family of sesquiterpenoid mycotoxins which include some of the most potent protein synthesis inhibitors known (Ueno, 1983). More than 180 trichothecenes have been identified and are categorized into four types based on functional groups attached to the trichothecene ring (Grove 1993;1996). Mechanisms of toxicity of foodborne Type A (e.g., T-2 toxin) and Type B (e.g., vomitoxin or deoxynivalenol) trichothecenes have been studied extensively (Bondy and Pestka, 2000). On the other hand, toxicological studies on environmentally encountered Type D (e.g., satratoxins, verrucarin, and roridin) macrocyclic trichothecenes are limited.

Macrocyclic trichothecenes are characterized by a cyclic diester or triester ring which connects C-4 to C-15 of the trichothecene structure (Figure 3.1). These trichothecenes are 10 to 100 times more toxic than Type A and Type B trichothecenes in mouse $LD_{50}$ studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays (Ueno, 1983). Several potentially important macrocyclic trichothecenes are produced by *Stachybotrys atra* (or also known as *S. chartarum* ). This mold grows well on cellulose substrates with a high moisture content such as wet hay and straw (Bata *et al.*, 1985; Harrach *et al.*, 1981). Macrocyclic trichothecenes have been etiologically associated with stachybotryotoxicosis in horse and sheep in Central Europe (Bata *et al.* 1985; Harrach *et al.*, 1981; 1983; Hintikka 1978). Clinical signs of this disease include leukopenia, thrombocytopenia, hemorrhage, arrhythmic heartbeat, and death (Forgacs, 1972).

Humans can also be exposed to *Stachybotrys* and macrocyclic trichothecenes via the environment. Outbreaks of *Stachybotrys*-associated human disease have been

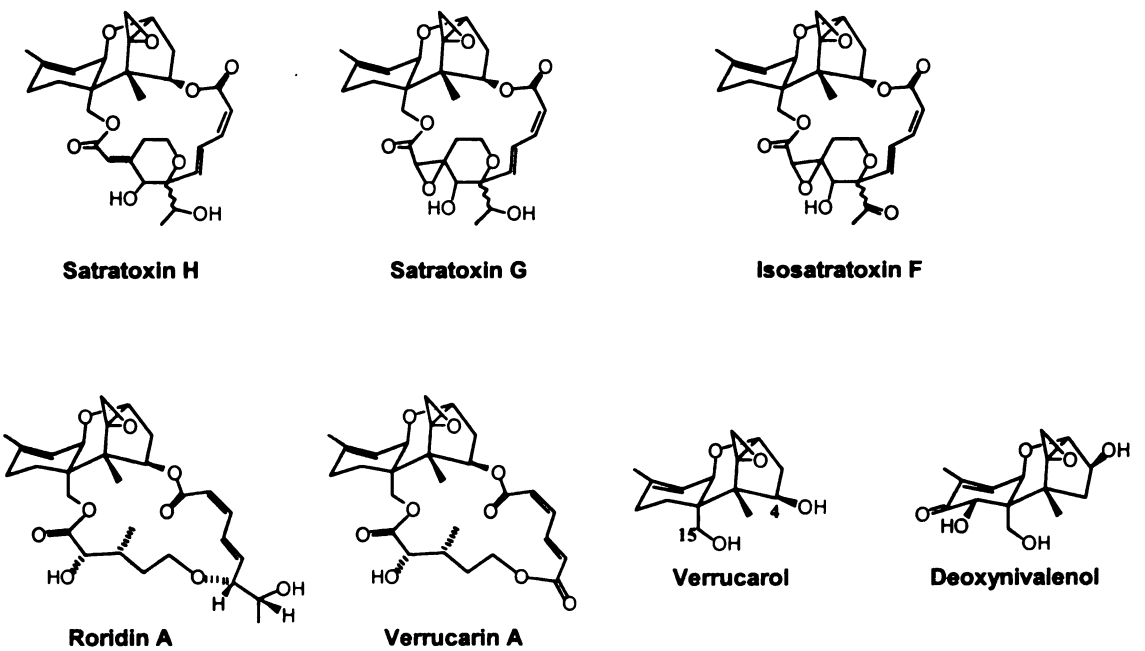


Figure 3.1. Structures of macrocyclic trichothecenes

epidemiologically associated with water-damaged buildings (Dearborn, 1997; Etzel *et al.*,1998; Johanning *et al.*, 1996). Symptoms reported by exposed individuals include recurrent cough, irritation of the eyes and skin, mucous membrane disorders, headache, and fatigue, which are also characteristics of indoor air illnesses. Notably, pulmonary hemorrhage and hemosiderosis in infants have been associated with toxigenic *S. atra* contaminated homes (Etzel *et al.*, 1998). Recently, spores of this fungus were isolated from the lungs of a child with pulmonary hemorrhage (Elidemir *et al.*, 1999). Although this was the first report of the fungal organism in human tissue, the specific involvement of macrocyclic trichothecenes was not determined.

Leukocytes are particularly sensitive to trichothecenes (Bondy and Pestka 2000). Depending on dose and duration of exposure, these toxins can be immunostimulatory as well as immunosuppressive. Trichothecene-induced immunostimulatory effects include increased cytokine production and upregulation of immunoglobulin (Ig) production. Immunosuppressive effects of trichothecenes include apoptosis and impairment of humoral immunity. To date, most work on the immunostimulatory effects of trichothecenes has focused on the Type A and Type B subgroups. However, macrocyclic trichothecenes have been shown to superinduce IL-2 production in EL-4 thymoma cells as well as induce and impair blastogenesis of lymphocytes in the presence of mitogen (Hughes *et al.*, 1990; Pestka and Forsell, 1988). Given the potential importance of macrocyclic trichothecenes to human health, it is important to better understand their effects on immune function.

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are proinflammatory cytokines which are secreted by activated macrophages. Although these cytokines are

important in host defense, overproduction of the cytokines may be important in the pathogenesis of several inflammatory diseases such as septicemia and fatal circulatory shock (Chantry and Feldman, 1990; Vogel and Hogan, 1990). These cytokines are also highly expressed by inflammatory cells in the airways (Noah *et al.*, 1995) and can potentiate allergic inflammation (Gosset *et al.*, 1993). The purpose of this investigation was to test a hypothesis that macrocyclic trichothecenes can alter TNF-α and IL-6 production and viability in a murine macrophage model.

## MATERIALS AND METHODS

**Chemicals**. All cell culture reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Satratoxin G (SG), satratoxin H (SH), and isosatratoxin F(iSF) were prepared from cultures as described by Jarvis *et al.* (1995). All toxins were dissolved in ethanol, dried at a stock concentration of 10 μg, and stored at 4°C for long term storage. Stock solutions were dissolved in ethanol and further diluted to 200 ng/ml in Dulbecco's Modified Eagles Medium (DMEM, Sigma) with a final ethanol concentration of < 0.1% of the diluent. Lipopolysaccharide (LPS) from *Salmonella typhimurium* was dissolved in DMEM at 250 ng/μl and stored at -20°C.

**Cell Culture and ELISA**. The murine macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in a 7% $CO_2$ humidified incubator in DMEM supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD), 1% (v/v) National Cancer Institute Medium NCTC 135 supplement, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml

streptomycin. For toxin treatment, RAW 264.7 cells ($2.5 \times 10^5$ cells/ml) were cultured for 24 hr in flat-bottomed 48-well tissue culture plates (Fisher Scientific Co., Corning, NV) with each well containing 500 µl of cell suspension. Then, supernatants were removed and fresh medium containing various concentrations (0 to 10 ng/ml) of macrocyclic trichothecenes and LPS (50 ng/ml) was added to culture. Supernatants were collected at various time points and assayed for TNF-α and IL-6 by enzyme-linked immunosorbent assay (ELISA) (Wong *et al.*, 1998).

**MTT Assay**. The MTT assay was performed as described by Marin *et al.* (1996). Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide) (Sigma) was dissolved in 0.01 M phosphate-buffer saline (PBS) at pH 7.4 with a concentration of 5 mg/ml. Filter sterilized MTT reagent was stored in the dark at 4°C no longer than 1 wk. RAW 264.7 cells ($2.5 \times 10^5$ cells/ml) were cultured for 24 hr in flat-bottomed 96-well tissue culture plates (Fisher Scientific) with each well containing 200 µl of cell suspension. Then, supernatants were removed and fresh medium containing macrocyclic trichothecenes and LPS was added to the culture. After 48 hr incubation, MTT reagent (20 µl/well) was added to the 96 well plate and incubated for an additional 3 hr in a $CO_2$ incubator at 37°C. Then, plates were centrifuged at 450 x g for 15 min and supernatants were carefully removed from wells by slow aspiration through a 28-gauge needle. Dimethyl sulfoxide (DMSO) (150 µl/well) was added to dissolve purple formazan crystals. After complete solubilization of the crystals by shaking, absorbance was measured on a microplate reader (Molecular Devices, Menlo Park, CA) using 570 nm as the test wavelength and 690 nm as the reference wave length.

**Real Time PCR**. Total RNA was extracted from RAW 264.7 cells by the method of Chomczynski and Sacchi (1987) using Trizol™ reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA content was determined spectrophotometrically at 260 nm.

Total RNA was analyzed to detect TNF-α mRNA level using an ABI Prism™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The levels of 18S rRNA were also measured as an indigenous control. Specific primers and probes for TNF-α and 18S were obtained from PE Appllied Biosystems. Concentrations used for primers and probes were 200 and 100 nM, respectively, in each reaction. Sixteen μl of master mix comprising 20 x TNF-α primers and probe, 20 x 18S primers and probe, predeveloped assay reagents for PCR and RT (PE Applied Biosystems) was added to 9 μl of samples containing 100 ng total RNA. Reverse transcriptase and polymerase reactions were performed in the same tubes according to manufacturer's recommendations. Relative quantitatiies of TNF-α mRNA and 18S rRNA were calculated using the comparative threshold cycle number for each sample fitted to standard curves for TNF-α mRNA and 18S rRNA, respectively, generated according to guideline (ABI Prism 7700 Use Bulletin #2, PE Applied Biosystems). Expression levels for TNF-α mRNA in each sample were normalized to 18S rRNA.

**Statistical Analysis**. One-way analysis of variance (ANOVA) using Dunnett's test for parametric data or Kruskal-Wallis one-way ANOVA for non parametric data were performed with Sigma Statistical Analysis System (Jandel Scientific, San Rafael, CA). A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

**Effects of macrocyclic trichothecenes on TNF-α and IL-6 production**. RAW 264.7 cells were exposed to macrocyclic trichothecenes in the presence of a sub-optimal concentration of LPS (50 ng/ml). After 48 hr, TNF-α levels in supernatant were significantly increased by SG, SH, and roridin A (RA) at 2.5, 2.5, and 1 ng/ml, respectively, while iSF and verrucarin A (VA) did not significantly induce TNF-α production at similar concentrations (Figure 3 2). At 12 and 24 hr with the same concentrations or lower of each macrocyclic trichothecene, TNF-α production in toxin-treated cells was not different from LPS-treated cells (data not shown) although TNF-α level gradually increased over time. At 10 ng/ml, all macrocyclic trichothecenes were cytotoxic, resulting in significant decrease of TNF-α production in LPS-treated cells. At 10 ng/ml, all macrocyclic trichothecenes significantly suppressed TNF-α production in LPS-treated cells. In the absence of LPS stimulation, TNF-α production was not induced in macrophages by macrocyclic trichothecenes at 12, 24, and 48 hr (data not shown).

The effect of macrocyclic trichothecenes on TNF-α production was further related to TNF-α gene expression. SG was chosen as a representative of macrocyclic trichothecenes for mRNA expression study. RAW 264.7 cells were exposed to 2.5 ng/ml of SG in the presence of LPS (50 ng/ml) and levels of TNF-α mRNA and 18S rRNA were assayed by real time-PCR. After 24, 36, and 48 hr incubation, relative expression of TNF-α mRNA in SG (2.5 ng/ml)-treated cells were higher than that of LPS-treated cells (Figure 3.3). These results suggest that the increased TNF-α production was due, in part, to increased TNF-α mRNA

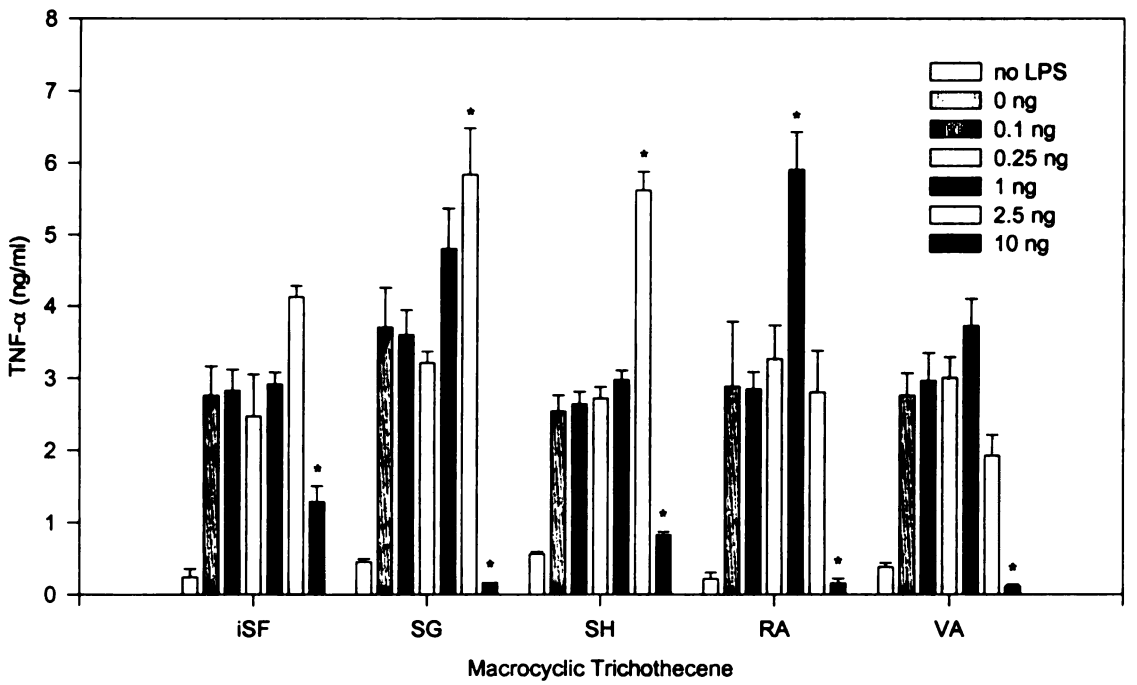


Figure 3.2. Effect of macrocyclic trichothecenes on TNF-α production in RAW 264.7 cells. Cells (2.5 x $10^5$ cells/ml) were cultured for 24 hr and then medium was replaced with medium containing various concentrations of macrocyclic trichothecene in the presence of LPS (50 ng/ml) and incubated for 48 hr. Supernatants from cell cultures were subjected to TNF-α assay by ELISA. Data are mean ± SE of triplicate cultures. Bars marked with an asterisk differ significantly from values of no toxin (0 ng/ml) treatment (* $p < 0.05$). Results are representative of three separate experiments. iSF stands for isosatratoxin F, SG satratoxin G, SH satratoxin H, RA roridin A, and VA verrucarin A.

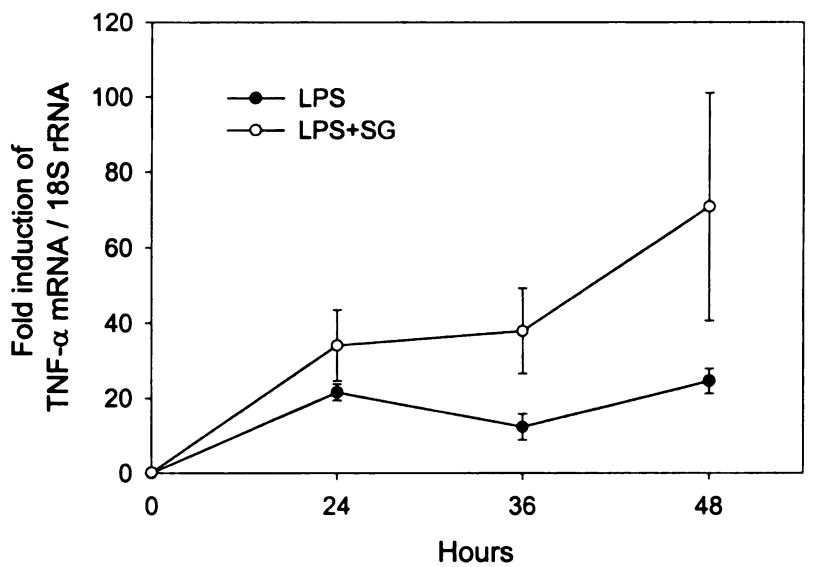


Figure 3.3. Effect of satratoxin G (SG) on TNF-α mRNA level in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were incubated with LPS (50 ng/ml) and LPS +SG (2.5 ng/ml) for the indicated time periods. After isolation of total RNA, real-time PCR was performed for TNF-α mRNA and 18S rRNA. The results were expressed in relative fold induction to non-stimulated cells after normalization against 18S rRNA. Values are given as mean ± S.E.M. of duplicate samples. Results are representative of two separate experiments.

expression in toxin-treated cells. Another important cytokine involved in regulation of inflammation, IL-6, was assayed in this system. In LPS- treated cells, IL-6 production was significantly impaired by 10 ng/ml iSF, SG, and SH, and by 2.5 ng/ml RA and VA at 48 hr (Figure 3.4). At 12 and 24 hr, the same concentrations of each macrocyclic trichothecene inhibited IL-6 production in LPS-treated cells (data not shown). Exposure to lower doses of macrocyclic trichothecenes did not alter IL-6 production in LPS-stimulated cells (Figure 3.4). In the absence of LPS stimulation, IL-6 production was not induced in the macrophage cells by macrocyclic trichothecenes at 12, 24, and 48 hr (data not shown).

**Effect of macrocyclic trichothecenes on MTT response**. The MTT assay was also performed because cytotoxicity might also affect cytokine production. At 48 hr, viability of RAW 264.7 cells treated with macrocyclic trichothecenes with stimulation by LPS was significantly reduced at 10 ng/ml iSF, SG, and SH, at 2.5 ng/ml RA, and 1 ng/ml VA (Figure 3.5) whereas viability was not affected by the toxin concentrations that induced TNF-α production. These data suggest that proliferation and viability of the cells were impaired at the same toxin concentrations that impaired TNF-α production.

## DISCUSSION

Proinflammatory cytokines are key mediators in regulating immune and inflammation responses to microbes, toxins, trauma, and ischemia (Dinarello, 2000). Cytokine production in cloned cell lines has been used as an endpoint to assess key modulatory effects of toxicants on immune function. The results presented herein indicate that low levels of macrocyclic trichothecenes superinduced TNF-α production in a murine macrophage model whereas high

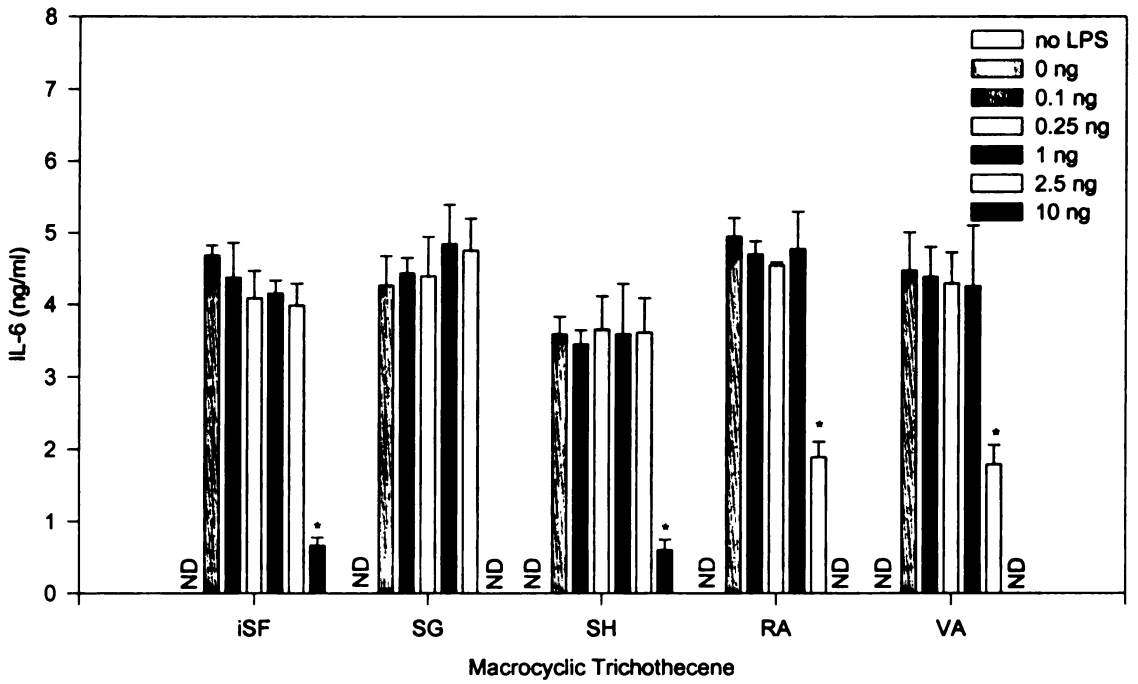


Figure 3.4. Effect of macrocyclic trichothecenes on IL-6 production in RAW 264.7 cells. Cells (2.5 x $10^5$ cells/ml) were cultured for 24 hr and medium was replaced with medium containing various concentrations of macrocyclic trichothecene in the presence of LPS (50 ng/ml) and incubated for 48 hr. Supernatants from cell cultures were subjected to IL-6 assay by ELISA. Data are mean ± SE of triplicate cultures. Bars marked with an asterisk differ significantly from values of no toxin (0 ng/ml) treatment (* $p < 0.05$). ND; not detectable. Results are representative of three separate experiments. Abbreviations are identified in Fig.3.2 legend.

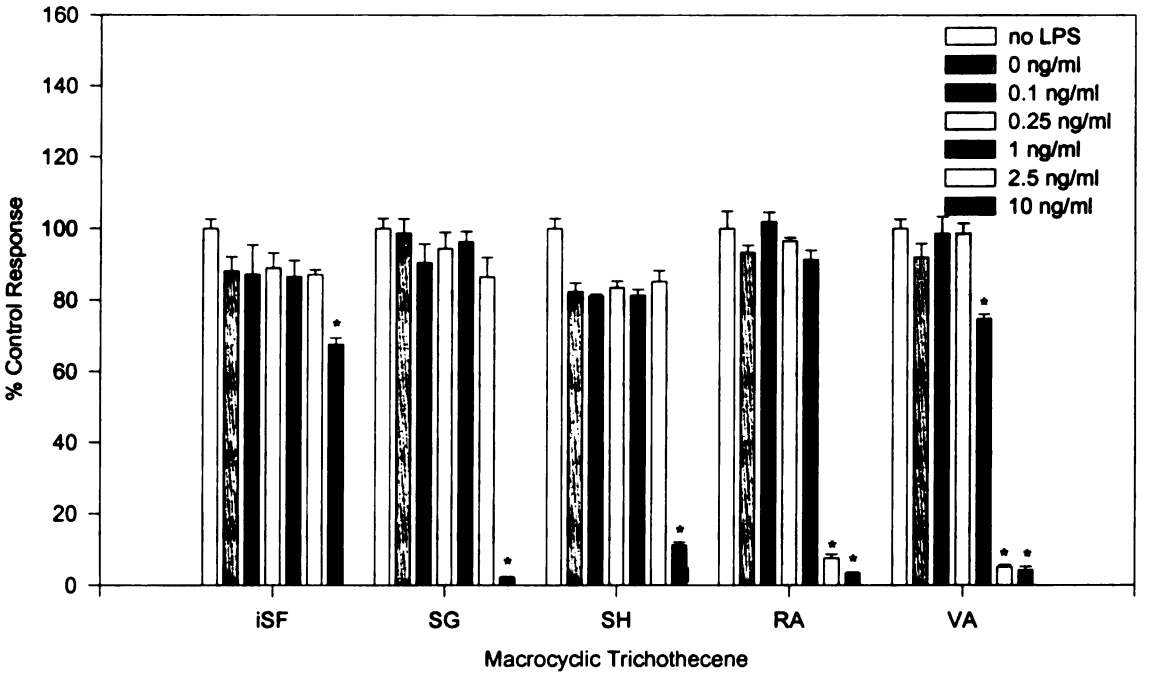


Figure 3.5. Effect of macrocyclic trichothecene on viability of RAW 264.7 cells by MTT assay. Cells (2.5 $\times 10^5$ cells/ml) were cultured for 24 hr in 96-well tissue culture plates containing 200 µl of cell suspension. Then, supernatants were removed and fresh medium containing macrocyclic trichothecenes and LPS was added to the culture and incubated for 48 hr. Then, MTT assays were performed as written in Materials and Methods. Data are mean ± SD of triplicate cultures. Bars marked with an asterisk differ significantly from values of no toxin (0 ng/ml) treatment (* $p < 0.05$). Results are representative of two separate experiments. Abbreviations are identified in Fig.3.2 legend.

concentrations decreased both TNF-α and IL-6 production due to their cytotoxicity.

The concentrations of macrocyclic trichothecenes required for superinduction of TNF-α were approximately 100 times less than that found for deoxynivalenol(vomitoxin) in the same cell line (Wong *et al.*, 1998). Molar concentrations of macrocyclic trichothecenes required to significantly superinduce TNF-α were 1.8 - 4.6 nM (1-2.5 ng/ml) whereas 338 - 845 nM (100-250 ng/ml) of deoxynivalenol is required for the same effect. Similar macrocyclic trichothecene concentrations superinduce IL-2 secretion in EL-4 thymoma cells (Lee *et al.*, 1999). Remarkable sensitivity of lymphocytes to macrocyclic trichothecenes, roridin A, and verrucarin A, has been previously observed in mitogen-induced blastogenesis (Hughes *et al.*, 1990; Pestka and Forsell, 1988). The potency of macrocyclic trichothecenes might be due to their extremely high ribosome binding affinity as compared to affinities of other trichothecenes (Cannon *et al.*, 1976).

In this study, sub-optimal concentration of LPS (50 ng/ml) was chosen to investigate maximal effect of macrocyclic trichothecenes for cytokine production in less stimulated cells. With less activated cells, effects of the toxins on cytokine production can be more readily discerned (Sugita-Konishi and Pestka, 2001). The observation that TNF-α and IL-6 production was impaired by high concentrations of the macrocyclic trichothecenes may relate to cytotoxicity. Macrocyclic trichothecenes (≥2.5 ng/ml) significantly decreased MTT response in LPS-stimulated RAW 264.7 cells. Similar results have been reported in EL-4 T cells (Lee *et al.*, 1999). Impaired proliferation and cytotoxicity by these toxins may result from their capacity to induce apoptosis (Yang *et al.*, 2000). Interestingly, toxin concentrations that superinduced cytokine production produced slight (10~20%) inhibition

of MTT response, suggesting that the capacity of TNF-α production per cell might be increased. This observation is supported by similar findings in $CD4^+$ T cells that partially cytotoxic concentrations of deoxynivalenol can increase IL-2 secretion per cell (Ouyang *et al.*, 1996a).

Although specific mechanisms for increased TNF-α transcripts upon exposure to macrocyclic trichothecenes are unknown, it may relate to the capacity of the toxins to inhibit translation. Superinduction is modulated in part via a repressive system that mediates degradation of mRNA (Roger *et al.*, 1998). Degradation of mRNA including TNF-α transcripts is facilitated in genes containing AU rich elements (ARE) in the 3' untranslated region (UTR). AREs are known to be mRNA instability determinants (Chen and Shyu, 1995; Gillis and Malter, 1991). Specific binding proteins to ARE, such as AUF1, bind with high affinity to c-*myc*, c-*fos*, GM-CSF, and other early response gene mRNAs, and regulate degradation of those mRNAs (Nakamaki *et al.*, 1995; Sirenko *et al.*, 1997; Zhang *et al.*, 1993). Protein synthesis inhibitors may repress mRNA degradation by depleting the specific 3'-UTR binding proteins leading to increased mRNA half life and superinduction (Ohh and Takei, 1995; Pages *et al.*, 2000; Roger *et al.*, 1998). Notably, TNF-α, IL-6, and IL-2 mRNAs and resultant proteins are superinduced by deoxynivalenol and these observations are partly explained by increased mRNA stability (Li *et al.*, 1997; Wong *et al.*, 2001).

Cytokine mRNA levels might also be elevated through binding of transcription factors on a promoter region of these genes, resulting in increased transcriptional rates. For example, superinduction of TNF-α and IL-6 mRNAs by deoxynivalenol is partly explained by an increase of NF-κB, AP-1, and NF-IL6 (C/EBPβ) binding to the promoter regions in

RAW 264.7 cells (Wong, 2000) and EL-4 cells (Li *et al.*, 2000; Ouyang *et al.*, 1996b). Increased binding activity of these transcription factors may result from activation of mitogen activated protein kinases (MAPKs) (Guha and Mackman, 2001). For example, activation of MAPK families (p38, ERK1/2, and JNK1/2) is required for LPS-induced TNF-α gene transcription and TNF-α mRNA translation (Zhu *et al.*, 2000). All three MAPK families are known to be important for TNF-α mRNA induction and, furthermore, JNK1/2 is required for translation of TNF-α mRNA (Rutault *et al.*, 2001; Swantek *et al.*, 1997). Macrocyclic trichothecenes have previously been observed to activate all three MAPK families (Shifrin and Anderson, 1999; Yang *et al.*, 2000). The precise roles of MAPK activation in controlling cytokine transcript levels during superinduction by macrocyclic trichothecenes require further study.

Interestingly, the level of IL-6 was not increased despite a significant elevation in TNF-α production following stimulation by macrocyclic trichothecenes in RAW 264.7 cells. This observation was unexpected because exposure to deoxynivalenol up-regulates both TNF-α and IL-6 expression in vivo and in vitro (Wong *et al.* 2001; Zhou *et al.* 1998;1999). The differential effect might result from inherent differences in structures of these toxins. Deoxynivalenol, a type B trichothecene, does not possess a ring structure connected to C4 and C15 positions in the trichothecene nucleus that is found in macrocyclic trichothecenes. This additional ring structure might interfere with IL-6 induction.

The results found in this study may have relevance to *Stachybotrys*-induced lung disease. Exposure to *Stachybotrys* spores can cause inflammation in the lungs (Nikulin *et al.*, 1997; Rao *et al.*, 2000). When the spores are introduced to the lung, the number of

alveolar macrophages, which can not only phagocytose the spores but also secrete various immune mediators, are increased (Rao *et al.*, 2000). Relatedly, Rao *et al.* (2000a) demonstrated that removal of toxins from *Stachybotrys* spores reduces inflammatory effects in the lungs of mice. These studies imply that toxins from the spores may enhance the inflammatory response in this organ. The present study also supports the possibility that macrocyclic trichothecenes in *Stachybotrys* spores may induce inflammation in the lungs through TNF-α production.

An in vitro study by Ruotsalainen *et al.*, (1998) examined the effects of exposure to *Stachybotrys* spores in production of proinflammatory cytokines, TNF-α and IL-6, in a RAW 264.7 cell model. In that study, cytotoxicity of spores from 21 different strains was determined by MTT assay or Trypan blue exclusion test. With a concentration of $10^6$ spores per $10^6$ cells, all the tested strains of *Stachybotrys* sp. were cytotoxic producing a 31 to 97 % decrease in cell viability at 24 hr. Interestingly, *Stachybotrys* sp. strains with low cytotoxicity induce the production of TNF-α and IL-6 at the concentrations of $10^6$ spores/$10^6$ cells, whereas other strains that are highly cytotoxic do not induce the cytokine production in the macrophages. The results from these experiments are consistent with our findings because exposure to highly cytotoxic spores, presumably containing high concentrations of macrocyclic trichothecenes, would reduce cytokine production due to overt cytotoxicity. The ability of weakly cytotoxic strains of *Stachybotrys* sp. to induce cytokine production may be due to phagocytosis by macrophages or the presence of other metabolites in *Stachybotrys* spores. Other fungal metabolites such as atranones (Hinkley *et al.*, 2000), stachybotrylactones, and stachybotrylactams (Jarvis *et al.*, 1995) are found in *Stachybotrys*

sp. and might be responsible for the production of proinflammatory cytokines by *Stachybotrys* spores. Further study is necessary to determine the effect of other fungal metabolites as well as the combination of macrocyclic trichothecenes and metabolites on the immunomodulatory effects.

Taken together, the results show that at low cytotoxic concentrations, macrocyclic trichothecenes can superinduce the proinflammatory cytokine, TNF-α, in murine macrophages, whereas at higher concentrations, these compounds are cytotoxic and reduce cytokine production. These immunomodulatory effects were observed at relatively low (ng/ml) concentrations, suggesting that macrocyclic mycotoxins may pose a hazard to humans exposed to *Stachybotrys*. Further studies on immunomodulatory effects of macrocyclic trichothecenes should improve understanding of possible mechanisms for *Stachybotrys* spore-induced lung injury.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants ES-09521 (JJP) and ES-03358 (JJP) from the National Institute for Environmental Health Sciences, and by the Michigan State University Agricultural Experimental Station.

# CHAPTER IV

# UP-REGULATION OF MACROPHAGE INFLAMMATORY PROTEIN-2 AND COMPLEMENT 3A RECEPTOR BY THE TRICHOTHECENES DEOXYNIVALENOL AND SATRATOXIN G[1]

[1] This work has been submitted for publication in a similar form with authors Chung, Y.J., Yang, G.H., Islam, Z., and Pestka, J.J. in Toxicology.

## ABSTRACT

The trichothecenes are a group of mycotoxins that target leukocytes and have a wide range of immunomodulatory effects. Differential display analysis was applied to assess the effects of the trichothecenes deoxynivalenol (vomitoxin, VT) and satratoxin G (SG), on mRNA in the RAW 264.7 macrophage cell line. Cells were incubated with VT (1 μg/ml) or SG (5 ng/ml) for 2 h and total RNA then subjected to RT-PCR with a set of oligo(dT) primers. Resultant cDNA was amplified using an oligo (dT) downstream primer and an arbitrary decanucleotide upstream primer to make $^{35}$S-labeled PCR products. After separation of the products in denaturing polyacrylamide gels, 23 differentially expressed cDNA fragments were isolated and sequenced. Two of these were identified as known genes, namely, macrophage inflammatory protein-2 (MIP-2), a potent neutrophil chemoattractant involved in tissue injury and inflammation, and complement 3a receptor (C3aR), a proinflammatory mediator. Both MIP-2 and C3aR mRNAs were up-regulated by VT while only MIP-2 mRNA was induced by SG. Using commercially available antibodies, MIP-2 protein was also found to be induced by both VT and SG in RAW 264.7 cell cultures. When mice were treated with VT (12.5 mg/kg), splenic MIP-2 mRNA and serum MIP-2 levels were increased. MIP-2 mRNA and serum MIP-2 levels were synergistically increased when mice were co-treated with VT and LPS. Up-regulation of MIP-2 and C3aR are consistent with previous reports of trichothecene-induced inflammatory gene up-regulation and suggest that the specific genes affected may depend on trichothecene structures.

## INTRODUCTION

The trichothecene mycotoxins are a group of sesquiterpenoid fungal metabolites that are commonly found as contaminants of grain-based foods and indoor air. These mycotoxins include some of the most potent protein synthesis inhibitors known (Ueno, 1983a). Exposures of farm animals and humans to trichothecenes have been reported worldwide and have been related to outbreaks of alimentary toxic aleukia, vomiting, gastroenteritis, leukocytosis, and circulatory shock (Yagen and Joffe, 1976; Ueno, 1983b; Luo et al., 1990 Arnold et al., 1986; Li et al., 1999). Trichothecenes can markedly alter immune function in experimental animals (Bondy and Pestka, 2000).

Deoxynivalenol (VT, vomitoxin) is produced by *Fusarium graminearum*, and frequently found in cereals (Yuwai et al., 1994; Rotter et al., 1996; Ryu et al., 1996). Previous work in our laboratory has demonstrated that VT modulates immune function in mice. One of the most prominent effects of dietary VT is dysregulation of immunoglobulin A (IgA) production (Pestka et al., 1989; Dong et al., 1991), which is highly analogous to human IgA nephropathy (D'Amico, 1987). Overproduction of IgA by VT, observed in vivo, is mediated through alteration of cytokine production by macrophages and helper T cells (Azcona-Olivera et al., 1995a; Li et al., 1997; Yan et al., 1997, Zhou et al., 1997; Wong et al., 1997; Yan et al., 1998). Expression of various cytokine genes including interleukin (IL)-6, IL-2, IL-4, IL-5, IL-1β, and TNF-α is also up-regulated by VT. The mechanisms by which VT induces cytokine gene expression involve increased binding of transcription factors (Ouyang et al., 1996; Li et al., 2000; Wong et al., 2002) and increased mRNA stability (Li *et al.*, 1997; Wong *et al.*, 2001). At high exposure levels, VT induces apoptosis in lymphoid

organs (Pestka *et al.*, 1994; Zhou *et al.*, 2000) and in macrophage and leukemic cell lines (Yang *et al.*, 2000a). Down-regulation of GRP78/BiP (a 78-kDa glucose-regulated protein) and cochaperone P58 (IPK) by VT may also be involved in VT-induced apoptosis (Yang *et al.*, 2000b).

Satratoxins, a group of macrocyclic trichothecenes, are mainly produced by *Stachybotrys atra*, which grows on cellulose materials in water-damaged buildings (Bata *et al.*, 1985; Johanning *et al.*, 1996). Satratoxins are 10 to 100 times more toxic than are other groups of trichothecenes in mouse $LD_{50}$ studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays (Ueno, 1983a). These toxins have been etiologically associated with stachybotryotoxicosis in horse and sheep in Central Europe (Hintikka, 1978; Harrach et al., 1981; 1983; Harrach et al.; Bata et al., 1985). Clinical signs of this disease include leukopenia, thrombocytopenia, hemorrhage, arrhythmic heartbeat, and death (Forgacs, 1972). Outbreaks of *Stachybotrys*-associated diseases in water-damaged homes have been reported (Johanning et al., 1996; Etzel et al., 1998). Recently, the toxigenic *S. atra* was reported to be involved in pulmonary hemorrhage and hemosiderosis in infants living in *S. atra*-contaminated homes (Elidemir *et al.*, 1999). Leukocytes are remarkably sensitive to macrocyclic trichothecenes. At low exposure levels, these toxins have been shown to superinduce IL-2 production in EL-4 thymoma cells (Lee *et al.*, 1999) and TNF-α production in RAW 264.7 murine macrophage cells (Chung *et al.*, 2002). In addition, these toxins impair blastogenesis of lymphocytes in the presence of mitogens (Pestka and Forsell, 1988; Hughes et al., 1990) as well as induce apoptosis in macrophages (Yang *et al.*, 2000a).

In order to expand understanding of how trichothecenes affect gene regulation,

murine RAW 264.7 macrophage cells were treated with VT or satratoxin G (SG) and expressed genes were assessed by differential display PCR (DD-PCR) (Liang and Pardee, 1992). This cell line was employed since it has been previously used for studies on VT or SG-induced cytokine production as well as apoptosis studies (Wong et al., 1998; Yang et al., 2000a; Chung et al., 2002). The results indicate that both toxins up-regulated expression of macrophage inflammatory protein-2 (MIP-2), which is a chemokine responsible for chemotaxis of neutrophils to inflammation site, whereas VT selectively up-regulated complement 3a receptor (C3aR), which is a receptor involved in activation through complement. Induction of these two genes might contribute to trichothecene-induced immunotoxicity.

This study was to test the hypothesis that VT or SG induces MIP-2 and/or C3aR expression in RAW 264.7 murine macrophage cells

## MATERIALS AND METHODS

**Reagents and cell culture.** All chemicals were reagent grade or better and were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Satratoxin G was kindly provided by Dr. Bruce Jarvis (U. Of Maryland). The murine macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in a 7% $CO_2$ humidified incubator in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 1% (v/v) NCTC 135 (Gibco BRL), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

**RNA preparation.** Total RNA was extracted from RAW 264.7 cells by the method of Chomczynski and Sacchi (1987) using Trizol™ reagent (Gibco BRL) according to the manufacturer's instructions. Total RNA content was determined spectrophotometrically at 260 nm.

**Differential display analysis.** Differential display (Display Systems Biotech, Vista, CA) was performed on total RNA isolated from RAW 264.7 cell cultures treated with or without VT (1 μg/ml) or SG (5 ng/ml) for 2 h according to the manufacturer's instructions with some modifications. Briefly, 7 down stream primers were used in combination with 12 upstream primers. For first-strand cDNA synthesis, cDNA master mix was prepared by mixing 24 μl of 5X first strand cDNA synthesis buffer, 39 μl of 500 μM dNTP mix, and 3 μl of RNasin (40 units/μl). A mixture (11 μl) containing total RNA (1 μg), oligo (dT) primer (75 pmol, 5-T11VV-3, where V represents A, C, or G), and dNTP (2.5 nmol) was heated to 70 °C for 10 min, then put on ice. cDNA master mix (16.5 μl) and reverse transcriptase M-MLV (1.5 μl, 30 units) were added to the mixture, which was subsequently incubated at 42 °C for 1 h, and for 5 min at 95 °C, then put on ice. $H_2O$ (61 μl) was added and the resultant cDNA solution was aliquoted to 15 μl per vial for following PCR.

For differential display RT-PCR (DDRT-PCR), a master mix for 72 DDRT-PCR reactions was prepared by mixing 175.5 μl of 10X PCR buffer, 35.1 μl of 25 mM $MgCl_2$, 8.85 μl of [α-$^{35}$S]-dATP (12.5 mCi/ml; NEN™ Life Science Products, Boston, MA), 21.2 μl of 500 μM dNTP mix, 16.5 μl of TAQ FL DNA Polymerase (Display System Biotech; 5 units/μl), and 796.1 μl of $H_2O$. To six tubes containing 13 μl of cDNA (each two tubes for control, VT treatment, and SG treatment) and 27 μl of 25 μM downstream primer 5-T11VV-

3 (where V represents A, C, or G), 162 μl of the DDRT-PCR master mix were added. Then, these premixed components (15 μl) were mixed with 5 μl of 2 μM arbitrary decanucleotide upstream primer. PCR reactions were performed in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA) using the following parameters: 40 cycles of 94 °C for 30 s, 40 °C for 90 s, and 72 °C for 60 s for amplification followed by a final extension step at 72 °C for 7 min.

For electrophoresis, the DD-PCR sample (10 μl) was mixed with 8 μl of denaturing loading dye (95% (v/v) formamide, 20 mM EDTA (pH 8), 0.05% (w/v) bromphenol blue, and 0.05% (w/v) Xylene Cyanol FF), and were denatured by incubating at 75 °C for 3 min. Each sample (4 μl) was applied to a denaturing 5% (w/v) polyacrylamide gel (Long Ranger gel solution, FMC Bioproducts, Rockland, ME) in 1.2X TBE buffer, and the gel was run at 50 Watt in 0.6X TBE buffer. The gel was dried and subjected to autoradiography using Kodak BioMax MR film (Eastman Kodak, Rochester, NY).

**Cloning and sequence analysis of differential display bands.** cDNA bands of interest in dried gel were superimposed on autoradiography film and the corresponding gel slice was incubated in 400 μl of $H_2O$ at 100 °C for 15 min. Eluted cDNA was further amplified by PCR in 50 μl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 250 μM dNTP, 10 pmol of each downstream and upstream primer used in DDRT-PCR, 2.5 U *Taq* polymerase, and 2 μl of eluted DNA. PCR parameters were the same as described above. Amplified DNA fragments were purified with Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA), and were cloned into the plasmid vector pCRII using a TA Cloning Kit (Invitrogen, San Diego, CA). The cloned vectors were purified with a Wizard® Plus

Miniprep Kit (Promega, Madison, WI) and sequenced using *Taq*-cycle sequencing and dye terminator chemistry at the Michigan State University Sequencing Facility. Similarity of DNA sequences was compared with the GenBank and the EMBL databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, National Center for Biotechnology Information, NCBI).

**RT-PCR for mRNA expression for in vitro study.** For first-strand cDNA synthesis, 10 μl of total cellular RNA (0.50 μg) was heated to 70 °C for 5 min and chilled on ice. cDNA master mix containing 5 μg of oligo$(dT)_{12\text{-}18}$ primer (Gibco BRL), 1 mM dNTPs, 1X first-strand cDNA synthesis buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3mM $MgCl_2$, Gibco BRL), 10 mM DTT, 20 U of RNase inhibitor (Boehringer-Mannheim, Indianapolis, IN), and 100 U of M-MLV reverse-transcriptase (Gibco BRL) was added to make a final volume of 30 μl/reaction. This reaction mixture was incubated at 37 °C for 60 min, then heated at 70 °C for 15 min and chilled on ice. The RT product was diluted 10-fold with distilled $H_2O$ and stored at -20 °C.

Specific primer sets used for cDNAs of MIP-2 and C3aR were synthesized from Macromolecule Synthesis Facility at MSU (forward primer for MIP-2 5'-GAACAAAGGCAAGGCTAACTGA-3', reverse primer for MIP-2 5'-AACATAACAACATCTGGGCAAT-3', forward primer for C3aR 5'-CCTATGATTTCCAGGGGGAT-3', reverse primer for C3aR 5'-CCAAGAGGGCATACAGGAAA-3', forward primer for β-actin 5'-CACACCCGCCACCAGTTC-3', and reverse primer for β-actin 5'-ACGCACGATTTCCCTCTCA-3'). PCR was performed using a GeneAmp PCR system

9600 (Perkin-Elmer) in a final volume of 25 μl. A master mixture was made to contain 5 pmol of each primer, 125 μM dNTPs, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Gibco BRL) per each reaction tube. cDNA template (5 μl) was added to 20 μl of master mixture. PCR amplification for MIP-2, C3aR, and β-actin was performed using 23 to 31 cycles of denaturation (30 s at 94 °C), annealing (45 s at 59 °C), and extension (60 s at 72 °C) with a final extension for 10 min at 72°C. The PCR products were electrophoretically separated on 2 % (w/v) agarose gel containing 0.2 μg/ml ethidium bromide. Fluorescence intensity of each band was captured with a Molecular Imager® FX (Bio-Rad, Hercules, CA). Data were presented for multiple cycles to verify that endproduct plateau was not attained.

**MIP-2 protein quantitation.** Production of MIP-2 in RAW 264.7 supernatants and mouse sera was quantified by ELISA using modifications of the procedure of Wong et al. (1998). Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories Inc., Chantilly, VA) were coated with 50 μl/well of 1μg/ml purified antibody to MIP-2 (rabbit anti-recombinant murine; PeproTech Inc., Rocky Hill, NJ) in 0.1 M sodium bicarbonate buffer (pH 8.2) overnight at 4 °C. Plates were washed three times with 0.01M phosphate-buffered (pH 7.2) saline containing 0.2% (v/v) Tween 20 (PBST) to remove unbound capture antibodies. Wells were then incubated with 300 μl of PBST containing 3% (w/v) bovine serum albumin (Amresco, Solon, OH) (BSA-PBST) for 30 min at 37°C to block nonspecific protein binding and washed four times with PBST. Fifty μl of standard recombinant murine MIP-2 (PeproTech) or samples diluted in 10% (v/v) FBS DMEM were added to appropriate wells and plates were incubated for 1 h at 37°C. After washing four

times with PBST, 50 µl of biotinylated rabbit anti-mouse MIP-2 antibody (PeproTech), diluted in BSA-PBST (1 µg/ml), was added to each well and plates were incubated for 1 h at room temperature. After washing 6 times with PBST, 50 µl of streptavidin-horseradish peroxidase conjugate (1.5 µg/ml in BSA-PBST; Sigma) was added and incubated for 1 h at room temperature. After washing 8 times with PBST and 2 times with deionized $H_2O$, bound peroxidase conjugate was detected by adding 100 µl/well substrate solution consisting of 25 ml of 0.1 M citric-phosphate buffer (pH 5.5), 400 µl of tetramethylbenzidine (TMB) (Fluka Chemical Corp., Ronkonkoma, NY; 6 mg/ml in dimethylsulfoxide, Sigma), 100 µl of 1% $H_2O_2$. To stop the reaction, 100 µl/well of 2N $H_2SO_4$ was added and absorbance was measured at 450 nm on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). MIP-2 was quantified from standard curve using the Softmax curve-fitting program (Molecular Devices).

**MIP-2 expression in mouse.** All animal handling was conducted in accordance with recommendations established by the National Institutes of Health. Experiments were designed to minimize the number of animals required to adequately test the proposed hypothesis, and were approved by the Michigan State University Laboratory Animal Research Committee. Male B6C3F1 (C57B1/6J×C3H/HeJ) mice (7 weeks) obtained from Charles River (Portage, MI) were used for experiments. Mice were acclimated for at least 1 wk, housed 3 per cage under a 12 h light/dark cycle, and provided standard rodent chow and water *ad libitum*.

Food and water were withdrawn from cages 1 h before toxin administration. In a typical experiment, mice were given vehicle (VH) (i.p.) + VH (p.o.) [VH], VH (i.p.) + VT

(p.o.) [VT], LPS (i.p.) + VH (p.o.) [LPS], LPS (i.p.) + VT (p.o.) [LPS + VT]. LPS was dissolved in tissue culture-grade, endotoxin-free water (Sigma), aliquoted and stored at -80 °C. VT was also dissolved in tissue culture grade, endotoxin-free water and stored at 4 °C. *Escherichia coli* LPS was injected i.p. (0.1 mg/kg bw: 250 µl/mouse). VT was gavaged p.o. (12.5 mg/kg bw: 250 µl/mouse) 5 min after LPS injection.

For serum collection, blood was collected and allowed to clot overnight at 4 °C. Serum was collected and stored at -80 °C for MIP-2 ELISA.

Total RNA was extracted from spleen with Trizol™ reagent (Gibco BRL) according to the manufacturer's instructions. RNA (100 ng) from each sample was reverse transcribed to cDNA and MIP-2 cDNA was amplified as described previously.

**Statistics.** A one-way analysis of variance (ANOVA) using Bonferoni's test for parametric data was performed with Sigma Statistical Analysis System (Jandel Scientific, San Rafael, CA). A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

**mRNA differential display analysis of VT- and SG-treated RAW 264.7 cells.** Genes differentially expressed after VT or SG treatment of RAW 264.7 cells were identified using DD-PCR. RNAs derived from VT (1 µg/ml)- or SG (5 ng/ml)- treated cells for 2 h were compared to those of untreated cells. These concentrations were used because these partially (50-75%) inhibit protein synthesis and have been found effective at inducing cytokine gene expression. Using seven degenerate anchored oligo (dT) primers, two pools of cDNA from each treatment were generated. Then, PCR was performed with cDNA pools

using the same oligo (dT) downstream primers in combination with 12 short arbitrary upstream primers. A total of 140 bands were identified as potentially responsive genes following toxin treatments. Of 140 bands, twenty-three bands with high band intensities were chosen, reamplified, subcloned into a T/A vector, and sequenced. After comparisons of cDNA sequences to nucleic acid sequences in EMBL databases at NCBI, two cDNA fragments (Figure 4.1) were identified as known genes with >99% homology. The others showed no strong homology (i.e., >85%) to any existing nucleic acid sequences. The two known genes were macrophage inflammatory protein-2 (MIP-2), a chemokine that attracts neutrophils to inflammation sites, and complement 3a receptor (C3aR), which is transmembrane receptor activated during complement activation.

**VT and SG induction of MIP-2 and C3aR mRNA expression in RAW 264.7 cells.** RT-PCR was used to confirm MIP-2 and C3aR gene expression. Total RNA was isolated from control and VT or SG-treated RAW 264.7 cells and subjected to RT-PCR. For dose-response experiments, cells were treated for 3 h with a range of concentrations of VT (0 to 1000 ng/ml) or SG (0 to 25 ng/ml). Lipopolysaccharide (LPS, 500 ng/ml) induction was used as a positive control for MIP-2 (Kopydlowski *et al.*, 1999) and C3aR (Drouin *et al.*, 2001) mRNA expression. MIP-2 and C3aR cDNAs were dose-dependently increased by VT over a range of 100 to 1000 ng/ml (Figure 4.2). MIP-2 cDNAs were dose-dependently increased by SG over a range of 10 to 25 ng/ml (Figure. 4.3) whereas C3aR cDNAs were not induced over a range of 2.5 to 25 ng/ml of SG (Figure 4.3).

For time course experiments, cells were treated with VT (250 ng/ml) or SG (10 ng/ml) and mRNAs were analyzed over a six hour period. MIP-2 cDNAs were time-

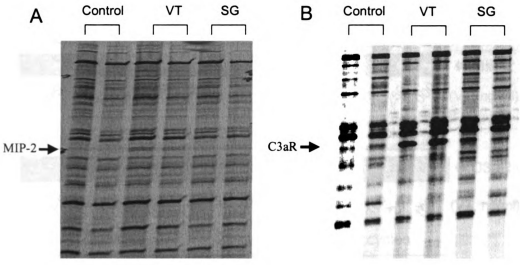


Figure 4.1. Sequencing gel section from mRNA DD-PCR. RAW 264.7 cells were cultured in the presence of VT (1 μg/ml) or SG (5 ng/ml) for 2 h. After cDNA synthesis from total RNA, $^{35}$S-labeled PCR products were synthesized using a combination of upstream and downstream primers. Resultant PCR products were subjected to electrophoretic separation. (A) Primers used: #3 downstream primer (T11AG) and #6 upstream primer (B) Primers used: #8 downstream primer (T11GC) and #5 upstream primer. Arrows indicate mRNA transcripts that are highly expressed in RAW 264.7 cells.

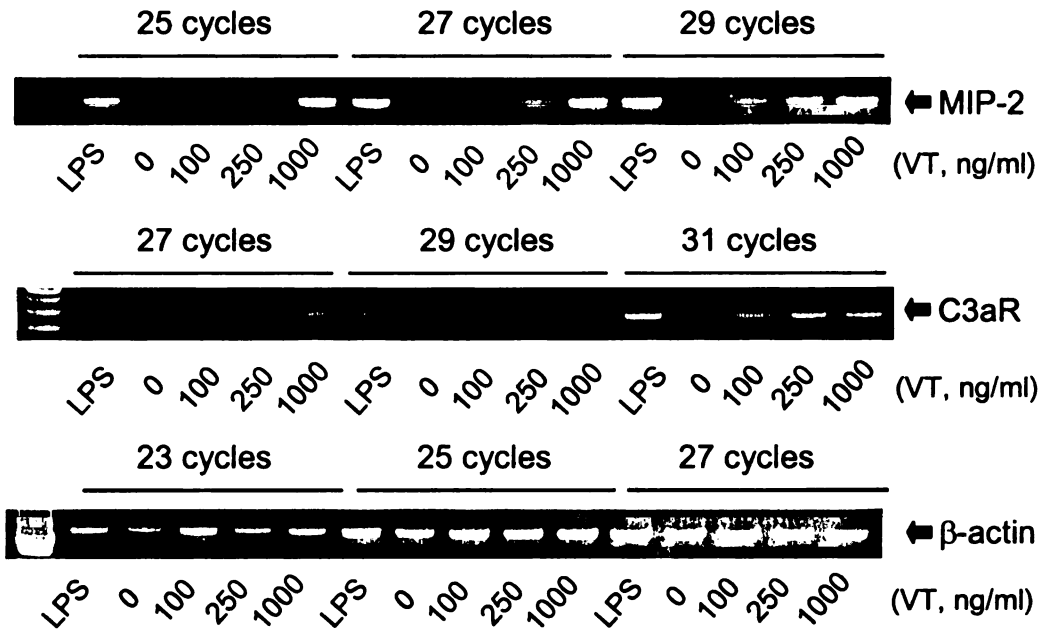


Figure 4.2. Dose-dependent induction of MIP-2 and C3aR mRNAs expression by VT in RAW 264.7 cells. RAW 264.7 cells (2.5 x $10^5$ cells/ml) were treated with various concentrations of VT for 3 h. Total RNA was subjected to RT-PCR for variable cycle periods as shown using murine specific MIP-2 C3aR and β-actin primers, respectively. PCR products were separated on 2% (w/v) agarose gel and visualized using ethidium bromide (0.2 μg/ml). LPS (500 ng/ml) was used as a positive control for MIP-2 and C3aR. Data are a representative of two independent experiments.

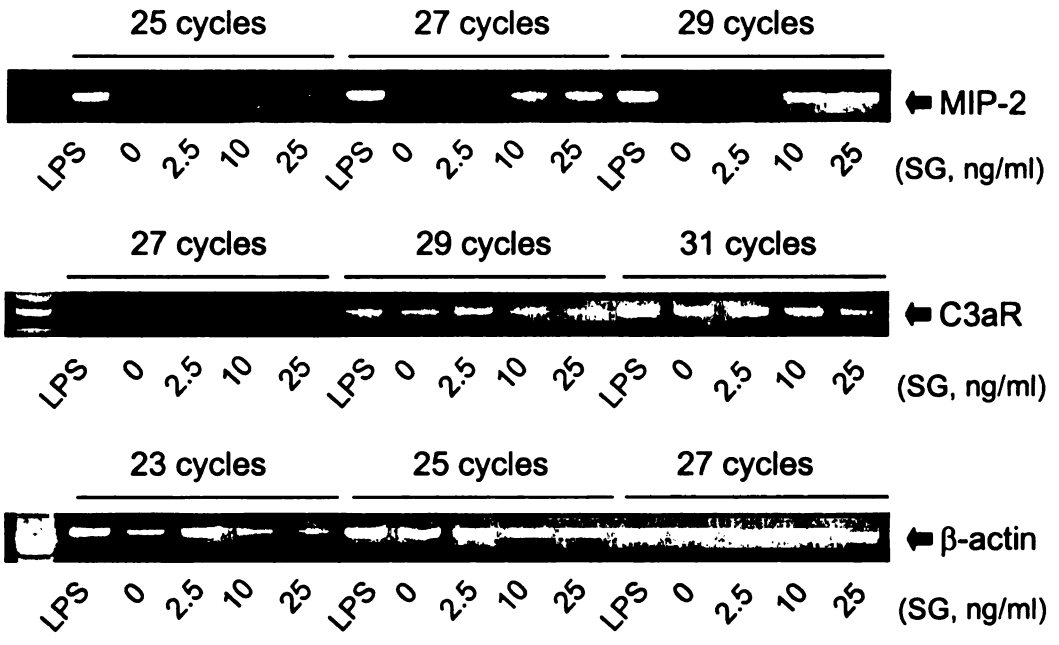


Figure 4.3. Dose-dependent induction of MIP-2 mRNAs expression by SG in RAW 264.7 cells. RAW 264.7 cells (2.5 x $10^5$ cells/ml) were treated with various concentrations of SG for 3 h. Total RNA was subjected to RT-PCR for variable cycle periods as shown using murine specific MIP-2 C3aR and β-actin primers. Detection of agarose gel separation of cDNA bands of PCR products were explained in Figure 2 legend. LPS (500 ng/ml) was used as a positive control for MIP-2 and C3aR mRNA. Data are a representative of two independent experiments.

dependently increased by VT and over a 6 h period while C3aR cDNAs were increased by VT at 2 h and remained constant until 6 h (Figure 4.4). MIP-2 cDNAs were also time-dependently increased by SG over a 6 h period, whereas C3aR cDNAs were not affected by SG (Figure 4.5).

Further studies for MIP-2 protein expression by VT or SG were performed using commercially available antibodies whereas examination of C3aR protein expression was not possible due to unavailability of specific antibodies.

**VT and SG induce MIP-2 protein production in RAW 264.7 cells.** ELISA was used to quantify induction of MIP-2 protein expression by VT and SG in RAW 264.7 cells. After 12 h, VT significantly induced production of MIP-2 between 100 and 1000 ng/ml with peak levels observed at 250 ng/ml of the toxin (Figure 4.6A). SG at concentrations between 2.5 and 10 ng/ml also induced production of MIP-2 with peak effects observed at 5 ng/ml (Figure 4.6B). Maximum levels of MIP-2 (32 ng/ml) induced by VT were 8 times higher than maximum levels of MIP-2 (3.8 ng/ml) induced by SG. This result suggests that VT appeared to be a more potent inducer of MIP-2 than SG when presented at optimal concentrations for stimulation.

For time course experiments, cells were treated with VT (250 ng/ml) or SG (5 ng/ml) for 6, 12, 24 h, then MIP-2 protein in cell culture supernatant was quantified by ELISA. MIP-2 production by VT (Figure 4.7A) and SG (Figure 4.7B) was significantly increased up to 24 h. Maximum levels of MIP-2 produced by VT at 24 h incubation were 12 times higher than those levels induced by SG at the same incubation time, which also supports the observation that VT was a more potent inducer of MIP-2 than SG.

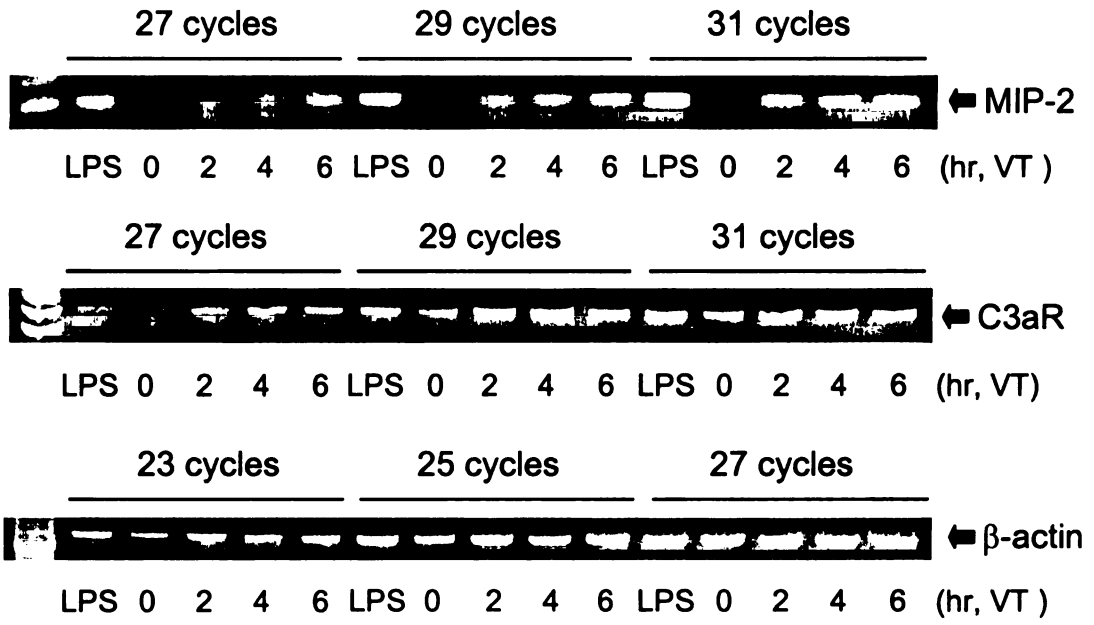


Figure 4.4. Time-dependent induction of MIP-2 and C3aR mRNA expression by VT in RAW 264.7 cells. RAW 264.7 cells (2.5 x $10^5$ cells/ml) were treated with VT (250 ng/ml) over a 6 h period. Total RNA was subjected to RT-PCR using murine specific MIP-2, C3aR, and β-actin primers, respectively. LPS (500 ng/ml) at 2 h was used as a positive control for MIP-2. Detection of cDNA bands and agarose gel separation of PCR products were explained in Figure 2 legend. Data are a representative of two independent experiments.

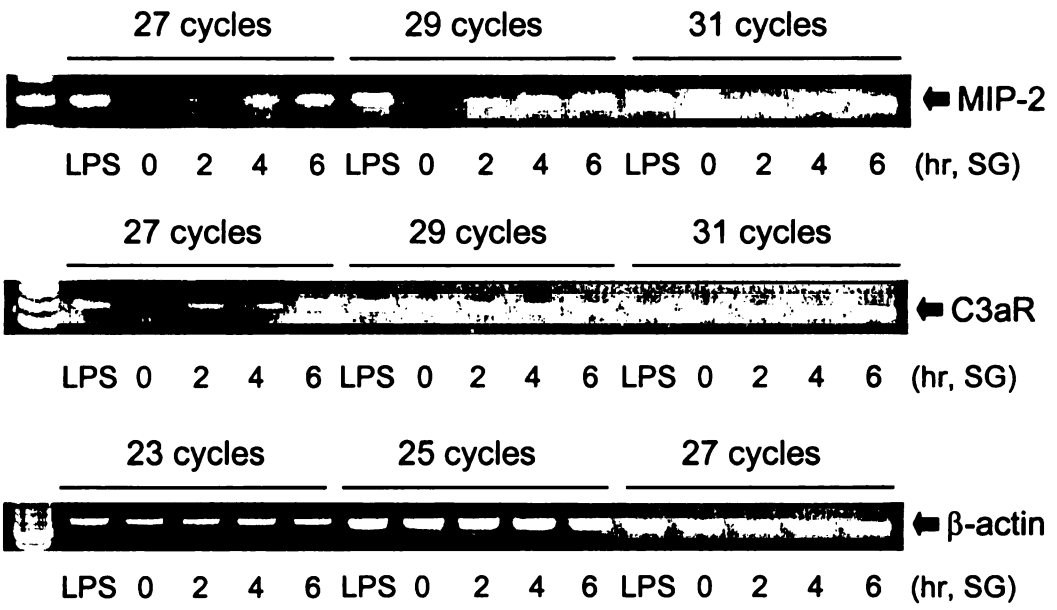


Figure 4.5. Time-dependent induction of MIP-2 mRNA expression by SG in RAW 264.7 cells. RAW 264.7 cells (2.5 x $10^5$ cells/ml) were treated with SG (10 ng/ml) over a 6 h period. Total RNA was subjected to RT-PCR using murine specific MIP-2, C3aR and β-actin primers, respectively. LPS (500 ng/ml) at 2 h was used as a positive control for MIP-2. Detection of cDNA bands and agarose gel separation of PCR products were explained in Figure 2 legend. Data are a representative of two independent experiments.

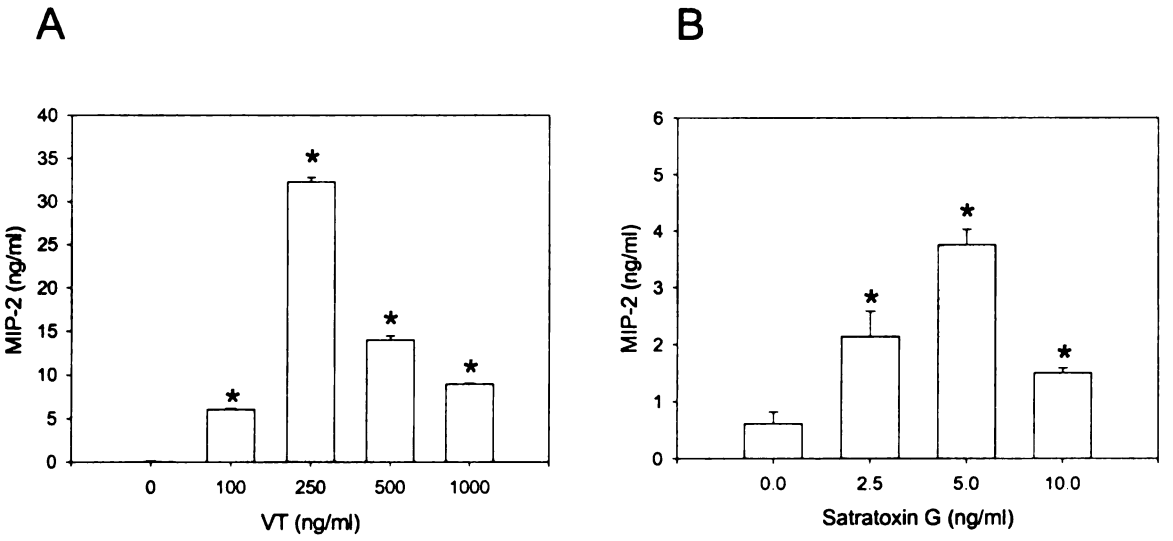


Figure 4.6. Effect of VT or SG on MIP-2 protein production by RAW 264.7 cells for dose-dependent study. Cells (2.5 x $10^5$ cells/ml) were cultured with various concentrations of (A) VT or (B) SG for 12 h. MIP-2 production in supernatant was assayed by ELISA. Data are mean ± SEM of triplicate cultures. Values marked with an asterisk (*) differ significantly from control values ($p < 0.05$). Data are a representative of two separate experiments.

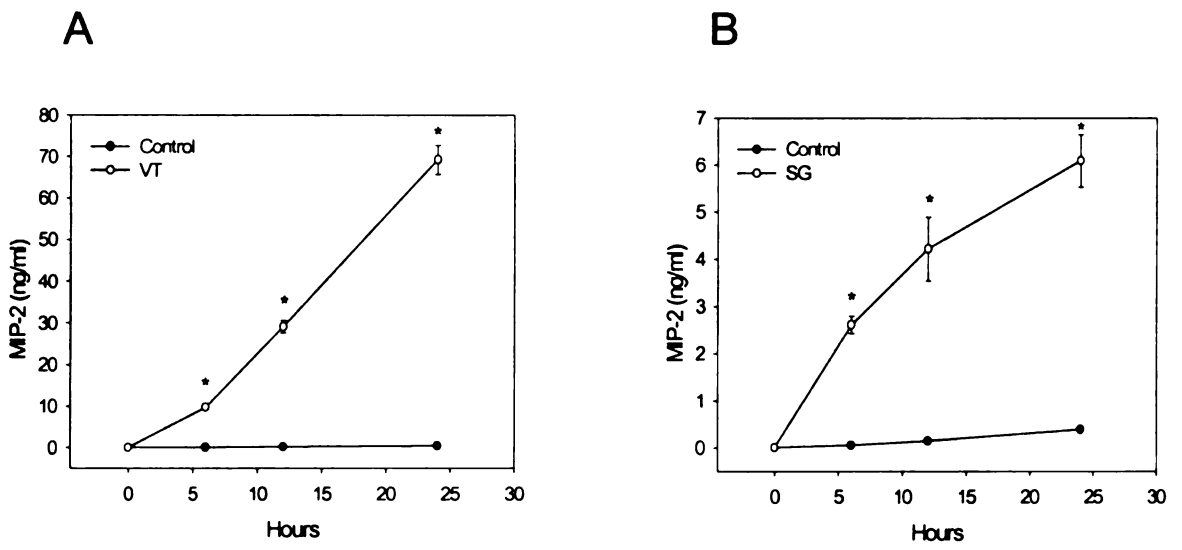


Figure 4.7. Effect of VT or SG on MIP-2 protein production by RAW 264.7 cells for time-course study. Cells (2.5 x $10^5$ cells/ml) were cultured for various time periods with (A) VT (250 ng/ml) or (B) SG (5 ng/ml). MIP-2 production in supernatant was assayed by ELISA. Data are mean ± SEM of triplicate cultures. Values marked with an asterisk (*) differ significantly from vehicle ($p < 0.05$). Data are a representative of two separate experiments.

**MIP-2 expression by VT, LPS and VT+LPS in vivo.** Mice were treated with VH, LPS, VT or VT + LPS and splenic expression for MIP-2 mRNA was assayed 3 h later. As observed by Kopydlowski et. al. (1999), MIP-2 mRNA expression was induced by the treatment of LPS and this served as a positive control. VT also independently induced MIP-2 mRNA at this time point (Figure 4.8A). When mice were co-treated with LPS and VT, more MIP-2 mRNA was induced than single treatment in spleen.

After 3 h toxin treatment, sera were collected and subjected to MIP-2 protein assay by ELISA (Figure 4.8B). VT significantly induced MIP-2 protein (20 ng/ml) at 3 h and LPS also significantly induced MIP-2 protein (14 ng/ml). When mice were treated with both LPS and VT, MIP-2 concentrations in sera was significantly higher than expected additive responses.

## DISCUSSION

VT, SG and other trichothecenes are protein synthesis inhibitors that target actively dividing cells such as leukocytes (Ueno, 1983a). Trichothecene mycotoxins can dose-dependently cause cytokine up-regulation and apoptosis in lymphoid tissues in mouse (Bondy and Pestka, 2000). Here, we employed differential display to identify additional genes that might be involved in immune dysregulation by these toxins in a murine macrophage model. The capacity of trichothecenes to induce MIP-2 and C3aR mRNA may contribute to immunotoxicity.

Macrophage inflammatory proteins (MIP) are structurally related chemokines that induce migration of specific subsets of leukocytes (Driscoll, 1994). MIPs are approximately

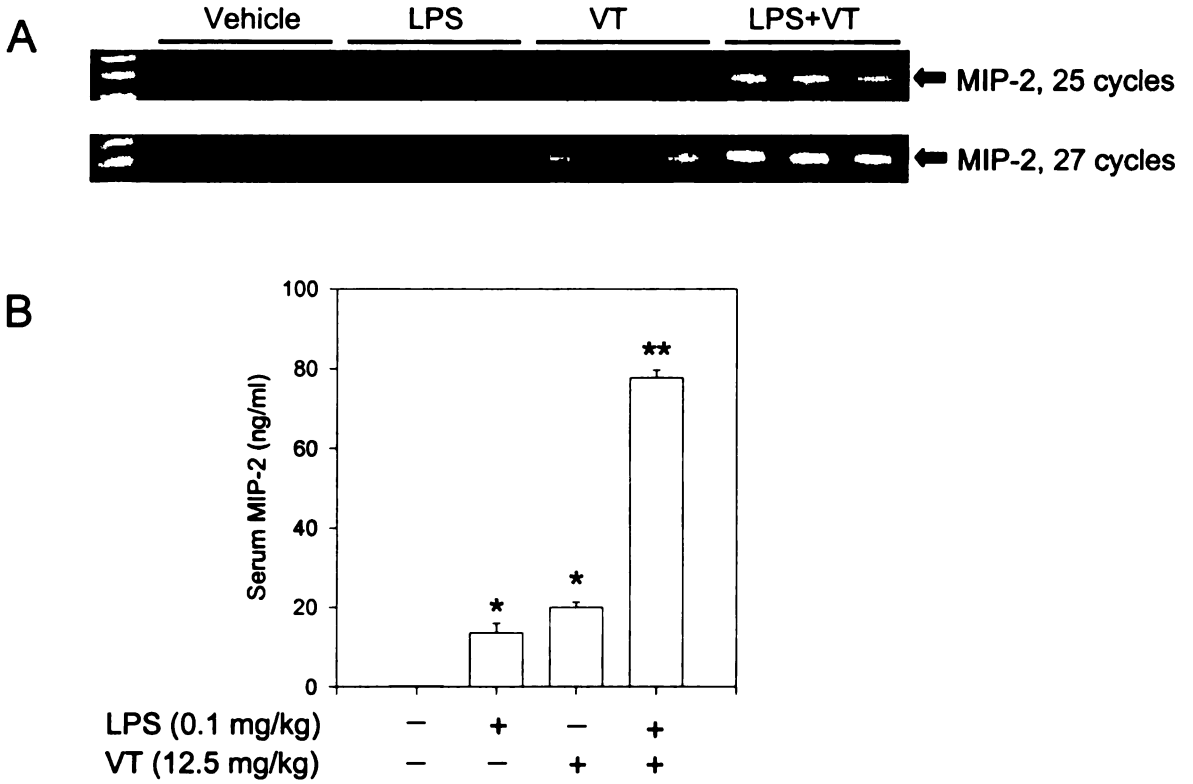


Figure 4.8. Effect of VT on MIP-2 expression in vivo. Mice were treated with vehicle, LPS + vehicle, vehicle + VT or LPS + VT for 3h. (A) Spleen was collected after treatment. Total RNA was subjected to RT-PCR (n = 3) using murine MIP-2 primers for 25 and 27 cycles of PCR. (B) Blood was collected at 3 h treatment and MIP-2 concentrations (mean ± SEM, n = 3) in serum were measured by ELISA. Values marked with an asterisk (*) differ significantly ($p < 0.05$) from vehicle values. Values marked with an double asterisk (**) differ significantly ($p < 0.05$) from additive expected responses of LPS + VT. Data are a representative of two separate experiments.

6-8 kd, heparin binding proteins and three MIPs (MIP-1α, MIP-1β and MIP-2) have been identified according to their similar structure and function (Sherry *et al.*, 1988; Wolpe *et al.*, 1988; Wolpe *et al.*, 1989). MIPs are induced by various stimuli such as LPS, proinflammatory cytokines, and oxidative stress in a wide range of cells including alveolar macrophages, mast cells, peritoneal macrophages, epithelial cells, and fibroblasts (Burd *et al.*, 1989; Driscoll *et al.*, 1993; Kopydlowski *et al.*, 1999; Shi *et al.*, 1999). MIP-1α and MIP-1β can activate both neutrophils and mononuclear cells and they possess proinflammatory properties (Driscoll, 1994). MIP-2 is a potent neutrophil chemoattractant and epithelial cell mitogen and it is involved in acute pulmonary inflammation (Huang et al., 1992; Driscoll et al., 1995).

Several in vivo studies demonstrate that MIP-2 is involved in tissue injury and inflammation. Expression of MIP-2 mRNA and accumulation of neutrophils in the lung were associated with development of lung edema during hepatic ischemia/repercussion in mice. Passive immunization against MIP-2 with neutralizing antibody reduced lung edema indicating that MIP-2 is involved in tissue injury (Yoshidome *et al.*, 1999). LPS instillation into rat lung up-regulates MIP-2 mRNA in a time dependent manner. Furthermore, MIP-2 is responsible for neutrophil accumulation in lungs (Schmal *et al.*, 1996). When the effect of elevated MIP-2 was also studied in a cecal ligation and puncture (CLP) model of septic peritonitis in CD-1 mice, the chemokine was significantly increased in peritoneal fluid, serum, liver and lung after CLP and this increase correlated with severity of sepsis. Passive immunization prior to CLP with antibody to MIP-2 decreased both mortality and neutrophil percentage in peritoneal fluid (Walley *et al.*, 1997). Overall, these studies demonstrate that

increased MIP-2 expression and neutrophil accumulation can mediate tissue damage and inflammation. The previous observation by our laboratory that ingestion of VT by mice significantly increases neutrophil levels in blood (Forsell et al., 1986) may relate to MIP-2 induction.

In this experiment, satratoxin G, a major macrocyclic trichothecene produced by *S. atra*, induced MIP-2 expression of both mRNA and protein levels in RAW 264.7 macrophage model. Since neutrophils induced by MIP-2 are involved in acute pulmonary inflammation and hemorrhage-induced acute lung injury (Huang et al., 1992; Driscoll et al., 1995; Abraham et al., 2000), the capacity of SG to induce MIP-2 may be important in relation to *Stachybotrys*-associated diseases. Pulmonary hemorrhage and hemosiderosis in infants have been associated with toxigenic *S. atra* contaminated homes (Etzel *et al.*, 1998). Exposure of *S. atra* to humans resulted in various disorders in respiratory, immune, and central nervous systems (Johanning *et al.*, 1996). Although the specific etiologic involvement of macrocyclic trichothecenes has not yet been verified, the capacity to induce MIP-2 as well as other cytokines (Lee et al., 1999; Chung et al., 2002) provides a feasible mechanism for *Stachybotrys*-associated lung injury.

Induction of MIP-2 expression by VT and SG might be mediated through activation of mitogen-activated protein kinases (MAPKs). SG at 2.5 to 10 ng/ml activates all three major MAPKs: the extracellular signal-regulated kinase (ERKs or p42/p44), the c-Jun amino-terminal kinase (JNKs, also known as stress-activated protein kinases, SAPK), and p38 MAPK in RAW 264.7 murine macrophage cells (Yang *et al.*, 2000a). VT at 250 ng/ml also induces phosphorylation of the three MAPKs in RAW 264.7 cells (Moon and Pestka,

2002). MIP-2 induction by soluble intercellular adhesion molecule-1 in mouse astrocytes is mediated through activation of ERKs (Otto *et al.*, 2002). Production of MIP-2 by staurosporine in rat peritoneal neutrophils is mediated through both p38 and ERK pathways (Xiao *et al.*, 1999). Also, p38 kinase plays an important role in LPS-induced murine pulmonary inflammation in which MIP-2 draws neutrophils into airways (Nick *et al.*, 2000). In that study, inhibition of p38 kinase by M39, a novel p38 kinase inhibitor, blocked MIP-2 and TNF-α production in vitro and, furthermore, systemic inhibition of p38 kinase significantly decreased in neutrophil accumulation and the release of TNF-α in the airspaces. Thus, further study is needed to relate MAPK activation to trichothecene-induced MIP-2 expression and potential downstream pathologic effects.

MIP-2 is considered to be a functional analog of human IL-8 (Sherry *et al.*, 1992). Interestingly, VT readily induces IL-8 production in a clonal human macrophage U-937 cells (Sugita-Konishi and Pestka, 2001). Neutrophil recruitment to sites of inflammation due to IL-8 is involved in adult respiratory stress syndrome, idiopathic pulmonary fibrosis, rheumatoid arthritis, and hepatic ischemia-repercussion injury (Lynch et al., 1992; Donnelly et al., 1994; Koch et al., 1994; Colletti et al., 1995). Increased IL-8 expression may be related to progression of immunoglobulin A nephropathy (IgAN). For example, a significant increase of IL-8 in sera of IgAN patients was detected compared to healthy controls. IgA autoantibodies to IL-8 are frequently detected in IgAN patients (Lai *et al.*, 1996). In addition, Sekikawa et al., (1998) observed the expression of IL- 8 both in its transcript and the protein levels in renal biopsy specimens obtained from patients with IgAN and lupus nephritis. The expression of IL-8 mRNA was significantly correlated to the number of neutrophils in the

glomerulus. In our laboratory, a VT-induced mouse model of IgAN has been used to study progress of this disease as well as cellular and molecular mechanisms of trichothecene-induced immunotoxicity. As a functional analog of human IL-8, MIP-2 may play an important role in progression of VT-induced IgAN in the mouse.

C3aR is a transmembrane receptor that is functionally coupled to G proteins (Roglic *et al.*, 1996), and is distributed in both myeloid cells (monocytes/macrophage, neutrophils and eosinophils) and nonmyeloid cells (mast cells, glial cells, astrocytes, and smooth muscle cells) throughout various tissues including lung, heart, kidney, liver, testis, and brain (Legler et al., 1996; Martin et al., 1997; Gasque et al., 1998; Davoust et al., 1999; Sayah et al., 1999). C3aR is activated upon binding C3a ligand, which is one of the anaphylatoxins produced by proteolytic cleavage during complement activation (Hugli, 1990). C3a ligand, on binding the C3aR, mediates numerous biological responses including lysosomal enzyme secretion from neutrophils, histamine release from mast cells, smooth muscle contraction, and modulation of the humoral and cellular immune responses (Glovsky et al., 1979; Showell et al., 1982; Stimler et al., 1983; Morgan, 1986).

C3a can modulate immune response by stimulating or suppressing cytokine production. When the effects of C3a on IL-6 gene expression and protein production were studied in the presence of LPS in human PBMC (Fischer et al., 1999), both IL-6 mRNA and its protein were increased with a co-treatment of C3a and LPS whereas IL-6 was not enhanced with C3a alone. In a study with human astrocytomas, stimulation by C3a enhanced IL-6 mRNA level and protein level while IL-1β, TNF-α and TGF-β mRNA expression remained unchanged (Sayah *et al.*, 1999). IL-6 mRNA expression was blocked by treatment

of pertussis toxin or C3aR antibody, which indicates that the IL-6 response was related to C3a. Regulation of B cell functions by C3a was studied by measuring IgG, TNF-α, and IL-6 production in *Staphylococcus aureus* Cowan strain I (SAC)/IL-2-activated B cells (Fischer and Hugli, 1997). Incubation of SAC/IL-2-activated B cells with C3a suppressed polyclonal immune response, IL-6 and TNF-α production. Monsinjon et al. (2001) studied the effect of C3a on IL-8 production in the epithelial cell line ECV 304. Binding of C3a to the epithelial cells resulted in IL-8 production in time- and dose dependent manners. Pretreatment of ECV 304 cells with pertussis toxin inhibited C3a-induced IL-8 mRNA and its protein. Taken together, C3a has immunomodulatory effects upon binding C3aR in various cell types.

C3aR expression may potentiate or attenuate pathologic responses. For example, expression of C3aR plays a role in lung inflammation. When mice are treated with LPS or ovalbumin (OVA), both C3aR protein and mRNA significantly increase in bronchial epithelial and smooth muscle cells from mouse lung (Drouin *et al.*, 2001). These observations suggest the possible involvement of C3aR in the pathology of diseases such as sepsis and asthma. On the other hand, in a study with targeted-disruption of C3aR in mice, C3aR played an anti-inflammatory role in endotoxemia (Kildsgaard *et al.*, 2000). In that study, $C3aR^{-/-}$ mice were i.v. challenged with increasing doses of LPS and displayed increased mortality to endotoxin-induced shock compared to their wild-type mice. Plasma IL-1β levels were significantly elevated in the $C3aR^{-/-}$ mice compared with wild-type littermates after LPS challenge. This latter result indicates that C3aR attenuates the pathological effects of septic shock. Thus, the role of C3aR may function in both pro- and

anti-inflammatory manners in models of shock.

Although the observation that VT and SG, two protein synthesis inhibitors (PSIs), increased MIP-2 protein levels is paradoxical, several studies support the capability of translational arrest by PSI to induce early response genes including cytokines. For example, VT enhances expression of cytokine mRNA in vivo (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997) and in vitro (Dong *et al.*, 1994; Wong *et al.*, 1998). Cycloheximide, a prototype protein synthesis inhibitor, also induces cytokine gene expression (Dong et al., 1994; Azcona-Olivera et al., 1995b). In those studies with VT and/or cycloheximide, elevated cytokine mRNA levels by PSI contribute to subsequent increase in cytokine protein levels. Increased cytokine gene expression by PSIs can be partly explained by both transcriptional and post-transcriptional mechanisms. Transcriptional up-regulation might result from inhibition of labile negative regulator synthesis (Fong et al., 1995; Ye and Young, 1997; Yang and Pestka, 2002). At the post-transcriptional level, increased cytokine mRNA stabilities by VT have been reported (Li *et al.*, 1997; Wong *et al.*, 2001). This phenomenon can be explained by inhibition of synthesis of labile RNases that facilitate RNA degradation (Shaw and Kamen, 1986) and/or labile proteins that bind 3'-untranslated region to regulate mRNA degradation (Baker *et al.*, 2000; Chambers and Kacinski, 1994; Madireddi *et al.*, 2000). Similarly, VT and SG-induced MIP-2 production in this study may be related to transcriptional and post-transcriptional controls by the PSIs.

In summary, we have isolated VT- and SG-responsive genes, MIP-2 and C3aR, from a murine macrophage cell line using DD-PCR. These findings provide further insight into potential mechanisms of trichothecene-induced tissue injury and immunotoxicity. Additional

studies of the mechanisms MIP-2 and C3aR induction by trichothecenes are necessary to understand their roles in immunopathological effects of the toxins. In addition, DD-PCR is subject to a number of difficulties, most notably the use of many multiple primer combinations as well as the generation of many clones with no known relationship to existing genes. Further study of trichothecene-altered gene regulation could be greatly enhanced by application of microarray technology which has evolved significantly since the onset of this DD-PCR study.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service Grants ES-03358 (JJP) and ES-09521 (JJP) and by the Michigan Agricultural Experiment Station.

# CHAPTER V

# ROLE OF MITOGEN-ACTIVATED PROTEIN KINASES IN VOMITOXIN-INDUCED TNF-α MESSENGER RNA EXPRESSION IN MURINE MACROPHAGE RAW 264.7 CELLS

## ABSTRACT

Upregulation of proinflammatory cytokine gene expression by vomitoxin (VT, deoxynivalenol) is transcriptionally and post-transcriptionally controlled. To better understand the molecular basis for this observation, the effects of VT-induced mitogen-activated protein kinases (MAPKs) on TNF-α gene expression were studied in murine macrophage RAW 264.7 cells. VT was found to induce phosphorylation of p38 kinase, extracellular signal-regulated kinases (ERKs), and c-Jun amino terminal kinases (JNKs) in a dose-dependent manner. Induction of TNF-α mRNA expression was significantly reduced in lipopolysaccharide (LPS) plus VT treated cells at 3 hr in the presence of the p38 kinase inhibitor, SB 203580 (2 μM), or the MEK-ERK inhibitor, PD 98059 (10 μM), whereas the induction of TNF-α mRNA was not affected in the presence of JNK inhibitor, SP 600125 (1 μM). To investigate the role of MAPKs on VT-enhanced TNF-α promoter activity, a luciferase reporter gene driven by murine TNF-α promoter was used. LPS+VT-induced TNF reporter gene expression was significantly reduced at 12 hr in the presence of 0.2 to 2 μM of SB 203580, 100 μM of PD 98059, and 10 μM of SP 600125. To study the effects of MAPKs on VT-induced TNF-α mRNA stability, cells were exposed to VT and LPS in asynchronous fashion. In the asynchronous model, cells were pretreated with LPS (1 μg/ml) for 4 hr, medium removed, and medium containing a transcription inhibitor, 5,6-dichloro-beta-D-ribofuranosyl-benzimidazole (DRB), MAPK inhibitors, and/or VT (250 ng/ml) were added. VT-induced TNF-α mRNA stabilization was abolished in the presence of SB 203580 (2 μM) while the stabilization by VT was not affected in the presence of PD 98059 (10 μM)

or SP 600125 (1 μM). To determine whether activation of MAPKs was involved in the regulation of LPS+VT-induced TNF-α production, cells were incubated with LPS (100 ng/ml), VT (250 ng/ml) or LPS (100 ng/ml)+VT(250 ng/ml) for 18 hr in the presence of inhibitors. ELISA of supernatant revealed that LPS+VT-induced TNF-α production was significantly reduced with 0.2 to 20 μM of SB 203580, 1 to 100 μM of PD 98059, or 0.1 to 10 μM of SP 600125. These results demonstrate that activation of MAPKs by VT are critical in VT-induced TNF-α expression in macrophage cells.

## INTRODUCTION

Trichothecenes are a group of structurally related sesquiterpenoid metabolites produced by various strains of fungi including *Fusarium* and *Stachybotrys* (Grove, 1988; Grove, 1993; Grove, 1996). These mycotoxins bind to eukaryotic ribosomes and consequently inhibit protein synthesis (Ueno, 1983, 1984). More than 180 trichothecenes have been identified, and of these, deoxynivalenol (vomitoxin, VT) is one of the most common contaminants in cereal grains (Rotter *et al.*, 1996). VT is resistant to degradation during processing (Jackson and Bullerman, 1999) and, therefore, is often encountered in human and animal food. Notably, VT can alter immune function experimentally (Bondy and Pestka, 2000).

Depending on dose and frequency of exposure, VT can be both immunosuppressive and immunostimulatory. Immunosuppression by high doses of VT is manifested by rapid onset of leukocyte apoptosis (Islam *et al.*, 2002; Pestka *et al.*, 1994; Yang *et al.*, 2000; Zhou *et al.*, 2000; Zhou *et al.*, 1999). On the other hand, low doses of VT exposure enhance

expression of various cytokines in vitro and in vivo (Yan *et al.*, 1997; Yan *et al.*, 1998; Zhou *et al.*, 1997; Azcona-Olivera *et al.*, 1995a; Li *et al.*, 1997; Wong *et al.*, 1998). Exposure to VT increases IL-2, IL-4, IL-5 and IL-6 mRNA and protein levels in T cells (Ouyang *et al.*, 1996; Warner *et al.*, 1994). Superinduction of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β has also been observed in VT-treated macrophages (Miller and Atkinson, 1986; Wong *et al.*, 1998). Acute exposure of mice to VT increases IL-6, TNF-α, IL-1β, and interferon (IFN)-γ mRNA expression (Azcona-Olivera *et al.*, 1995b; Zhou *et al.*, 1997, 1998).

Upregulation of proinflammatory cytokines such as TNF-α and IL-6 plays an important role in VT-induced pathologic consequences. TNF-α is a key cytokine in triggering various physiological and pathological processes. For example, TNF-α mediates biological responses such as cell death, cytokine and chemokine induction, antiviral activity, and sepsis (Beutler, 1999; Sedgwick *et al.*, 2000; Smith *et al.*, 1990). IL-6 produced by VT-stimulated macrophages in Peyer's patch drives IgA production from B cells (Yan *et al.*, 1998) and this overproduction of IL-6 is strongly suspected to contribute to dysregulation of IgA production (Dong *et al.*, 1991; Pestka *et al.*, 1989). Furthermore, IL-6-deficient mice are refractory to IgA dysregulation by dietary VT (Pestka and Zhou, 2000). This dysregulation of IgA production is highly analogous to human IgA nephropathy (D'Amico, 1987).

Although the exact mechanisms for superinduction of proinflammatory cytokine genes by VT are not fully understood, studies with RAW 264.7 murine macrophage cells to VT have indicated the following effects: increased binding activities of transcription factors

such as AP-1, NF-κB, NF-IL6 (Wong *et al.*, 2002); increased IL-6, TNF-α, and IL-1β mRNA expression (Wong *et al.*, 1998); stabilization of IL-6 and TNF-α mRNA (Wong *et al.*, 2001); and increased soluble IL-6 and TNF-α in culture supernatants (Wong *et al.*, 1998). Recently, VT was found to induce activation of mitogen-activated protein kinases (MAPKs) (Moon and Pestka, 2002; Yang *et al.*, 2000) which may contribute to both activation of transcription factors and mRNA stability.

MAPKs transduce extracellular signals to intracellular responses in leukocytes and other cells. Three distinct, but partially "cross-talking" MAPKs have been characterized in the mammalian system (Tibbles and Woodgett, 1999). These include the extracellular signal-regulated kinase (ERKs or p42/p44), the c-Jun amino-terminal kinase (JNKs, also known as stress-activated protein kinases, SAPK), and p38 MAPK. The MAPK signaling pathways consist of a series of kinases which sequentially activate and consequently phosphorylate downstream kinases to transduce extracellular stimuli into intracellular responses. Typical MAPK cascades involve the activation of a MAP kinase kinase kinase (MAPKKK), which phosphorylates and activates a MAP kinase kinase (MAPKK) which in turn phosphorylates MAPK(s). Then, activated MAPKs phosphorylate downstream cellular substrates such as transcription factors and other kinases (Kyriakis and Avruch, 2001). The major three MAPKs are known to be involved in TNF-α expression following treatment of macrophages with bacterial lipopolysaccharides (LPS) in transcriptional and post-transcriptional manners (Brook *et al.*, 2000; Mahtani *et al.*, 2001; Swantek *et al.*, 1997; Zhu *et al.*, 2000).

The purpose of this study was to test the hypothesis that TNF-α expression by VT is

mediated by differential activation of p38 kinase, ERKs and/or JNKs. Specifically, the effects of VT on MAPK activation, as well as the relationship of MAPKs to VT-induced mRNA expression, TNF-α promoter activity, TNF-α mRNA stability, and TNF-α protein production were assessed in RAW 264.7 murine macrophage cells.

## METHODS AND MATERIALS

**Reagents and plasmids.** All chemicals were reagent grade or better and were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. The p38 MAPK inhibitor SB203580, MEK1 inhibitor PD98059, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA). A transcriptional inhibitor, 5,6-dichloro-beta-D-ribofuranosyl-benzimidazole (DRB) was purchased from Fluka Co. (Madison, WI). Murine TNF-α promoter luciferase reporter construct (pCDNA-tnf-luc) was kindly provided by Dr. W. Zhu (The Scripps Research Institute, La Jolla, CA). The murine TNF promoter construct received was originally derived from a λEMBL3 genomic clone containing most of the mouse (strain C57B1/6) TNF locus (Shakhov et al., 1990). A genomic fragment (2,223-nt fragment containing the distal coding sequence of lymphotoxin [exon IV] and 28 nt upstream of the initiation site within the TNF 5'-untranslated region) was cloned to the luciferase coding sequence in pcDNA3 vector (Beutler and Brown, 1991; Zhu et al., 2000). Beta-galactosidase reporter construct (pCMV-gal) was kindly provided by Dr. T. Zacharewski (Michigan State University, East Lansing, MI). All plasmids were purified with Endo-Free Plasmid Prep Kit (Qiagen, Valencia, CA).

**Cell culture**. The murine macrophage RAW 264.7 cell line was obtained from the

American Type Culture Collection (Rockville, MD). Cells were maintained at 37 °C in a 6% $CO_2$ humidified incubator in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Atlanta Biologicals Inc., Norcross, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL).

For MAPK studies, cells (2.5 x $10^5$ cells/ml) were incubated in 60 mm tissue culture plates (Fisher Scientific Co., Corning, NV) in a volume of 6 ml for 24 hr. Supernatant was replaced with medium containing LPS (*Salmonella typhimurium*, 100 ng/ml), VT (250 ng/ml) or LPS (100 ng/ml) + VT (250 ng/ml). Cells were incubated for intervals, and then subjected to lysis for MAPK activation assay. For dose-dependency experiments, media containing LPS (100 ng/ml), VT (100 to 1000 ng/ml), and combination of LPS and VT were added and cells analyzed as described above.

For mRNA induction studies, cells (2.5 x $10^5$ cells/ml) were incubated in 6 well tissue culture plates (Fisher Scientific Co., Corning, NV) in a volume of 1.5 ml for 24 hr. Supernatant was replaced with media containing LPS (100 ng/ml), VT (100 - 500 ng/ml) or a combination of LPS and VT. Cells were incubated for 2 and 7 hr and total RNA was extracted.

For mRNA stability studies, cells were pretreated as described above. Supernatant was replaced with media containing LPS (1000 ng/ml) and incubated for 4 hr. Then, media containing DRB (50 μM), VT (250 ng/ml), and/or MAPK inhibitor were added. Cells were incubated for various time intervals and then subjected to total RNA isolation.

For TNF-α production studies, cells (2.5 ×$10^5$ cells/ml) were cultured for 24 hr in flat-bottomed 24-well tissue culture plates (Fisher Scientific Co.) with each well containing

500 μl of cell suspension. Then, supernatants were replaced with medium containing MAPK inhibitors and VT (250 ng/ml) and/or LPS (100 ng/ml). After 18 hr incubation, supernatants were subjected to enzyme-linked immunosorbent assay (ELISA).

**MAPK phosphorylation by Western blot analysis.** Cells were washed with ice-cold phosphate buffer, lysed in lysis buffer (1% [w/v] SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris, pH7.4), and sonicated for 10 seconds. After determination of protein concentration using Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA), equal amounts of protein were resolved by SDS-PAGE (10%[w/v] gel). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Du Pont, Boston, MA). Membranes were blocked either 1 hr at room temperature or overnight at 4 °C in 5% (w/v) bovine serum albumin (BSA) in TBST (20 mM Tris-HCl, pH 8, 137 mM NaCl containing 0.1% (v/v) Tween 20). After two washes in TBST, membranes were probed with specific phospho-p38, phospho-ERK, or phospho-JNK antibodies (New England Biolabs, Beverly, MA) diluted in 5% BSA in TBST overnight at 4 °C. After 4 washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr, and blots were developed using an ECL chemiluminescent detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**RNA extraction.** Total RNA was extracted from RAW 264.7 cells by the method of Chomczynski and Sacchi (1987) (Chomczynski and Sacchi, 1987) using Trizol™ reagent (Gibco BRL) according to the manufacturer's instructions.

**Ribonuclease protection assay.** Total RNA (5 or 10 μg) was used for cytokine expression analysis by multiprobe RPA (RiboQuant mCK3b template set; Pharmingen).

Assays were performed according to the manufacturer's instruction. Briefly, radioactive riboprobes were synthesized at 37 °C for 1 hr in vitro from 50 ng of linearized plasmids with T7 RNA polymerase in the presence of unlabeled ATP, CTP, GTP, UTP and [$\alpha$-$^{32}$P]UTP (PerkinElmer Life Sciences, Boston, MA) using an In Vitro Transcription Kit (Pharmingen). The probes were digested with DNase I (2000 units/ml) at 37 °C for 30 min, extracted with phenol: chloroform: isoamyl alcohol (50:50:1) and chloroform:isoamyl alcohol (50:1), precipitated with ammonium acetate and ethanol, and washed with ethanol. The purified antisense RNA probes were dissolved in hybridization buffer. A quantity of 5 or 10 μg of total RNA from samples was incubated at 56 °C for 12-16 hr in a hybridization buffer with more than 6 x $10^6$ cpm/reaction. After hybridization, RNase digestion with Pharmingen RPA RNase A (0.25 U/μl) and T1 (10 U/μl) was carried out for 45 min at 30 °C. The undigested (protected) RNA was precipitated with ethanol, separated on 6% (w/v) polyacrylamide gel electrophoresis with 8 M urea at 50 watts constant power for 1 hr. The gel was dried and subjected to band analysis using Molecular Imager FX (Bio-Rad). Bands were quantified using Quality One Software (Bio-Rad). The cytokine template set (mCK3b) included ten cytokines, TNF-β, lymphotoxin (LT)-β, TNF-α, IL-6, interferon (IFN)-γ, IFN-β, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, macrophage inflammatory factor (MIF), and two housekeeping genes, L32 and GAPDH, with the following lengths of RNA transcripts: 389, 351, 316, 284, 257, 232, 208, 191, 171, 154, 141, and 125 bases, respectively.

**Real Time PCR.** Total RNA was analyzed to detect TNF-α mRNA level using an ABI Prism™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

The levels of 18S rRNA were also measured as an indigenous control. Specific primers and probes for TNF-α and 18S were obtained from PE Appllied Biosystems. Concentrations used for primers and probes were 200 and 100 nM, respectively, in each reaction. Sixteen μl of master mix comprising 20 x TNF-α primers and probe, 20 x 18S primers and probe, predeveloped assay reagents for PCR and RT (PE Applied Biosystems) was added to 9 μl of samples containing 100 ng total RNA. Reverse transcriptase and polymerase reactions were performed in the same tubes. Relative quantities of TNF-α mRNA and 18S rRNA were calculated using the comparative threshold cycle number for each sample fitted to standard curves for TNF-α mRNA and 18S rRNA, respectively, generated according to manufacturer's recommendations. Expression levels for TNF-α mRNA in each sample were normalized to 18S rRNA.

**Cell transfection.** RAW 264.7 cells were grown to 80 % confluence and then transfected with plasmids by the electroporation method of Stacey et al., (1993). Briefly, cells were harvested and resuspended at a concentration of $3.0 \times 10^7$ cells/ml in DMEM with 10% (v/v) heat-inactivated FBS supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Two hundred microliter medium containing $5 \times 10^6$ cells was transferred to 0.4 cm electroporation cuvettes (Bio-Rad) with 44 μl of TE (10mM Tris, 1 mM EDTA, pH 7.4) buffer containing plamid DNA (3 μg of pCDNA-tnf-luc, 3 μg of pCMV-gal, and 5 μg of control vector [pCDNA]). Cuvettes were held at room temperature for 5 min and electroporated at 960 μF, 300 V using a Bio-Rad Gene Pulser with capacitance extender. These conditions generated a time constant of 46-48 ms.

**Luciferase and β-galactosidase assay**. Transfected cells were diluted in 12 ml culture medium, plated in 12 well culture plates (1 ml/well), and incubated 24 hr. Supernatant was removed and fresh media containing MAPK inhibitors and LPS (100 ng/ml) and/or VT (250 ng/ml) were replaced and incubated 12 hr. Cells were then washed with ice-cold PBS once and lysed with 200 μl of lysis buffer (Promega, Madison, WI). After a brief centrifugation, supernatant was subjected to luciferase and β-galactosidase assay. Luciferase activity was measured with a luminometer (Turner Designs Co. Model 20e, Sunnyvale, CA) after brief mixing of supernatant with luciferase assay substrate solution (Promega). For β-galactosidase assay, equal volumes of supernatant and 2x assay buffer (Promega) were mixed in a 96 well culture plate and incubated at 37 °C for 30-60 min. After stopping reaction by adding 1M Tris buffer, absorbance was read at 405 nm on a microplate reader (Molecular Devices, Menlo Park, CA). Luciferase activity was normalized against β-galactosidase activity by dividing activity of luciferase by activity of β-galactosidase.

**TNF-α production.** Supernatants were collected at 18 hr and assayed for TNF-α by ELISA using Opt EIA™ mouse TNF-α set (Pharmingen, San Diego, CA) according to manufacturer's instruction.

**Statistics.** A one-way analysis of variance (ANOVA) using Bonferroni's test for parametric data or a student's t-test was performed with Sigma Statistical Analysis System (Jandel Scientific, San Rafael, CA). A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

VT induces TNF-α, IL-6, and IL-1β mRNA in the presence of LPS in RAW 264.7 macrophage cells (Wong *et al.*, 1998). To assess the effect of VT+LPS on activation of p38 kinase, ERK1/2, and JNK1/2, RAW 264.7 cells were incubated with LPS (100 ng/ml), VT (250 ng/ml), or LPS+VT over a 8 hr period and whole cell lysates were subjected to Western blot analysis (Figure 5.1). Since activation of MAPKs requires specific phosphorylation at threonine and tyrosine residues, antibodies specific for phosphorylated p38, ERK1/2, and JNK1/2 were used to detect their activation. As shown in Fig 5.1, LPS induced phosphorylation of p38 kinase and JNK1/2 with a maximal activation at 30 min and diminished their activation at 2 hr. Activation of ERK1/2 was maximal at 30 min and prolonged with a lesser activation until 8 hr. These results were consistent with observations from Chen and Wang (1999). VT alone induced phosphorylation of ERK1/2 and JNK1/2 with a maximal activation of at 30 min and diminished their activation at 2 hr. Activation of p38 kinase was maximal at 30 min and prolonged with a lesser activation until 8 hr. Similar results were observed at a study of Moon and Pestka (2001) (Moon and Pestka, 2002). Cotreatment of LPS and VT induced activation of all three MAPKs with a peak activation at 30 min and prolonged their activation until 8 hr for p38 kinase and ERK1/2 and 2 hr for JNK1/2.

For dose-dependency experiments, RAW 264.7 cells were incubated with LPS (100 ng/ml), VT (100 -1000 ng/ml), or LPS (100 ng/ml)+VT(100 - 1000ng/ml) for 2 hr and whole cell lysates were subjected to Western blot analysis (Figure 5.2). VT induced dose-dependent activation of all three MAPKs whereas cotreatment of LPS and VT also induced

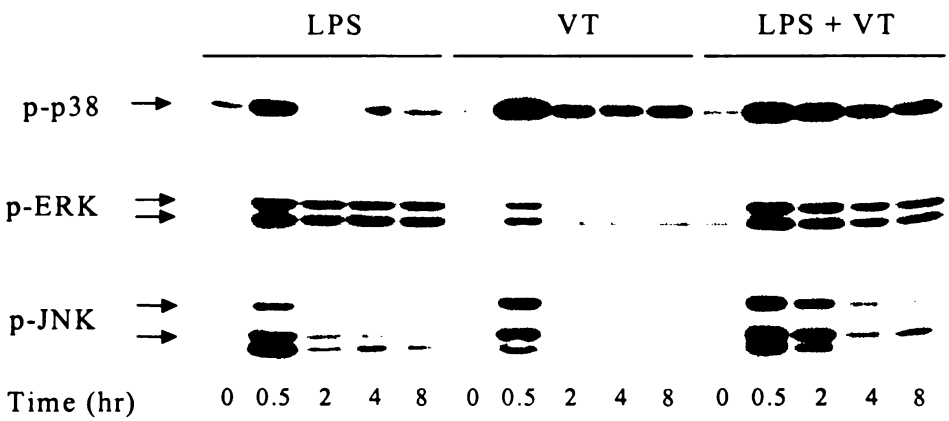


Figure 5.1. Time-dependent activation of p38, ERK1/2, and JNK1/2 by LPS, VT and LPS+VT in RAW 264.7 macrophage cells. Cells were treated with 100 ng/ml of LPS, 250 ng/ml of VT or cotreatment of LPS and VT for a period of times. Whole cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated form of p38, ERK1/2, or JNK1/2 as described in Materials and Methods. Data are a representative of two independent experiments.

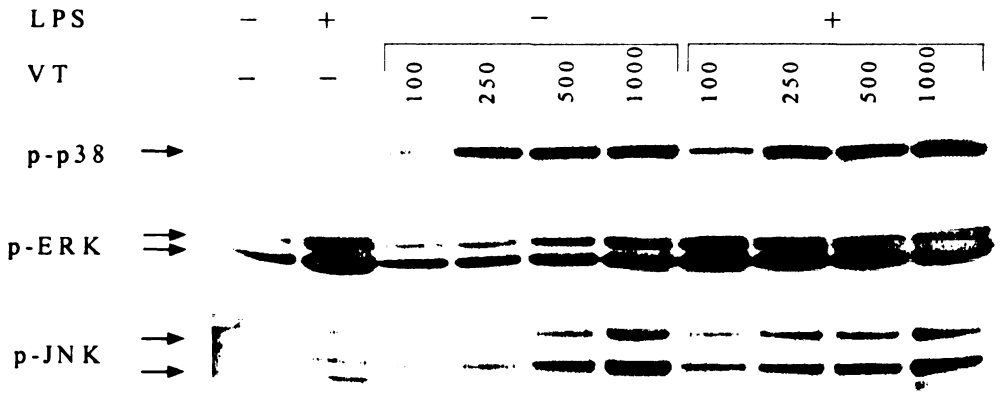


Figure 5.2. Dose-dependent activation of p38, ERK1/2, and JNK1/2 by VT and LPS+VT in RAW264.7 macrophage cells. Cells were treated with 100 ng/ml of LPS as control, VT (100-1000 ng/ml) or LPS (100 ng/ml)+VT (100-1000 ng/ml) for 2 hr. Whole cell lysates were subjected to Western blotting using antibodies specific for phosphorylated form of p38, ERK1/2, or JNK1/2 as described in Materials and Methods. Data are a representation of two independent experiments.

phosphorylation of three MAPKs with slightly increased activities compared to VT alone treatment.

To investigate and confirm genes affected by VT and cotreatment with LPS, RAW 264.7 cells were incubated with LPS, VT, and LPS+VT for 2 and 7 hr and total RNA was subjected to ribonuclease protection assay using multiprobes as described in material and methods (Figure 5.3A). LPS (100 ng/ml) alone induced LT-β, TNF-α, IL-6, IFN-γ, TGF-β1, and TGF-β3 mRNA at both 2 and 7 hr. Induction of these genes was consistent with other studies for LT-β (Browning *et al.*, 1997), TNF-α, IL-6 (Wong *et al.*, 1998), IFN-γ (Wang *et al.*, 2000), TGF-β1 , and TGF-β3 (Gosiewska *et al.*, 1999). VT alone also induced TNF-α, IL-6, IFN-γ, TGF-β1, and TGF-β3 mRNA at 2 hr but much less at 7 hr. These results are consistent with previous studies for TNF-α, (Azcona-Olivera *et al.*, 1995a; Wong *et al.*, 1998), IL-6, IFN-γ (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997), TGF-β (Azcona-Olivera *et al.*, 1995a). Cotreatment of LPS and VT enhanced the induction of TNF-α, IL-6, IFN-γ, TGF-β1, and TGF-β3 mRNA at both 2 and 7 hr. TNF-α mRNA was most profoundly induced by both LPS and VT at 2 and 7 hr in RAW 264.7 cells. After normalization with GAPDH mRNA, relative intensities of TNF-α mRNA were described in Figure 5.3B. VT dose-dependently induced TNF-α mRNA at 2 hr and lessened induction at 7 hr. When LPS was cotreated with VT, TNF-α mRNA levels at 7hr remained as high as the levels at 2 hr.

To assess the effect of MAPK inhibitors on LPS+VT induced TNF-α mRNA expression, RAW 264.7 cells were pretreated with SB 203580 (2 μM, p38 kinase inhibitor), PD 98059 (10 μM, MEK1-ERK inhibitor), or SP 600125 (1 μM, JNK inhibitor) for 30 min.

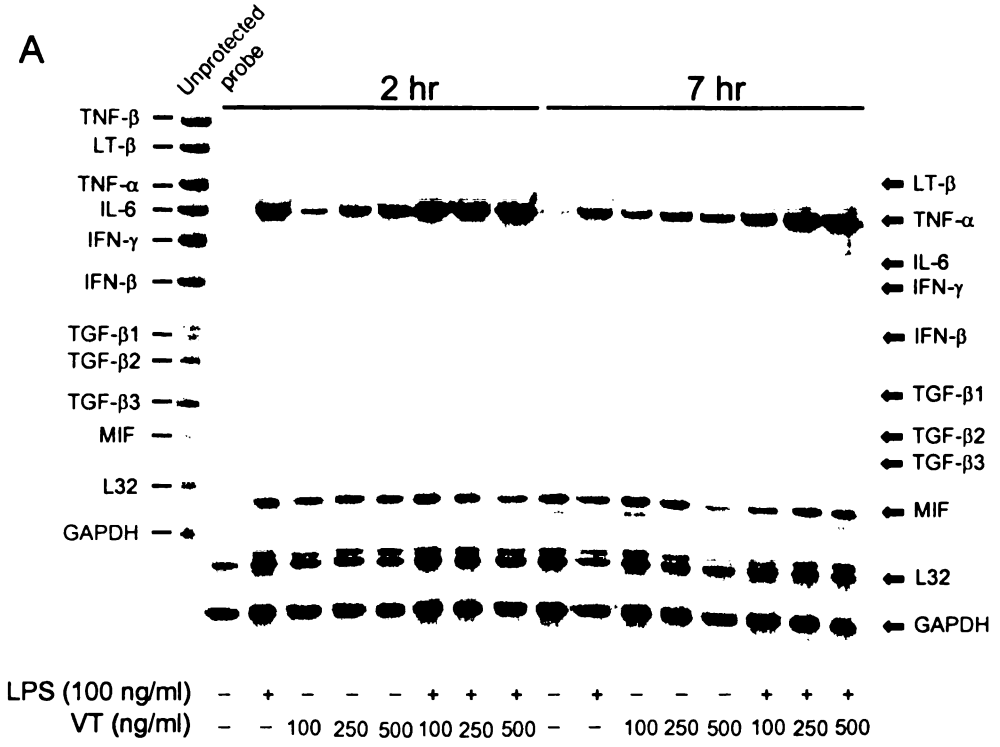


Figure 5.3.A. Cytokine mRNA expression in LPS-, VT-, and LSP+VT treated RAW264.7 cells. Cells were treated with LPS (100 ng/ml), VT (100-500 ng/ml), or LPS+VT for 2 and 7 hr. Total RNA was subjected to RNase protection assay using multiprobes as described in Material and Methods. The results are a representative of two separate experiments.

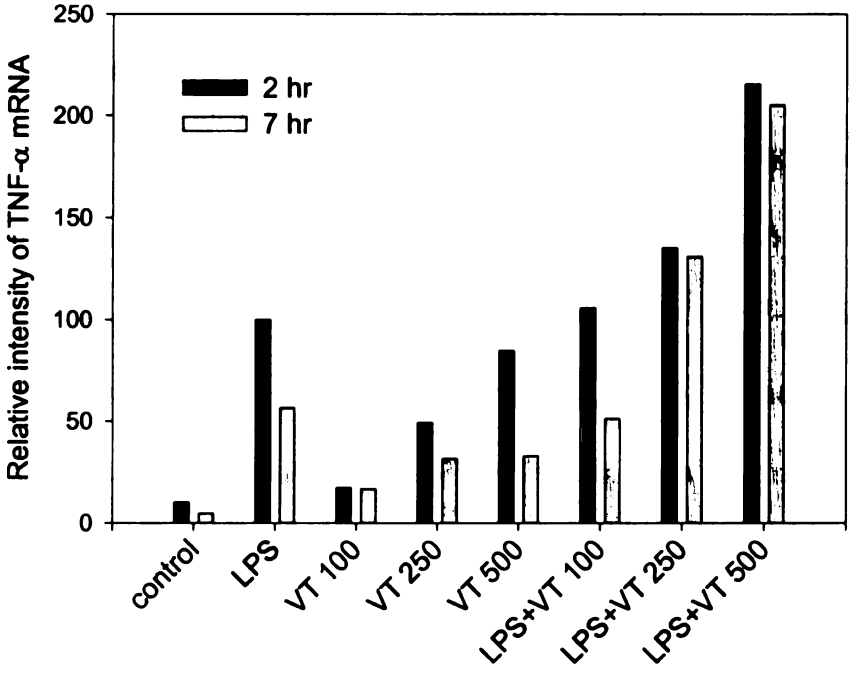


Figure 5.3.B. The bands of TNF-α and GAPDH mRNA were quantified using PhosphoImager and are shown as a relative intensity of control sample at 2 hr (set at 100 with LPS treatment at 2 hr).

Then, cells were incubated with LPS (100 ng/ml), VT (250 ng/ml), or LPS+VT for 3 hr. Total RNAs were isolated and subjected to real time PCR. In the presence of SB 203580 and PD 98059, LPS+VT-induced TNF-α mRNA expression was significantly suppressed while LPS-induced TNF-α mRNA level was not affected (Figure 5.4). VT-induced TNF-α mRNA expression was significantly suppressed in the presence of PD 98059.

The effects of MAPKs on TNF-α gene expression were further studied by investigating TNF-α promoter activity. TNF-α promoters have binding sites for AP-1 and other transcription factors which are known downstream targets of MAPKs (Garcia *et al.*, 1998; Hazzalin *et al.*, 1998; Minden and Karin, 1997). VT induces AP-1, NF-κB, and NF-IL6 binding activities in vitro (Li *et al.*, 2000; Wong *et al.*, 2002). These increased binding activities can potentially be regulated by MAPK activation (Chakraborti and Chakraborti, 1998; Hambleton *et al.*, 1996; Koj, 1996). To investigate the role of MAPKs on VT-enhanced TNF-α promoter activity, a luciferase reporter gene driven by murine TNF promoter was used. LPS, VT, and LPS+VT all significantly induced TNF-α promoter luciferase activity in RAW 264.7 cells (Figure 5.5A, 5.5B, and 5.5C). Cotreatment of LPS and VT significantly increased TNF-α promoter activity compared to mean additive response. As shown in Figure 5.5A, the p38 kinase inhibitor, SB203580 (0.2, 2, and 20 μM), significantly inhibited LPS-, VT- and LPS+VT- induced TNF reporter gene expression in a dose-dependent manner. Interestingly, MEK-ERK inhibitor, PD98059, significantly increased LPS-induced TNF reporter gene expression (Figure 5.5B). VT-, LPS+VT-induced TNF reporter gene expression were significantly decreased with 100 μM of PD98059. The

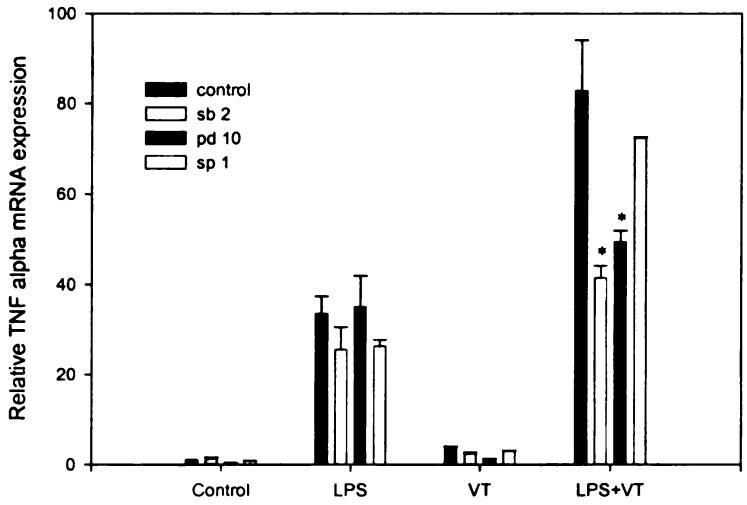


Figure 5.4. Effect of MAPK inhibitors on LPS-, VT-, and LPS+VT- induced TNF-α mRNA expression in RAW 264.7 macrophage cells. Cells were pretreated with SB 203580 (2 μM), PD 98059 (10 μM), or SP 600125 (1 μM) for 30 min, and then incubated with LPS (100 ng/ml), VT (250 ng/ml), or LPS+VT for 3 hr. Total RNAs were isolated and subjected to Real Time PCR. Data are representative of two separate experiments. An asterisk indicates a significant difference from control with p value < 0.05.

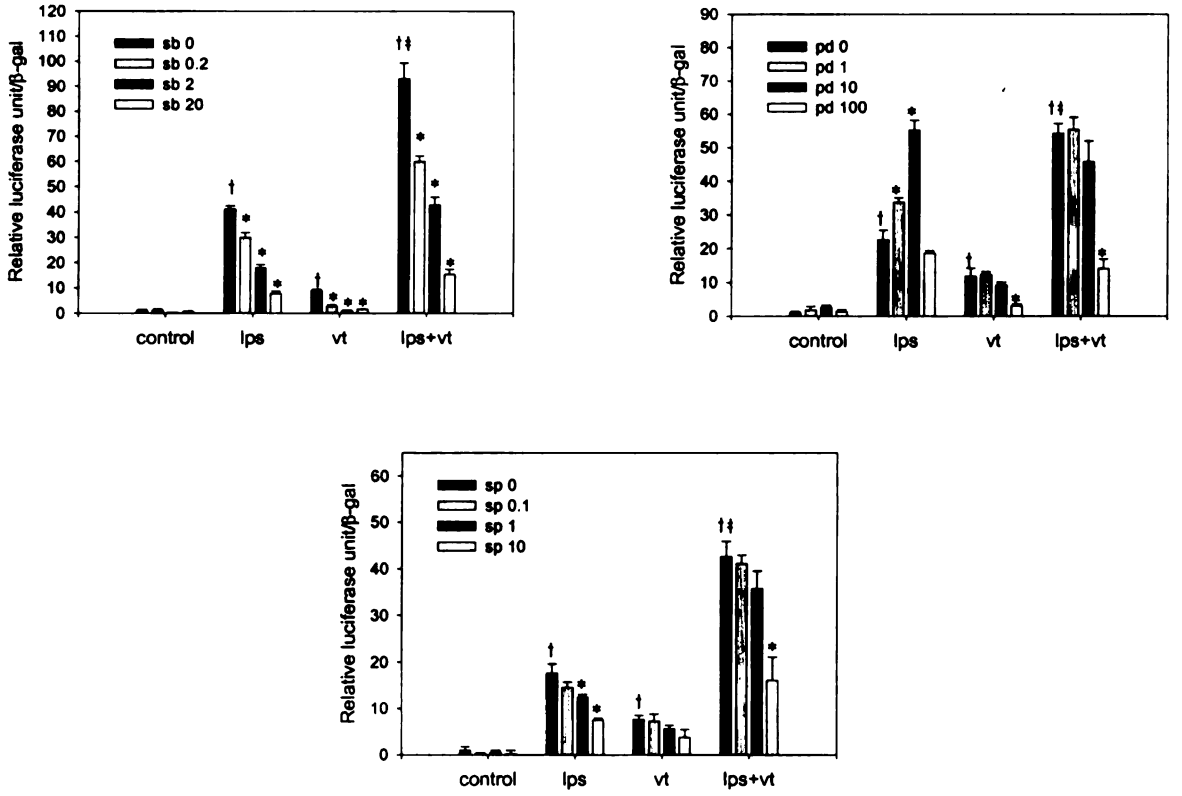


Figure 5.5. Effect of MAPK inhibitors on LPS-, VT-, and LPS+VT- induced TNF reporter gene expression in RAW 264.7 macrophage cells. A, cells were transfected with murine TNF reporter. After 24 hr, cells were incubated with LPS (100 ng/ml), VT (250 ng/ml), or LPS (100 ng/ml)+VT (250 ng/ml) in the presence of p38 inhibitor, SB203580 (0.2, 2, and 20 mM) for 12 hr. Luciferase activities were measured. Cotransfected β-galactosidase was used to normalize the transfection efficiency. Data are representative of two separate experiments. B, the same conditions as in A except using PD98059 (1, 10, and 100 μM). C, the same conditions as in A except SP600125 (0.1, 1, and 10 μM). An asterisk, †, or ‡ indicates a significant difference from control group (no inhibitor), from control (no treatment), from an expected mean additive effect with p value $<0.05$, respectively.

JNK inhibitor, SP600125, significantly reduced LPS-induced TNF reporter gene expression at 1 and 10 μM while it reduced LPS+VT-induced TNF reporter gene expression at 10 μM (Figure 5.5C). VT-induced TNF reporter gene expression was not changed by the JNK inhibitor.

VT can increase the stability of TNF-α mRNA (Wong *et al.*, 2001). To determine whether activation of MAPKs contributes to this increased stability, RAW 264.7 cells were pretreated with LPS (1 μg/ml) for 4 hr. After washing with culture medium, cells were treated with VT (250 ng/ml), p38 inhibitor (SB203580, 2 μM) in the presence of DRB, a transcription inhibitor, over several time intervals. DRB treatment suppressed TNF-α mRNA expression at 30, 90, and 180 min, resulting in TNF-α mRNA half-life ($t_{1/2}$) of 45 min (Figure 5.6A). GAPDH mRNA expression was not changed with DRB treatment because of their long half life. VT at 250 ng/ml in the presence of DRB increased TNF-α mRNA stability, resulting in TNF-α mRNA $t_{1/2}$ of more than 180 min. This result was consistent with the finding of Wong et al., (2001). When p38 inhibitor, SB203580 (2 μM), was coincubated with DRB and without VT treatment, TNF-α mRNA levels were rapidly reduced, resulting in TNF-α mRNA $t_{1/2}$ of 11 min. In other words, p38 inhibitor reduced TNF-α mRNA $t_{1/2}$ from 45 min to 11 min. This result indicates that activation of p38 kinase is required to stabilize TNF-α mRNA. This observation is consistent with findings from other studies indicating that p38 kinase is involved in stabilization of LPS-induced TNF-α mRNA (Brook *et al.*, 2000; Wang *et al.*, 1999). However, increased TNF-α mRNA $t_{1/2}$ (> 180 min) by VT was reduced to 22 min in the presence of p38 inhibitor. When comparing

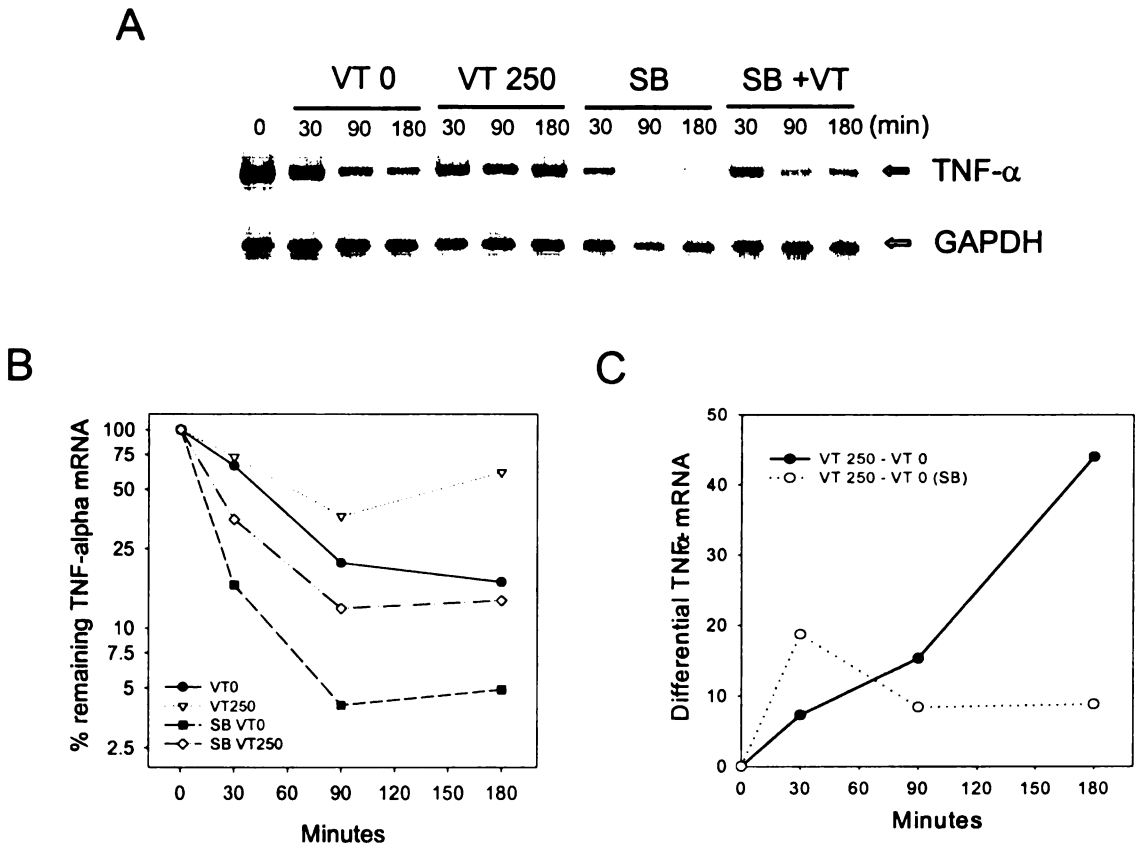


Figure 5.6. Effect of SB203580 on VT-induced TNF-α mRNA expression in LPS-treated RAW 264.7 cells. Cells were pretreated with 1 μg/ml of LPS for 4 hr and medium was then removed. Fresh medium containing VT (250 ng/ml), DRB (50 μM), and/or SB203580 (2μM) was added to cultures and incubated for a period of times. Total RNA was subjected to RNase protection assay using multiprobes including TNF-α. A, blot from RNase protection assay. B, relative TNF-α mRNA levels after normalization with GAPDH gene expression determined by densitometry of blot from RNase protection assay. C, differential induction at each time points by subtracting percent remaining TNF-α mRNA of DRB-treated cells from percent remaining TNF-α mRNA of VT-treated cells. Data are representative of two separate experiments.

differential percent remaining TNF-α mRNA in the absence of p38 inhibitor to those of TNF-α mRNA in the presence of p38 inhibitor over time, the difference due to VT were abrogated over a period of time in the presence of p38 inhibitor (Figure 5.6C), indicating that VT-induced TNF-α mRNA stability was impaired by p38 inhibitor.

The MEK1 inhibitor, PD98059 (10μM), was used to investigate whether the ERK1/2 pathway is involved in mRNA stabilization. DRB treatment resulted in TNF-α mRNA half-life ($t_{1/2}$) of 45 min (Figure 5.7A). VT increased TNF-α mRNA half life to greater than 180 min. When MEK1 inhibitor was coincubated with DRB and without VT treatment, TNF-α mRNA stability were slightly reduced, resulting in TNF-α mRNA $t_{1/2}$ of 38 min. This observation indicates that activation of ERK1/2 might be involved in stabilizing TNF-α mRNA. Similar findings were reported that MEK1 inhibitor destabilizes TNF-α mRNA (Dumitru *et al.*, 2000; Rutault *et al.*, 2001). Increased TNF-α mRNA $t_{1/2}$ (> 180min) by VT was reduced to 60 min in the presence of MEK1 inhibitor. When comparing differential percent remaining TNF-α mRNA in the absence of MEK1 inhibitor to those of TNF-α mRNA in the presence of MEK1 inhibitor over time, the difference due to VT were not changed over a period of time (Figure 5.7C), indicating that VT-induced TNF-α mRNA stability was not likely affected by the presence of MEK1 inhibitor.

The JNK inhibitor, SP600125 (1μM), was used to investigate whether JNK1/2 pathway is involved in mRNA stabilization. DRB treatment resulted in TNF-α mRNA half-life ($t_{1/2}$) of 60 min (Figure 5.8A). VT increased TNF-α mRNA stability, resulting in $t_{1/2}$ of more than 180 min. When JNK inhibitor was coincubated with DRB and without VT

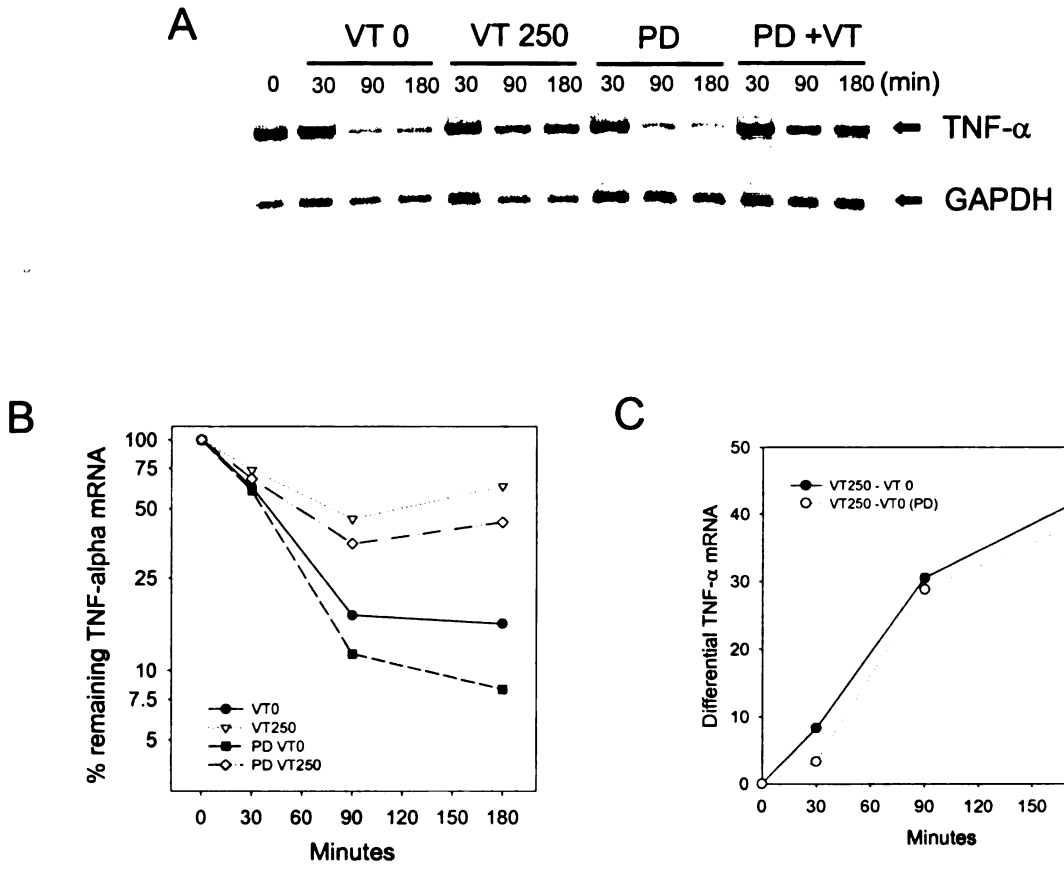


Figure 5.7. Effect of PD98059 on VT-induced TNF-α mRNA expression in LPS-treated RAW264.7 cells. All experimental conditions were the same as described in figure 5.6 legend except the inhibitor. PD98059, 10 μM was used instead. A, blot from RNase protection assay. B, relative TNF-α mRNA levels after normalization with GAPDH gene expression determined by densitometry of blot from RNase protection assay. C, differential induction at each time points by subtracting percent remaining TNF-α mRNA of DRB-treated cells from percent remaining TNF-α mRNA of VT-treated cells. Data are representative of two separate experiments.

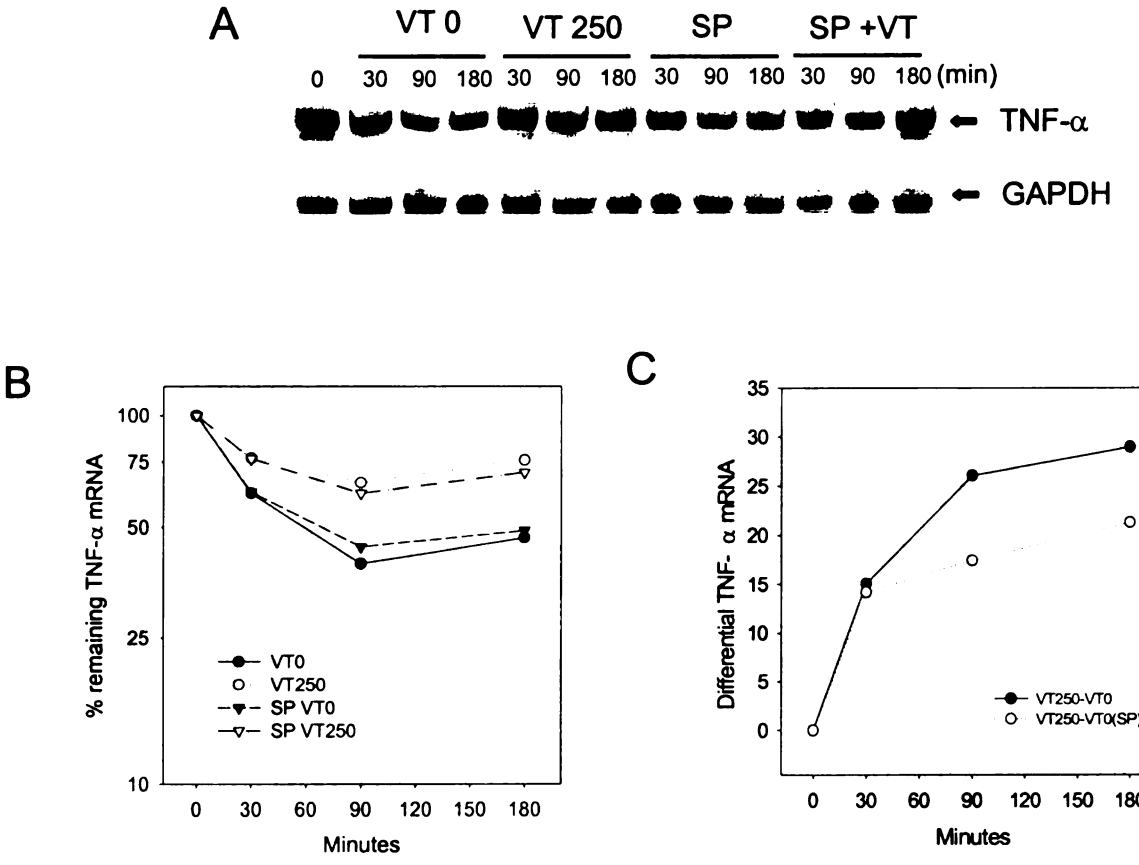


Figure 5.8. Effect of SP600125 on VT-induced TNF-α mRNA expression in LPS-treated RAW264.7 cells. All experimental conditions were the same as described in figure 5.6 legend except the inhibitor. SP600125, 1 μM was used instead. A, blot from RNase protection assay. B, relative TNF-α mRNA levels after normalization with GAPDH gene expression determined by densitometry of blot from RNase protection assay. C, differential induction at each time points by subtracting percent remaining TNF-α mRNA of DRB-treated cells from percent remaining TNF-α mRNA of VT-treated cells. Data are representative of two separate experiments.

treatment, TNF-α mRNA stability was not affected. This observation indicates that activation of JNK1/2 might not be required for stabilization of TNF-α mRNA. When comparing differential percent remaining TNF-α mRNA in the absence of JNK inhibitor to those of TNF-α mRNA in the presence of JNK inhibitor over time, the difference due to VT were not much changed over a period of time (Figure 5.8C), indicating that VT-induced TNF-α mRNA stability was not affected by the presence of JNK inhibitor.

To determine whether activation of p38 kinase, ERK1/2, or JNK1/2 was involved in the regulation of VT-induced and LPS+VT-induced TNF-α secretion, the p38 inhibitor (SB203580), the MEK1 inhibitor (PD98059), or the JNK inhibitor (SP600125) was used, respectively. RAW 264.7 cells were incubated with LPS (100 ng/ml), VT (250 ng/ml) or LPS (100 ng/ml)+VT(250 ng/ml) for 18 hr in the presence of inhibitors. Cell culture supernatants were subjected to TNF-α assay by ELISA. As shown in Figure 5.9A and 5.9B, LPS-, VT-, LPS+VT- induced TNF-α productions were significantly inhibited dose-dependently at 18 hr in the presence of SB203580 and PD98059 in RAW 264.7 cells. In the presence of JNK inhibitor, SP600125, LPS- and LPS+VT-induced TNF-α production were significantly and dose-dependently reduced at 18 hr whereas VT-induced TNF-α production was not affected.

## DISCUSSION

VT superinduces various inflammatory mediators such as proinflammatory cytokines, chemokines, and prostaglandins which have been suggested as major contributors to

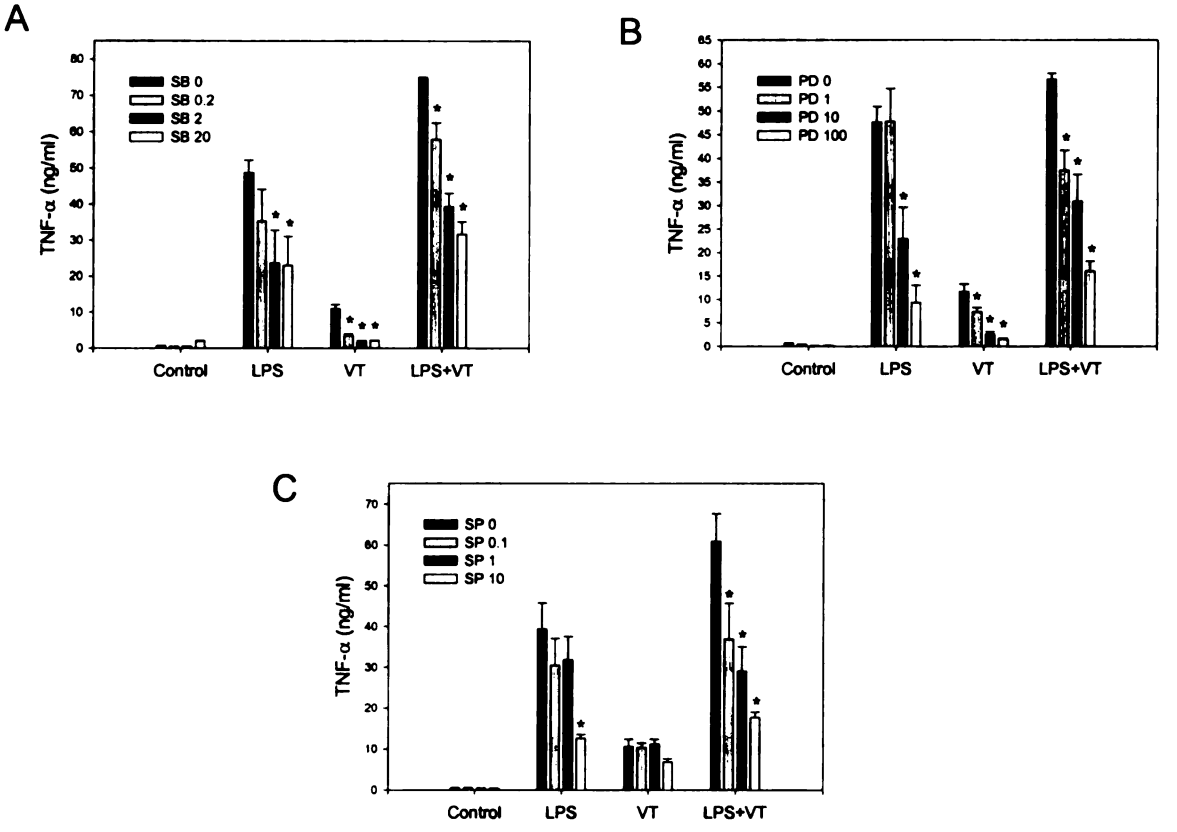


Figure 5.9. Effect of MAPK inhibitors on LPS-, VT-, and LPS+VT- induced TNF-α production in RAW 264.7 macrophage cells. A, cells were incubated with LPS (100 ng/ml), VT (250 ng/ml) or LPS (100 ng/ml)+VT(250 ng/ml) for 18 hr in the presence of 0.2, 2, and 20 μM of SB203580. TNF-α levels in the supernatant were measured by ELISA. Data are the means of triplicate culture supernatants ± SEM. The results are representative of two separate experiments. B, the same conditions as in A except using PD98059 (1, 10, and 100 μM). C, the same conditions as in A except SP600125 (0.1, 1, and 10 μM). An asterisk indicates a significant difference from control with p value < 0.05.

immunopathogenic events induced by the toxin (Bondy and Pestka, 2000; Chung *et al.*, 2002; Moon and Pestka, 2002). Induction of the inflammatory mediators are closely related to activation of MAPKs (Dong *et al.*, 2002). Activation of MAPKs by VT has been observed previously in RAW 264.7 cells (Moon and Pestka, 2002; Yang *et al.*, 2000). In this study, activation of MAPKs by VT was prolonged in the presence of LPS and contributed to superinduction of proinflammatory cytokine, TNF-α, in both transcriptional and post-transcriptional manners. These data suggest that MAPKs play a major role in TNF-α superinduction by VT in RAW 264.7 cells.

Cycloheximide (CHX), a prototype protein synthesis inhibitor, also superinduces the inflammatory mediators. For example, cytokines such as interleukin (IL)-2, IL-5, and IL-6 mRNA and proteins are superinduced by CHX in phorbol 12-myristate 13-acetate (PMA) stimulated EL-4 cells (Azcona-Olivera *et al.*, 1995b; Dong *et al.*, 1994). IL-8 is a potent neutrophil chemoattractant and its mRNA and protein levels are superinduced by TNF-α and LPS in the presence of CHX in lung epithelial cells and bone marrow-derived mononuclear cells, respectively (Dibb *et al.*, 1992; Roger *et al.*, 1998). Those results are further explained by increased transcript and increased mRNA stability followed by enhanced binding activity of transcription factors such as AP-1 and NF-κB (Roger *et al.*, 1998). Cyclooxygenase-2 (COX-2) is an important enzyme required for synthesis of prostaglandin which play an important role in inflammation (Vane *et al.*, 1998). CHX mediates superinduction of COX-2 expression by increased transcript but not by mRNA stabilization in IL-1β-stimulated pulmonary type II A549 cells (Newton *et al.*, 1997). In that study, increased COX-2 transcript is suggested to be due to NF-κB binding to COX-2 promoter κB sites and

activation of JNK. Interestingly, VT superinduces the inflammatory mediators in a similar manner; increased transcripts (Azcona-Olivera *et al.*, 1995b; Dong *et al.*, 1994; Wong *et al.*, 1998), extended mRNA half life (Li *et al.*, 1997; Wong *et al.*, 2001), enhanced binding activity of transcription factors (Li *et al.*, 2000; Wong, 2000), and activation of MAPKs (Moon and Pestka, 2002; Yang *et al.*, 2000). Thus, protein synthesis inhibitors seem to share common pathways, increased binding of transcription factors and increased mRNA transcript stabilization that ultimately contribute to superinduction of inflammatory mediators.

VT-induced MAPK activation may relate to a"ribotoxic stress response". Iordanov et al., (1997) proposed that translational inhibitors such as anisomycin, ricin, and α-sarcin activate JNK1 by binding to or altering the structure of 28S ribosomal RNA, resulting in a "ribotoxic stress response". Trichothecene-induced ribotoxic stress response is known to activate JNK and p38 kinase and consequently induces apoptosis (Shifrin and Anderson, 1999). In addition, activation of ERK1/2, p38 kinase, and JNK1/2 by VT and satratoxins is related to induction of COX-2 expression and apoptosis (Moon and Pestka, 2002; Yang et al., 2000). Shiga toxin also triggers the ribotoxic stress response resulting from activation of JNK1/2 and p38 kinase and consequently produce TNF-α in the human monocyte cell line THP-1 (Foster and Tesh, 2002). Taken together, the ribotoxic stress response by translational inhibitors initiates activation of MAPKs resulting in apoptosis or inflammatory responses. These observations are consistent with our data that activation of MAPK by VT contributes to superinduction of TNF-α through the ribotoxic stress response.

One of major functions of MAPK is to activate transcription factors (Chang and Karin, 2001; Davis, 2000). For example, p38 kinase phosphorylates activating transcription

factor-2 (ATF-2) (Zhu and Lobie, 2000) and Elk-1(Zhu and Lobie, 2000). JNK can also phosphorylate many transcription factors including Elk-1 (Cavigelli *et al.*, 1995), ATF-2 (Botteron and Dobbelaere, 1998), and c-Jun (Hambleton *et al.*, 1996). ERK can induce activation of Elk-1(Babu *et al.*, 2000) and C/EBP-β (Zhu *et al.*, 2002). ATF-2 binds to the cAMP response element (CRE) (Yamada *et al.*, 1997). Elk-1 is a c-fos regulatory transcription factor which recognizes the AP-1 binding site (Papavassiliou, 1994). c-Jun is one of Jun family transcription factors which bind to the AP-1 site (Hambleton *et al.*, 1996). Activation of C/EBP-β leads to enhanced transactivation potential of the factor (Ramji and Foka, 2002). In addition, activation of nuclear factor κB (NF-κB) through phosphorylation of IκBα is related to activation of all three MAPKs (Lee *et al.*, 1997; Schwenger *et al.*, 1998; Zhao and Lee, 1999). The aforementioned AP-1, CRE, and NF-κB binding sites are known to be important in cytokine gene expression in leukocytes. Consistent with these possibilities, our laboratory recently reported that VT increased binding activity of AP-1 through activation of c-Jun and c-Fos, of NF-IL6 through activation of C/EBP-β, of NF-κB through activation of p50 and c-Rel subunits (Wong *et al.*, 2002). Therefore, increased TNF-α promoter reporter activity by VT and/or LPS in this study might be related to increased binding activity of the transcription factors such as AP-1, NF-IL6, and NF-κB resulting from activation of MAPKs since TNF-α promoter is known to possess binding sites for these factors (Pauli, 1994).

Activation of MAPKs by VT might also contribute to stabilization of TNF-α mRNA. The sequences in the 3'-untranslated region (3'-UTR) of TNF-α mRNA is responsible for

mRNA stability and translational repression (Chen and Shyu, 1995; Han *et al.*, 1991). This region contains multiple repeats of an AUUUA motif (AU-rich element, ARE), which are also found in early response genes such as cytokines and protooncogenes (Chambers and Kacinski, 1994; Schiavi *et al.*, 1992; Wang *et al.*, 1997). The AREs are known to be recognized by several RNA binding proteins (Bohjanen *et al.*, 1992; Zhang *et al.*, 1993). Recently, proteins, which bind to ARE of TNF-α mRNA, have been identified; as TIAR (Gueydan *et al.*, 1999), HuR (Sakai *et al.*, 1999), and tristetraprolin (TTP) (Lai *et al.*, 1999). TIAR is cytoplasmic protein and binds to TNF ARE independently of LPS stimulation of macrophages. This protein might be involved in TNF translational repression (Gueydan *et al.*, 1999). HuR, a member of ELAV-like protein family, is important in translocation and protection of ARE-containing RNA (Fan and Steitz, 1998). It is a TNF ARE binding protein which stabilized TNF-α mRNA (Dean *et al.*, 2001). In that study, HuR-mediated stabilization was found to be independent on activation of p38 kinase. TTP is a zinc finger RNA binding protein that is responsible for destabilization of TNF-α mRNA (Lai *et al.*, 1999). This protein is inducible by LPS in macrophages and phosphorylated by MAPK-activated protein kinase-2 (MAPKAPK2 or MK-2) through activated p38 kinase (Mahtani *et al.*, 2001). The phosphorylated form of TTP is suggested to have less binding activity to ARE RNA probe than dephosphorylated TTP (Carballo *et al.*, 2001). In our study, treatment with the p38 inhibitor, SB 203580, greatly decreased TNF-α mRNA stability. This suggests that inhibition of p38 kinase may block phosphorylation of TTP, causing avid binding of dephosphorylated TTP to TNF ARE, resulting in rapid degradation of TNF-α mRNA.

MAPK inhibitors, SB 203580, PD 98059, or SP 600125 have been used to block

activation of p38 kinase, MEK1/2 (upstream of ERK1/2), or JNK1/2, respectively. Efficacy of these inhibitors was tested by checking phosphorylated state of their substrate, ATF-2, $RSK^{90}$, or c-Jun for p38 kinase, ERK1/2 or JNK1/2, respectively, by Western blot analysis on total cell lysate. At highest concentrations of inhibitors used (20 μM of SB 203580, 100 μM of PD 98059, and 10 μM of SP 600125), phosphorylation of these substrates following LPS treatment was reduced by 40 to 50 %. Since the substrates of p38 kinase, ERK1/2, and JNK1/2 can be also phosphorylated by alternate routes such as JNK for ATF-2 activation (Tindberg et al., 2000), p38 kinase for RSK90 activation (Merienne et al., 2000), p38 kinase for c-Jun activation (Yamagishi et al., 2001), it would not be possible to completely block their phosphorylation. In order to accurately measure activities of MAPKs, immunoprecipitation from whole cell extracts using MAPK-specific antibodies could be applied.

Taken together, the results presented here indicate that VT may superinduce TNF-α expression by increasing its transcripts and mRNA stability through activation of MAPK. Trichothecene-induced activation of MAPK may play a critical role in superinduction of proinflammatory cytokine, TNF-α, and succeeding pathologic events. Further research on RNA binding protein, TTP, for stabilization is desirable to better understand VT-induced cytokine superinduction.

# CHAPTER VI

# SUMMARY AND CONCLUSIONS

In this dissertation, firstly, high antibody titers to macrocyclic trichothecene, satratoxin G (SG), were readily achieved upon immunization of rabbits with SG bis-hemisuccinate conjugated to BSA. The antibodies could detect free SG using a CD-ELISA with a range of detection from 0.1 to 100 ng/ml. The observed antibody cross-reactivity may facilitate simultaneous detection of other satratoxins and to a lesser extent, other macrocyclic trichothecenes. Methanol content up to 20% in samples did not largely affect the immunoassay and this stability expands applicability of the antibodies. Secondly, the capacity of representative macrocyclic trichothecenes to alter TNF-α and IL-6 production and viability was assessed in a murine macrophage model. Macrocyclic trichothecenes can superinduce the proinflammatory cytokine, TNF-α, at low cytotoxic concentrations, whereas these compounds are cytotoxic and reduce cytokine production at higher concentrations. These immunomodulatory effects were observed at relatively low (ng/ml) concentrations, suggesting that macrocyclic mycotoxins may pose a hazard to humans exposed to *Stachybotrys*. Thirdly, in order to expand understanding of how trichothecenes affect gene regulation, the VT- and SG-responsive genes, MIP-2 and C3aR, were isolated from a murine macrophage cell line using DD-PCR. These findings provide further insight into potential mechanisms of trichothecene-induced tissue injury and immunotoxicity. Additional studies of the mechanisms MIP-2 and C3aR induction by trichothecenes are necessary to understand their roles in immunopathological effects of the toxins. In addition, DD-PCR is subject to a number of limitations, most notably the use of many multiple primer combinations as well as the generation of many clones with no known relationship to existing genes. Further study of trichothecene-altered gene regulation could be greatly enhanced by application of

microarray technology which has evolved significantly since the onset of this DD-PCR study. Lastly, the effects of VT on MAPK activation, as well as the relationship of MAPKs to VT-induced mRNA expression, TNF-α promoter activity, TNF-α mRNA stability, and TNF-α protein production were assessed in RAW 264.7 murine macrophage cells. The results indicate that VT may superinduce TNF-α expression by increasing its transcripts and mRNA stability through activation of MAPK. Trichothecene-induced activation of MAPK may play a critical role in superinduction of proinflammatory cytokine, TNF-α, and succeeding pathologic events. Further research on RNA binding protein, TTP, for stabilization is necessary to better understand VT-induced cytokine superinduction.

Overall, the studies in this dissertation will contribute to understanding how trichothecenes can modulate immune function. In the long run, results of these studies would be used to reduce a health risk due to exposure to trichothecenes.

# BIBLIOGRAPHY

Abouzied, M. M., Azcona-Olivera, J. I., Yoshizawa, T., and Pestka, J. J. (1993). Production of polyclonal antibodies to the trichothecene mycotoxin 4,15-diacetylnivalenol with the carrier-adjuvant cholera toxin. *Appl Environ Microbiol* **59**, 1264-1268.

Abraham, E., Carmody, A., Shenkar, R., and Arcaroli, J. (2000). Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* **279**, L1137-1145.

Agriculture, C. (1989). Mycotoxins: Economic and Health Risks.

Arnold, D. L., McGuire, P. F., Nera, E. A., Karpinski, K. F., Bickis, M. G., Zawidzka, Z. Z., Fernie, S., and Vesonder, R. F. (1986). The toxicity of orally administered deoxynivalenol (vomitoxin) in rats and mice. *Food Chem Toxicol* **24**, 935-941.

Azcona-Olivera, J. I., Ouyang, Y. L., Warner, R. L., Linz, J. E., and Pestka, J. J. (1995b). Effects of vomitoxin (deoxynivalenol) and cycloheximide on IL-2, 4, 5 and 6 secretion and mRNA levels in murine CD4+ cells. *Food Chem Toxicol* **33**, 433-441.

Azcona-Olivera, J. I., Ouyang, Y., Murtha, J., Chu, F. S., and Pestka, J. J. (1995a). Induction of cytokine mRNAs in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): relationship to toxin distribution and protein synthesis inhibition. *Toxicol Appl Pharmacol* **133**, 109-120.

Azcona-Olivera, J. I., Abouzied, M. M., Plattner, R. D., Norred, W. P., and Pestka, J. J. (1992). Generation of antibodies reactive with fumonisins B1, B2, and B3 by using cholera toxin as the carrier-adjuvant. *Appl Environ Microbiol* **58**, 169-173.

Babu, G. J., Lalli, M. J., Sussman, M. A., Sadoshima, J., and Periasamy, M. (2000). Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. *J Mol Cell Cardiol* **32**, 1447-1457.

Baker, D. M., Wang, S. L., Bell, D. J., Drevon, C. A., and Davis, R. A. (2000). One or more labile proteins regulate the stability of chimeric mRNAs containing the 3'-untranslated region of cholesterol-7alpha - hydroxylase mRNA. *J Biol Chem* **275**, 19985-19991.

Barna-Vetro, I., Gyongyosi, A., and Solti, L. (1994). Monoclonal antibody-based enzyme-linked immunosorbent assay of Fusarium T-2 and zearalenone toxins in cereals. *Appl Environ Microbiol* **60**, 729-731.

Bata, A., Harrach, B., Ujszaszi, K., Kis-Tamas, A., and Lasztity, R. (1985). Macrocyclic trichothecene toxins produced by Stachybotrys atra strains isolated in Middle Europe. *Appl Environ Microbiol* **49**, 678-681.

Beagley, K. W., and Elson, C. O. (1992). Cells and cytokines in mucosal immunity and inflammation. *Gastroenterol Clin North Am* **21**, 347-366.

Beagley, K. W., Eldridge, J. H., Aicher, W. K., Mestecky, J., Di Fabio, S., Kiyono, H., and McGhee, J. R. (1991). Peyer's patch B cells with memory cell characteristics undergo terminal differentiation within 24 hours in response to interleukin-6. *Cytokine* **3**, 107-116.

Beutler, B., and Brown, T. (1991). A CAT reporter construct allows ultrasensitive estimation of TNF synthesis, and suggests that the TNF gene has been silenced in non-macrophage cell lines. *J Clin Invest* **87**, 1336-1344.

Beutler, B., and van Huffel, C. (1994). Unraveling function in the TNF ligand and receptor families [comment]. *Science* **264**, 667-668.

Beutler, B. A. (1999). The role of tumor necrosis factor in health and disease. *J Rheumatol* **26 Suppl 57**, 16-21.

Bhat, R. V., Beedu, S. R., Ramakrishna, Y., and Munshi, K. L. (1989). Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat production in Kashmir Valley, India. *Lancet* **1**, 35-37.

Biabani, S., and Dowling, T. (1996). Vomitoxin reports continue in the Midwest. In PR Newswire.

Bohjanen, P. R., Petryniak, B., June, C. H., Thompson, C. B., and Lindsten, T. (1992). AU RNA-binding factors differ in their binding specificities and affinities. *J Biol Chem* **267**, 6302-6309.

Bondy, G. S., and Pestka, J. J. (2000). Immunomodulation by fungal toxins. *J Toxicol Environ Health B Crit Rev* **3**, 109-143.

Bondy, G. S., and Pestka, J. J. (1991). Dietary exposure to the trichothecene vomitoxin (deoxynivalenol) stimulates terminal differentiation of Peyer's patch B cells to IgA secreting plasma cells. *Toxicol Appl Pharmacol* **108**, 520-530.

Botteron, C., and Dobbelaere, D. (1998). AP-1 and ATF-2 are constitutively activated via the JNK pathway in Theileria parva-transformed T-cells. *Biochem Biophys Res Commun* **246**, 418-421.

Brinkman, B. M., Telliez, J. B., Schievella, A. R., Lin, L. L., and Goldfeld, A. E. (1999). Engagement of tumor necrosis factor (TNF) receptor 1 leads to ATF-2- and p38

mitogen-activated protein kinase-dependent TNF-alpha gene expression. *J Biol Chem* **274**, 30882-30886.

Brook, M., Sully, G., Clark, A. R., and Saklatvala, J. (2000). Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS Lett* **483**, 57-61.

Browning, J. L., Sizing, I. D., Lawton, P., Bourdon, P. R., Rennert, P. D., Majeau, G. R., Ambrose, C. M., Hession, C., Miatkowski, K., Griffiths, D. A., Ngam-ek, A., Meier, W., Benjamin, C. D., and Hochman, P. S. (1997). Characterization of lymphotoxin-alpha beta complexes on the surface of mouse lymphocytes. *J Immunol* **159**, 3288-3298.

Burd, P. R., Rogers, H. W., Gordon, J. R., Martin, C. A., Jayaraman, S., Wilson, S. D., Dvorak, A. M., Galli, S. J., and Dorf, M. E. (1989). Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J Exp Med* **170**, 245-257.

Cannon, M., Jimenez, A., and Vazquez, D. (1976). Competition between trichodermin and several other sesquiterpene antibiotics for binding to their receptor site(s) on eukaryotic ribosomes. *Biochem J* **160**, 137-145.

Cano, E., Hazzalin, C. A., and Mahadevan, L. C. (1994). Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. *Mol Cell Biol* **14**, 7352-7362.

Carballo, E., Cao, H., Lai, W. S., Kennington, E. A., Campbell, D., and Blackshear, P. J. (2001). Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. *J Biol Chem* **276**, 42580-42587.

Cavigelli, M., Dolfi, F., Claret, F. X., and Karin, M. (1995). Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *Embo J* **14**, 5957-5964.

Chakraborti, S., and Chakraborti, T. (1998). Oxidant-mediated activation of mitogen-activated protein kinases and nuclear transcription factors in the cardiovascular system: a brief overview. *Cell Signal* **10**, 675-683.

Chambers, S. K., and Kacinski, B. M. (1994). Messenger RNA decay of macrophage colony-stimulating factor in human ovarian carcinomas in vitro. *J Soc Gynecol Investig* **1**, 310-316.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* **410**,

37-40.

Chantry, D., and Feldman, M. (1990). The role of cytokines in autoimmunity. *Biotechnol. Ther.* **1**, 361-409.

Chen, C. Y., Del Gatto-Konczak, F., Wu, Z., and Karin, M. (1998). Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science* **280**, 1945-1949.

Chen, C. Y., and Shyu, A. B. (1995). AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* **20**, 465-470.

Chen, C. C., and Wang, J. K. (1999). p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol Pharmacol* **55**, 481-488.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal.Biochem.* **162**, 156-159.

Chung, Y. J., Jarvis, B. B., and Pestka, J. J. (2002). Modulation of lipopolysaccharide-induced proinflammatory cytokine production by satratoxins and other macrocyclic trichothecenes in the murine macrophage. *J Toxicol environ Health* **In press**.

Chung, Y. J., Yang, G., Islam, Z., and Pestka, J. J. (2002). Up-regulation of macrophage inflammatory protein-2 and complement 3a receptor by the trichothecenes deoxynivalenol and satratoxin G. *Toxicology* **submitted**.

Colletti, L. M., Kunkel, S. L., Walz, A., Burdick, M. D., Kunkel, R. G., Wilke, C. A., and Strieter, R. M. (1995). Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat. The role of epithelial neutrophil activating protein. *J Clin Invest* **95**, 134-141.

Czerkinsky, C., Koopman, W. J., Jackson, S., Collins, J. E., Crago, S. S., Schrohenloher, R. E., Julian, B. A., Galla, J. H., and Mestecky, J. (1986). Circulating immune complexes and immunoglobulin A rheumatoid factor in patients with mesangial immunoglobulin A nephropathies. *J Clin Invest* **77**, 1931-1938.

D'Amico, G. (1987). The commonest glomerulonephritis in the world: IgA nephropathy. *Q J Med* **64**, 709-727.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252.

Davoust, N., Jones, J., Stahel, P. F., Ames, R. S., and Barnum, S. R. (1999). Receptor for the C3a anaphylatoxin is expressed by neurons and glial cells. *Glia* **26**, 201-211.

de Vries, J. E., Punnonen, J., Cocks, B. G., and Aversa, G. (1993). The role of T/B cell interactions and cytokines in the regulation of human IgE synthesis. *Semin Immunol* **5**, 431-439.

De Cesaris, P., Starace, D., Riccioli, A., Padula, F., Filippini, A., and Ziparo, E. (1998). Tumor necrosis factor-alpha induces interleukin-6 production and integrin ligand expression by distinct transduction pathways. *J.Biol.Chem.* **273**, 7566-7571.

Dean, J. L., Wait, R., Mahtani, K. R., Sully, G., Clark, A. R., and Saklatvala, J. (2001). The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. *Mol Cell Biol* **21**, 721-730.

Dearborn, D. G. (1997). Pulmonary hemorrhage in infants and children. *Curr Opin Pediatr* **9**, 219-224.

Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F., and Banchereau, J. (1992). Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* **175**, 671-682.

DeMaria, C. T., and Brewer, G. (1996). AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. *J Biol Chem* **271**, 12179-12184.

Diaz, B., and Lopez-Berestein, G. (2000). A distinct element involved in lipopolysaccharide activation of the tumor necrosis factor-alpha promoter in monocytes [In Process Citation]. *J.Interferon Cytokine Res.* **20**, 741-748.

Dibb, C. R., Strieter, R. M., Burdick, M., and Kunkel, S. L. (1992). Expression of interleukin-8 by lipopolysaccharide-stimulated bone marrow-derived mononuclear cells. *Infect Immun* **60**, 3052-3058.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest* **118**, 503-508.

Dong, W., Sell, J. E., and Pestka, J. J. (1991). Quantitative assessment of mesangial immunoglobulin A (IgA) accumulation, elevated circulating IgA immune complexes, and hematuria during vomitoxin-induced IgA nephropathy. *Fundam Appl Toxicol* **17**, 197-207.

Dong, W., Azcona-Olivera, J. I., Brooks, K. H., Linz, J. E., and Pestka, J. J. (1994). Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to

vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol Appl Pharmacol* **127**, 282-290.

Dong, W., and Pestka, J. J. (1993). Persistent dysregulation of IgA production and IgA nephropathy in the B6C3F1 mouse following withdrawal of dietary vomitoxin (deoxynivalenol). *Fundam Appl Toxicol* **20**, 38-47.

Dong, C., Davis, R. J., and Flavell, R. A. (2002). MAP kinases in the immune response. *Annu Rev Immunol* **20**, 55-72.

Donnelly, S. C., Strieter, R. M., Kunkel, S. L., Walz, A., Steedman, D., Grant, I. S., Pollok, A. J., Carter, D. C., and Haslett, C. (1994). Chemotactic cytokines in the established adult respiratory distress syndrome and at-risk patients. *Chest* **105**, 98S-99S.

Driscoll, K. E. (1994). Macrophage inflammatory proteins: biology and role in pulmonary inflammation. *Exp Lung Res* **20**, 473-490.

Driscoll, K. E., Hassenbein, D. G., Carter, J., Poynter, J., Asquith, T. N., Grant, R. A., Whitten, J., Purdon, M. P., and Takigiku, R. (1993). Macrophage inflammatory proteins 1 and 2: expression by rat alveolar macrophages, fibroblasts, and epithelial cells and in rat lung after mineral dust exposure. *Am J Respir Cell Mol Biol* **8**, 311-318.

Drouin, S. M., Kildsgaard, J., Haviland, J., Zabner, J., Jia, H. P., McCray, P. B., Jr., Tack, B. F., and Wetsel, R. A. (2001). Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol* **166**, 2025-2032.

Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* **103**, 1071-1083.

Elidemir, O., Colasurdo, G. N., Rossmann, S. N., and Fan, L. L. (1999). Isolation of Stachybotrys from the lung of a child with pulmonary hemosiderosis. *Pediatrics* **104**, 964-966.

Emancipator, S. N., and Lamm, M. E. (1989). IgA nephropathy: pathogenesis of the most common form of glomerulonephritis. *Lab Invest* **60**, 168-183.

Esnault, S., and Malter, J. S. (2002). Extracellular signal-regulated kinase mediates granulocyte-macrophage colony-stimulating factor messenger RNA stabilization in tumor necrosis factor-alpha plus fibronectin-activated peripheral blood eosinophils.

*Blood* **99**, 4048-4052.

Esser, C., and Radbruch, A. (1990). Immunoglobulin class switching: molecular and cellular analysis. *Annu Rev Immunol* **8**, 717-735.

Etzel, R. A., Montana, E., Sorenson, W. G., Kullman, G. J., Allan, T. M., Dearborn, D. G., Olson, D. R., Jarvis, B. B., and Miller, J. D. (1998). Acute pulmonary hemorrhage in infants associated with exposure to Stachybotrys atra and other fungi. *Arch Pediatr Adolesc Med* **152**, 757-762.

Fan, X. C., and Steitz, J. A. (1998). HNS, a nuclear-cytoplasmic shuttling sequence in HuR. *Proc Natl Acad Sci U S A* **95**, 15293-15298.

Feinberg, B., and MacLaughlin, C. S. (1989). Biochemical mechanism of action of trichethecene mycotoxins. In Trichothecene mycotoxins: pathophysiologic effects (V. R. Beasley, ed., pp. 27-36. CRC Press.

Fischer, W. H., and Hugli, T. E. (1997). Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. *J Immunol* **159**, 4279-4286.

Fischer, W. H., Jagels, M. A., and Hugli, T. E. (1999). Regulation of IL-6 synthesis in human peripheral blood mononuclear cells by C3a and C3a(desArg). *J Immunol* **162**, 453-459.

Fong, C. W., Siddiqui, A. H., and Mark, D. F. (1995). Characterization of protein complexes formed on the repressor elements of the human tumor necrosis factor alpha gene. *J Interferon Cytokine Res* **15**, 887-895.

Forgacs, J. (1972). Stachybotryotoxicosis. In Microbial Toxins (S. Kadis, A. Ciegler and S. J. Ajl, eds.), pp. 95-128. Academic Press, Inc.,, New York.

Forsell, J. H., Jensen, R., Tai, J. H., Witt, M., Lin, W. S., and Pestka, J. J. (1987). Comparison of acute toxicities of deoxynivalenol (vomitoxin) and 15-acetyldeoxynivalenol in the B6C3F1 mouse. *Food Chem Toxicol* **25**, 155-162.

Forsell, J. H., Witt, M. F., Tai, J. H., Jensen, R., and Pestka, J. J. (1986). Effects of 8-week exposure of the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. *Food Chem Toxicol* **24**, 213-219.

Forsyth, D. M., Yoshizawa, T., Morooka, N., and Tuite, J. (1977). Emetic and refusal activity of deoxynivalenol to swine. *Appl Environ Microbiol* **34**, 547-552.

Foster, G. H., and Tesh, V. L. (2002). Shiga toxin 1-induced activation of c-Jun NH(2)-terminal kinase and p38 in the human monocytic cell line THP-1: possible involvement in the production of TNF-alpha. *J Leukoc Biol* **71**, 107-114.

Friend, D. W., Trenholm, H. L., Elliot, J. I., Thompson, B. K., and Hartin, K. E. (1982). Effect of feeding vomitoxin-contaminated wheat to pigs. *Can.J.Anim Sci.* **62**, 1211-1222.

Garcia, J., Lemercier, B., Roman-Roman, S., and Rawadi, G. (1998). A Mycoplasma fermentans-derived synthetic lipopeptide induces AP-1 and NF-kappaB activity and cytokine secretion in macrophages via the activation of mitogen-activated protein kinase pathways. *J Biol Chem* **273**, 34391-34398.

Gasque, P., Singhrao, S. K., Neal, J. W., Wang, P., Sayah, S., Fontaine, M., and Morgan, B. P. (1998). The receptor for complement anaphylatoxin C3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. *J Immunol* **160**, 3543-3554.

Gillis, P., and Malter, J. S. (1991). The adenosine-uridine binding factor recognizes the AU-rich elements of cytokine, lymphokine, and oncogene mRNAs. *J Biol Chem* **266**, 3172-3177.

Glovsky, M. M., Hugli, T. E., Ishizaka, T., Lichtenstein, L. M., and Erickson, B. W. (1979). Anaphylatoxin-induced histamine release with human leukocytes: studies of C3a leukocyte binding and histamine release. *J Clin Invest* **64**, 804-811.

Gosiewska, A., Yi, C. F., Blanc-Brude, O., and Geesin, J. C. (1999). Characterization of a macrophage-based system for studying the activation of latent TGF-beta. *Methods Cell Sci* **21**, 47-56.

Gosset, P., Malaquin, F., Delneste, Y., Wallaert, B., Capron, A., Joseph, M., and Tonnel, A. B. (1993). Interleukin-6 and interleukin-1 alpha production is associated with antigen-induced late nasal response. *J Allergy Clin Immunol* **92**, 878-890.

Grove, J. F. (1996). Non-macrocyclic trichothecenes. Part 2. *Prog.Chem.Org.Nat.Prod.* **69**, 1-70.

Grove, J. F. (1988). Non-macrocyclic trichothecenes. *Nat.Prod.Rep.* **5**, 187-209.

Grove, J. F. (1993). Macrocyclic trichothecenes. *Nat. Prod. Rep.* **10**, 429-448.

Gueydan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D., and Kruys, V. (1999). Identification of TIAR as a protein binding to the translational regulatory AU-rich

element of tumor necrosis factor alpha mRNA. *J Biol Chem* **274**, 2322-2326.

Guha, M., and Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cell Signal* **13**, 85-94.

Habermehl, G. G., Busam, L., Heydel, P., Mebs, D., Tokarnia, C. H., Dobereiner, J., and Spraul, M. (1985). Macrocyclic trichothecenes: cause of livestock poisoning by the Brazilian plant Baccharis coridifolia. *Toxicon* **23**, 731-745.

Hambleton, J., Weinstein, S. L., Lem, L., and DeFranco, A. L. (1996). Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide- stimulated macrophages. *Proc Natl Acad Sci U S A* **93**, 2774-2778.

Han, J., Huez, G., and Beutler, B. (1991). Interactive effects of the tumor necrosis factor promoter and 3'- untranslated regions. *J Immunol* **146**, 1843-1848.

Harrach, B., Mirocha, C. J., Pathre, S. V., and Palyusik, M. (1981). Macrocyclic trichothecene toxins produced by a strain of Stachybotrys atra from Hungary. *Appl Environ Microbiol* **41**, 1428-1432.

Harrach, B., Bata, A., Bajmocy, E., and Benko, M. (1983). Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotryotoxicosis. *Appl Environ Microbiol* **45**, 1419-1422.

Hazzalin, C. A., Cano, E., Cuenda, A., Barratt, M. J., Cohen, P., and Mahadevan, L. C. (1996). p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. *Curr Biol* **6**, 1028-1031.

Hazzalin, C. A., Le Panse, R., Cano, E., and Mahadevan, L. C. (1998). Anisomycin selectively desensitizes signalling components involved in stress kinase activation and fos and jun induction. *Mol Cell Biol* **18**, 1844-1854.

Hinkley, S. F., Mazzola, E. P., Fettinger, J. C., Lam, Y. F., and Jarvis, B. B. (2000). Atranones A-G, from the toxigenic mold Stachybotrys chartarum. *Phytochemistry* **55**, 663-673.

Hinkley, S. F., and Jarvis, B. B. (2000). In *Method for Stachybotrys Toxins*. (Hinkley, S. F., and Jarvis, B. B.), pp 173-194. Humana Press, Totowa, NJ.

Hintikka, E. L. (1978). Mycotoxins, mycotoxicoses. In Mycotoxigenic Fungi (T. D. Wyllie and L. G. Morehouse, eds.), pp. 87-89. Marcer Dekker.

Huang, S., Paulauskis, J. D., Godleski, J. J., and Kobzik, L. (1992). Expression of

macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. *Am J Pathol* **141**, 981-988.

Hughes, B. J., Jarvis, B. B., and Sharma, R. P. (1990). Effects of macrocyclic trichothecene congeners on the viability and mitogenesis of murine splenic lymphocytes. *Toxicol Lett* **50**, 57-67.

Hugli, T. E. (1990). Structure and function of C3a anaphylatoxin. *Curr Top Microbiol Immunol* **153**, 181-208.

Hunder, G., Schumann, K., Strugala, G., Gropp, J., Fichtl, B., and Forth, W. (1991). Influence of subchronic exposure to low dietary deoxynivalenol, a trichothecene mycotoxin, on intestinal absorption of nutrients in mice. *Food Chem Toxicol* **29**, 809-814.

Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L., and Magun, B. E. (1997). Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol Cell Biol* **17**, 3373-3381.

Ishii, K. (1983). Chemistry of Trichothecenes. In Trichothecenes: Chemical, biological and toxicological aspects (Y. Ueno, ed. Elsevier, New York.

Islam, Z., Moon, Y. S., Zhou, H. R., King, L. E., Fraker, P. J., and Pestka, J. J. (2002). Endotoxin potentiation of trichothecene-induced lymphocyte apoptosis is mediated by up-regulation of glucocorticoids. *Toxicol Appl Pharmacol* **180**, 43-55.

Jackson, L. S., and Bullerman, L. B. (1999). Effect of processing on Fusarium mycotoxins. *Adv Exp Med Biol* **459**, 243-261.

Jarvis, B. B., Midiwo, J. O., Bean, G. A., Aboul-Nasr, M. B., and Barros, C. S. (1988). The mystery of trichothecene antibiotics in Baccharis species. *J Nat Prod* **51**, 736-744.

Jarvis, B. B., Salemme, J., and Morais, A. (1995). Stachybotrys toxins. 1. *Nat Toxins* **3**, 10-16.

Jarvis, B. B., Sorenson, W. G., Hintikka, E. L., Nikulin, M., Zhou, Y., Jiang, J., Wang, S., Hinkley, S., Etzel, R. A., and Dearborn, D. (1998). Study of toxin production by isolates of Stachybotrys chartarum and Memnoniella echinata isolated during a study of pulmonary hemosiderosis in infants. *Appl Environ Microbiol* **64**, 3620-3625.

Jarvis, B. B., and Wang, S. (1999). Stereochemistry of the roridins. Diastereomers of roridin

E. *J Nat Prod* **62**, 1284-1289.

Jijon, H. B., Panenka, W. J., Madsen, K. L., and Parsons, H. G. (2002). MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. *Am J Physiol Cell Physiol* **283**, C31-41.

Johanning, E., Biagini, R., Hull, D., Morey, P., Jarvis, B., and Landsbergis, P. (1996). Health and immunology study following exposure to toxigenic fungi (Stachybotrys chartarum) in a water-damaged office environment. *Int Arch Occup Environ Health* **68**, 207-218.

Kalish, D. E. (1995). FDA checking human food for dog-food toxin - Fungus byproduct may be in wheat crop. In The Seattle Times.

Kildsgaard, J., Hollmann, T. J., Matthews, K. W., Bian, K., Murad, F., and Wetsel, R. A. (2000). Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *J Immunol* **165**, 5406-5409.

Kincaid-Smith, P., Nicholls, K., and Birchall, I. (1989). Polymorphs infiltrate glomeruli in mesangial IgA glomerulonephritis. *Kidney Int* **36**, 1108-1111.

Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., Walz, A., and Strieter, R. M. (1994). Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis. *J Clin Invest* **94**, 1012-1018.

Koj, A. (1996). Initiation of acute phase response and synthesis of cytokines. *Biochim Biophys Acta* **1317**, 84-94.

Kopydlowski, K. M., Salkowski, C. A., Cody, M. J., van Rooijen, N., Major, J., Hamilton, T. A., and Vogel, S. N. (1999). Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol* **163**, 1537-1544.

Krishnamurthy, T., Beck, D. J., Isensee, R. K., and Jarvis, B. B. (1989). Mass spectral investigations on trichothecene mycotoxins. VII. Liquid chromatographic-thermospray mass spectrometric analysis of macrocyclic trichothecenes. *J Chromatogr* **469**, 209-222.

Kuby, J. (1997). Leukocyte migration and inflammation in immunology. In (J. Kuby, ed., pp. 357-378. W.H.Freeman and Company, New York.

Kyriakis, J. M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal

transduction pathways activated by stress and inflammation. *Physiol Rev* **81**, 807-869.

Lai, K. N., Leung, J. C., Li, P. K., and Lui, S. F. (1991). Cytokine production by peripheral blood mononuclear cells in IgA nephropathy. *Clin Exp Immunol* **85**, 240-245.

Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999). Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Mol Cell Biol* **19**, 4311-4323.

Lai, K. N., Shute, J. K., Lindley, I. J., Lai, F. M., Yu, A. W., Li, P. K., and Lai, C. K. (1996). Neutrophil attractant protein-1 interleukin 8 and its autoantibodies in IgA nephropathy. *Clin Immunol Immunopathol* **80**, 47-54.

Lai, K. N., Ho, R. T., Leung, J. C., Lai, F. M., and Li, P. K. (1994c). Increased mRNA encoding for transforming factor-beta in CD4+ cells from patients with IgA nephropathy. *Kidney Int* **46**, 862-868.

Lai, K. N., Ho, R. T., Lai, C. K., Chan, C. H., and Li, P. K. (1994a). Increase of both circulating Th1 and Th2 T lymphocyte subsets in IgA nephropathy. *Clin Exp Immunol* **96**, 116-121.

Lai, K. N., Ho, R. T., Leung, J. C., Chui, Y. L., Lim, P. L., Lui, S. F., and Li, P. K. (1994b). CD4-positive cells from patients with IgA nephropathy demonstrate increased mRNA of cytokines that induce the IgA switch and differentiation. *J Pathol* **174**, 13-22.

Lebman, D. A., and Edmiston, J. S. (1999). The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. *Microbes.Infect.* **1**, 1297-1304.

Lee, M. G., Li, S., Jarvis, B. B., and Pestka, J. J. (1999). Effects of satratoxins and other macrocyclic trichothecenes on IL-2 production and viability of EL-4 thymoma cells. *J.Toxicol.Environ.Health* **57**, 459-474.

Lee, F. S., Hagler, J., Chen, Z. J., and Maniatis, T. (1997). Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88**, 213-222.

Lee, R. C., Wei, R. D., and Chu, F. S. (1989). Enzyme-linked immunosorbent assay for T-2 toxin metabolites in urine. *J Assoc Off Anal Chem* **72**, 345-348.

Legler, D. F., Loetscher, M., Jones, S. A., Dahinden, C. A., Arock, M., and Moser, B. (1996). Expression of high- and low-affinity receptors for C3a on the human mast

cell line, HMC-1. *Eur J Immunol* **26**, 753-758.

Lesavre, P., Digeon, M., and Bach, J. F. (1982). Analysis of circulating IgA and detection of immune complexes in primary IgA nephropathy. *Clin Exp Immunol* **48**, 61-69.

Li, S., Ouyang, Y. L., Dong, W., and Pestka, J. J. (1997). Superinduction of IL-2 gene expression by vomitoxin (deoxynivalenol) involves increased mRNA stability. *Toxicol Appl Pharmacol* **147**, 331-342.

Li, F. Q., Luo, X. Y., and Yoshizawa, T. (1999). Mycotoxins (trichothecenes, zearalenone and fumonisins) in cereals associated with human red-mold intoxications stored since 1989 and 1991 in China. *Nat.Toxins.* **7**, 93-97.

Li, S., Ouyang, Y., Yang, G. H., and Pestka, J. J. (2000). Modulation of transcription factor AP-1 activity in murine EL-4 thymoma cells by vomitoxin (deoxynivalenol). *Toxicol Appl Pharmacol* **163**, 17-25.

Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction [see comments]. *Science* **257**, 967-971.

Liao, L. L., Grollman, A. P., and Horwitz, S. B. (1976). Mechanism of action of the 12,13-epoxytrichothecene, anguidine, an inhibitor of protein synthesis. *Biochim Biophys Acta* **454**, 273-284.

Liu, H., Sidiropoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pope, R. M. (2000). TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J.Immunol.* **164**, 4277-4285.

Luo, Y., Yoshizawa, T., and Katayama, T. (1990). Comparative study on the natural occurrence of Fusarium mycotoxins (trichothecenes and zearalenone) in corn and wheat from. *Appl.Environ.Microbiol.* **56**, 3723-3726.

Lynch, J. P., Standiford, T. J., Rolfe, M. W., Kunkel, S. L., and Strieter, R. M. (1992). Neutrophilic alveolitis in idiopathic pulmonary fibrosis. The role of interleukin-8. *Am Rev Respir Dis* **145**, 1433-1439.

Madireddi, M. T., Dent, P., and Fisher, P. B. (2000). Regulation of mda-7 gene expression during human melanoma differentiation. *Oncogene* **19**, 1362-1368.

Mahadevan, L. C., and Edwards, D. R. (1991). Signalling and superinduction [letter]. *Nature* **349**, 747-748.

Mahtani, K. R., Brook, M., Dean, J. L., Sully, G., Saklatvala, J., and Clark, A. R. (2001).

Mitogen-Activated Protein Kinase p38 Controls the Expression and Posttranslational Modification of Tristetraprolin, a Regulator of Tumor Necrosis Factor Alpha mRNA Stability. *Mol Cell Biol* **21**, 6461-6469.

Marin, M. L., Murtha, J., Dong, W., and Pestka, J. J. (1996). Effects of mycotoxins on cytokine production and proliferation in EL-4 thymoma cells. *J Toxicol Environ Health* **48**, 379-396.

Martin, U., Bock, D., Arseniev, L., Tornetta, M. A., Ames, R. S., Bautsch, W., Kohl, J., Ganser, A., and Klos, A. (1997). The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. *J Exp Med* **186**, 199-207.

Martlbauer, E., Gareis, M., and Terplan, G. (1988). Enzyme immunoassay for the macrocyclic trichothecene roridin A: production, properties, and use of rabbit antibodies. *Appl Environ Microbiol* **54**, 225-230.

McGhee, J. R., Mestecky, J., Elson, C. O., and Kiyono, H. (1989). Regulation of IgA synthesis and immune response by T cells and interleukins. *J Clin Immunol* **9**, 175-199.

McGhee, J. R., Beagley, K. W., and Taguch, T. (1990). In Molecular aspects of immune response and infectious disease (H. Kiyono, E. Jirillo and DeSimone, eds.), pp. 67-76.

Merienne, K., Jacquot, S., Zeniou, M., Pannetier, S., Sassone-Corsi, P., and Hanauer, A. (2000). Activation of RSK by UV-light: phosphorylation dynamics and involvement of the MAPK pathway. *Oncogene* **19**, 4221-4229.

Middlebrook, J. L., and Leatherman, D. L. (1989). Binding of T-2 toxin to eukaryotic cell ribosomes. *Biochem Pharmacol* **38**, 3103-3110.

Miller, K., and Atkinson, H. A. (1986). The in vitro effects of trichothecenes on the immune system. *Food Chem Toxicol* **24**, 545-549.

Minden, A., and Karin, M. (1997). Regulation and function of the JNK subgroup of MAP kinases. *Biochim Biophys Acta* **1333**, F85-104.

Ming, X. F., Kaiser, M., and Moroni, C. (1998). c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells. *EMBO J* **17**, 6039-6048.

Miyazawa, K., Mori, A., Miyata, H., Akahane, M., Ajisawa, Y., and Okudaira, H. (1998).

Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem* **273**, 24832-24838.

Monti, S. M., Fogliano, V., Randazzo, G., Peluso, G., Logrieco, A., and Ritieni, A. (1999). Polyclonal antibodies against fusaproliferin. *Can J Microbiol* **45**, 45-50.

Moon, Y., and Pestka, J. J. (2002). Vomitoxin-induced cyclooxygenase-2 gene expression in macrophage mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases. *Toxicol Sci* **in press**.

Morello, D., Lavenu, A., and Babinet, C. (1990). Differential regulation and expression of jun, c-fos and c-myc proto-oncogenes during mouse liver regeneration and after inhibition of protein synthesis. *Oncogene* **5**, 1511-1519.

Morgan, E. L. (1986). Modulation of the immune response by anaphylatoxins. *Complement* **3**, 128-136.

Murray, L. J., Lee, R., and Martens, C. (1990). In vivo cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-lpr/lpr mouse. *Eur J Immunol* **20**, 163-170.

Myer, V. E., Fan, X. C., and Steitz, J. A. (1997). Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. *Embo J* **16**, 2130-2139.

Nakamaki, T., Imamura, J., Brewer, G., Tsuruoka, N., and Koeffler, H. P. (1995). Characterization of adenosine-uridine-rich RNA binding factors. *J Cell Physiol* **165**, 484-492.

Namikoshi, M., Akano, K., Meguro, S., Kasuga, I., Mine, Y., Takahashi, T., and Kobayashi, H. (2001). A new macrocyclic trichothecene, 12,13-deoxyroridin E, produced by the marine-derived fungus Myrothecium roridum collected in Palau. *J Nat Prod* **64**, 396-398.

Newton, R., Stevens, D. A., Hart, L. A., Lindsay, M., Adcock, I. M., and Barnes, P. J. (1997). Superinduction of COX-2 mRNA by cycloheximide and interleukin-1beta involves increased transcription and correlates with increased NF- kappaB and JNK activation. *FEBS Lett* **418**, 135-138.

Nick, J. A., Young, S. K., Brown, K. K., Avdi, N. J., Arndt, P. G., Suratt, B. T., Janes, M. S., Henson, P. M., and Worthen, G. S. (2000). Role of p38 mitogen-activated protein kinase in a murine model of pulmonary inflammation. *J Immunol* **164**, 2151-2159.

Nikulin, M., Reijula, K., Jarvis, B. B., Veijalainen, P., and Hintikka, E. L. (1997). Effects of

intranasal exposure to spores of Stachybotrys atra in mice. *Fundam Appl Toxicol* **35**, 182-188.

Noah, T. L., Henderson, F. W., Henry, M. M., Peden, D. B., and Devlin, R. B. (1995). Nasal lavage cytokines in normal, allergic, and asthmatic school-age children. *Am J Respir Crit Care Med* **152**, 1290-1296.

Ohh, M., and Takei, F. (1995). Regulation of ICAM-1 mRNA stability by cycloheximide: role of serine/threonine phosphorylation and protein synthesis. *J Cell Biochem* **59**, 202-213.

Otto, V. I., Gloor, S. M., Frentzel, S., Gilli, U., Ammann, E., Hein, A. E., Folkers, G., Trentz, O., Kossmann, T., and Morganti-Kossmann, M. C. (2002). The production of macrophage inflammatory protein-2 induced by soluble intercellular adhesion molecule-1 in mouse astrocytes is mediated by src tyrosine kinases and p42/44 mitogen-activated protein kinase. *J Neurochem* **80**, 824-834.

Ouyang, Y. L., Azcona-Olivera, J. I., Murtha, J., and Pestka, J. J. (1996a). Vomitoxin-mediated IL-2, IL-4, and IL-5 superinduction in murine CD4+ T cells stimulated with phorbol ester calcium ionophore: relation to kinetics of proliferation. *Toxicol Appl Pharmacol* **138**, 324-334.

Ouyang, Y. L., Li, S., and Pestka, J. J. (1996b). Effects of vomitoxin (deoxynivalenol) on transcription factor NF-kappa B/Rel binding activity in murine EL-4 thymoma and primary CD4+ T cells. *Toxicol Appl Pharmacol* **140**, 328-336.

Pages, G., Berra, E., Milanini, J., Levy, A. P., and Pouyssegur, J. (2000). Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. *J Biol Chem* **275**, 26484-26491.

Papavassiliou, A. G. (1994). The role of regulated phosphorylation in the biological activity of transcription factors SRF and Elk-1/SAP-1. *Anticancer Res* **14**, 1923-1926.

Park, J. J., and Chu, F. S. (1996). Assessment of immunochemical methods for the analysis of trichothecene mycotoxins in naturally occurring moldy corn. *J AOAC Int* **79**, 465-471.

Pauli, U. (1994). Control of tumor necrosis factor gene expression. *Crit Rev Eukaryot Gene Expr* **4**, 323-344.

Pende, A., Tremmel, K. D., DeMaria, C. T., Blaxall, B. C., Minobe, W. A., Sherman, J. A., Bisognano, J. D., Bristow, M. R., Brewer, G., and Port, J. (1996). Regulation of the

mRNA-binding protein AUF1 by activation of the beta- adrenergic receptor signal transduction pathway. *J Biol Chem* **271**, 8493-8501.

Perkowski, J. (1998). Distribution of deoxynivalenol in barley kernels infected by Fusarium. *Nahrung* **42**, 81-83.

Pestka, J. J. (1991). High performance thin layer chromatography ELISAGRAM. Application of a multi-hapten immunoassay to analysis of the zearalenone and aflatoxin mycotoxin families. *J Immunol Methods* **136**, 177-183.

Pestka, J. J., and Forsell, J. H. (1988). Inhibition of human lymphocyte transformation by the macrocyclic trichothecenes roridin A and verrucarin A. *Toxicol Lett* **41**, 215-222.

Pestka, J. J., Yan, D., and King, L. E. (1994). Flow cytometric analysis of the effects of in vitro exposure to vomitoxin (deoxynivalenol) on apoptosis in murine T, B and IgA+ cells. *Food Chem Toxicol* **32**, 1125-1136.

Pestka, J. J., Moorman, M. A., and Warner, R. L. (1989). Dysregulation of IgA production and IgA nephropathy induced by the trichothecene vomitoxin. *Food Chem Toxicol* **27**, 361-368.

Pestka, J. J., Lin, W. S., and Miller, E. R. (1987). Emetic activity of the trichothecene 15-acetyldeoxynivalenol in swine. *Food Chem Toxicol* **25**, 855-858.

Pestka, J. J., and Zhou, H. R. (2000). Interleukin-6-deficient mice refractory to IgA dysregulation but not anorexia induction by vomitoxin (deoxynivalenol) ingestion. *Food Chem Toxicol* **38**, 565-575.

Peskta, J. J., and Zhou, H. R. (2002). Effect of tumor necrosis factor type 1 and 2 receptor deficiencies on anorexia, growth and IgA dysregulation in mice exposed to the trichothecene vomitoxin. *Food Chem Toxicol* **40**, 1623-1631.

Prelusky, D. B., and Trenholm, H. L. (1993). The efficacy of various classes of anti-emetics in preventing deoxynivalenol-induced vomiting in swine. *Nat Toxins* **1**, 296-302.

Ramji, D. P., and Foka, P. (2002). CCAAT/Enhancer Binding Proteins: Structure, Function and Regulation. *Biochem J* **365**, 561-575.

Rao, C. Y., Burge, H. A., and Brain, J. D. (2000). The time course of responses to intratracheally instilled toxic Stachybotrys chartarum spores in rats. *Mycopathologia* **149**, 27-34.

Rao, C. Y., Brain, J. D., and Burge, H. A. (2000). Reduction of pulmonary toxicity of

Stachybotrys chartarum spores by methanol extraction of mycotoxins. *Appl Environ Microbiol* **66**, 2817-2821.

Roger, T., Out, T. A., Jansen, H. M., and Lutter, R. (1998). Superinduction of interleukin-6 mRNA in lung epithelial H292 cells depends on transiently increased C/EBP activity and durable increased mRNA stability. *Biochim.Biophys.Acta* **1398**, 275-284.

Roger, T., Out, T., Mukaida, N., Matsushima, K., Jansen, H., and Lutter, R. (1998). Enhanced AP-1 and NF-kappaB activities and stability of interleukin 8 (IL-8) transcripts are implicated in IL-8 mRNA superinduction in lung epithelial H292 cells. *Biochem J* **330**, 429-435.

Roglic, A., Prossnitz, E. R., Cavanagh, S. L., Pan, Z., Zou, A., and Ye, R. D. (1996). cDNA cloning of a novel G protein-coupled receptor with a large extracellular loop structure. *Biochim Biophys Acta* **1305**, 39-43.

Rotter, B. A., Prelusky, D. B., and Pestka, J. J. (1996). Toxicology of deoxynivalenol (vomitoxin). *J Toxicol Environ Health* **48**, 1-34.

Ruotsalainen, M., Hirvonen, M. R., Hyvarinen, A., Meklin, T., Savolainen, K., and Nevalainen, A. (1998). Cytotoxicity, production of reactive oxygen species and cytokines induced by different strains of *Stachybotrys* sp. from moldy buildings in RAW264.7 macrophages. *Environmental Toxicology and Pharmacology* **6**, 193-199.

Rutault, K., Hazzalin, C. A., and Mahadevan, L. C. (2001). Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-alpha (TNF-alpha ) mRNA induction. Evidence for selective destabilization of TNF-alpha transcripts. *J Biol Chem* **276**, 6666-6674.

Ryu, J. C., Yang, J. S., Song, Y. S., Kwon, O. S., Park, J., and Chang, I. M. (1996). Survey of natural occurrence of trichothecene mycotoxins and zearalenone in Korean cereals harvested in 1992 using gas chromatography/mass spectrometry. *Food Addit Contam* **13**, 333-341.

Sachs, A. B. (1993). Messenger RNA degradation in eukaryotes. *Cell* **74**, 413-421.

Sakai, K., Kitagawa, Y., and Hirose, G. (1999). Binding of neuronal ELAV-like proteins to the uridine-rich sequence in the 3'-untranslated region of tumor necrosis factor-alpha messenger RNA. *FEBS Lett* **446**, 157-162.

Sayah, S., Ischenko, A. M., Zhakhov, A., Bonnard, A. S., and Fontaine, M. (1999). Expression of cytokines by human astrocytomas following stimulation by C3a and C5a anaphylatoxins: specific increase in interleukin-6 mRNA expression. *J*

*Neurochem* **72**, 2426-2436.

Schena, F. P., Mastrolitti, G., Jirillo, E., Munno, I., Pellegrino, N., Fracasso, A. R., and Aventaggiato, L. (1989). Increased production of interleukin-2 and IL-2 receptor in primary IgA nephropathy. *Kidney Int* **35**, 875-879.

Schiavi, S. C., Belasco, J. G., and Greenberg, M. E. (1992). Regulation of proto-oncogene mRNA stability. *Biochim Biophys Acta* **1114**, 95-106.

Schmal, H., Shanley, T. P., Jones, M. L., Friedl, H. P., and Ward, P. A. (1996). Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. *J Immunol* **156**, 1963-1972.

Schollenberger, M., Suchy, S., Jara, H. T., Drochner, W., and Muller, H. M. (1999). A survey of Fusarium toxins in cereal-based foods marketed in an area of southwest Germany. *Mycopathologia* **147**, 49-57.

Schwenger, P., Alpert, D., Skolnik, E. Y., and Vilcek, J. (1998). Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. *Mol Cell Biol* **18**, 78-84.

Scott, P. M., Kanhere, S. R., Dexter, J. E., Brennan, P. W., and Trenholm, H. L. (1984). Distribution of the trichothecene mycotoxin deoxynivalenol (vomitoxin) during the milling of naturally contaminated hard red spring wheat and its fate in baked products. *Food Addit.Contam* **1**, 313-323.

Sedgwick, J. D., Riminton, D. S., Cyster, J. G., and Korner, H. (2000). Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today* **21**, 110-113.

Sekikawa, T., Kashihara, N., Maruyama, K., Satoh, M., Okamoto, K., Kanao, K., Maeshima, Y., Sugiyama, H., Yamasaki, Y., and Makino, H. (1998). Expression of interleukin-8 in human glomerulonephritis. *Res Commun Mol Pathol Pharmacol* **99**, 217-224.

Service, D. J. C. (2000). USDA Reports on Israel grain production. Dow Jones & Company, Inc.

Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jongeneel, C. V. (1990). kB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor a gene in primary macrophages. *J Exp Med* **171**, 35-47.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region

of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667.

Sherry, B., Tekamp-Olson, P., Gallegos, C., Bauer, D., Davatelis, G., Wolpe, S. D., Masiarz, F., Coit, D., and Cerami, A. (1988). Resolution of the two components of macrophage inflammatory protein 1, and cloning and characterization of one of those components, macrophage inflammatory protein 1 beta. *J Exp Med* **168**, 2251-2259.

Sherry, B., Horii, Y., Manogue, K. R., Widmer, U., and Cerami, A. (1992). Macrophage inflammatory proteins 1 and 2: an overview. *Cytokines* **4**, 117-130.

Shi, M. M., Chong, I., Godleski, J. J., and Paulauskis, J. D. (1999). Regulation of macrophage inflammatory protein-2 gene expression by oxidative stress in rat alveolar macrophages. *Immunology* **97**, 309-315.

Shifrin, V. I., and Anderson, P. (1999). Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *J Biol Chem* **274**, 13985-13992.

Showell, H. J., Glovsky, M. M., and Ward, P. A. (1982). C3a-induced lysosomal enzyme secretion from human neutrophils: lack of inhibition by f met-leu-phe antagonists and inhibition by arachidonic acid antagonists. *Int Arch Allergy Appl Immunol* **67**, 227-232.

Sirenko, O. I., Lofquist, A. K., DeMaria, C. T., Morris, J. S., Brewer, G., and Haskill, J. S. (1997). Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1. *Mol Cell Biol* **17**, 3898-3906.

Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* **248**, 1019-1023.

Sorenson, W. G., Frazer, D. G., Jarvis, B. B., Simpson, J., and Robinson, V. A. (1987). Trichothecene mycotoxins in aerosolized conidia of Stachybotrys atra. *Appl Environ Microbiol* **53**, 1370-1375.

Stacey, K. J., Ross, I. L., and Hume, D. A. (1993). Electroporation and DNA-dependent cell death in murine macrophages. *Immunol Cell Biol* **71**, 75-85.

Stack, M. E., and Eppley, R. M. (1980). High pressure liquid chromatographic determination of satratoxins G and H in cereal grains. *J Assoc Off Anal Chem* **63**, 1278-1281.

Stimler, N. P., Bloor, C. M., and Hugli, T. E. (1983). C3a-induced contraction of guinea pig

lung parenchyma: role of cyclooxygenase metabolites. *Immunopharmacology* **5**, 251-257.

Subramaniam, M., Schmidt, L. J., Crutchfield, C. E., and Getz, M. J. (1989). Negative regulation of serum-responsive enhancer elements. *Nature* **340**, 64-66.

Sugita-Konishi, Y., and Pestka, J. J. (2001). Differential upregulation of TNF-alpha, IL-6, and IL-8 production by deoxynivalenol (vomitoxin) and other 8-ketotrichothecenes in a human macrophage model. *J Toxicol Environ Health A* **64**, 619-636.

Swantek, J. L., Cobb, M. H., and Geppert, T. D. (1997). Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol Cell Biol* **17**, 6274-6282.

Thirumala-Devi, K., Mayo, M. A., Reddy, G., Reddy, S. V., Delfosse, P., and Reddy, D. V. (2000). Production of polyclonal antibodies against ochratoxin A and its detection in chilies by ELISA. *J Agric Food Chem* **48**, 5079-5082.

Tibbles, L. A., and Woodgett, J. R. (1999). The stress-activated protein kinase pathways. *Cell Mol Life Sci* **55**, 1230-1254.

Tindberg, N., Porsmyr-Palmertz, M., and Simi, A. (2000). Contribution of MAP kinase pathways to the activation of ATF-2 in human neuroblastoma cells. *Neurochem Res* **25**, 527-531.

Trigo-Stockli, D., Sanchez-Marinez, R., Rocha-Cortez, M. O., and Pedersen, J. R. (1998). Comparison of the distribution and occurrence of Fusarium graminearum and deoxynivalenol in hard red winter wheat for 1993-1996. *Cereal chem.* **75**, 841-846.

Trucksess, M. W. (1995). General referee reports - Committee on natural toxins, Mycotoxins. *J Assoc Off Anal Chem* **78**, 135-141.

Trucksess, M. W., Thomas, F., Young, K., Stack, M. E., Fulgueras, W. J., and Page, S. W. (1995). Survey of deoxynivalenol in U.S. 1993 wheat and barley crops by enzyme-linked immunosorbent assay. *J AOAC Int* **78**, 631-636.

Tuomi, T., Saarinen, L., and Reijula, K. (1998). Detection of polar and macrocyclic trichothecene mycotoxins from indoor environments. *Analyst* **123**, 1835-1841.

Ueno, Y. (1983b). General Toxicology. In Trichothecenes: Chemical, biological and toxicological aspects (Y. Ueno, ed., pp. 135-162. Elsevier, New York.

Ueno, Y., Hosoya, M., Morita, Y., Ueno, I., and Tatsuno, T. (1968). Inhibition of the protein synthesis in rabbit reticulocyte by Nivalenol, a toxic principle isolated from Fusarium nivale-growing rice. *J Biochem (Tokyo)* **64**, 479-485.

Ueno, Y. (1984). Toxicological features of T-2 toxin and related trichothecenes. *Fundam Appl Toxicol* **4**, S124-132.

Ueno, Y. (1983a). Effect of Trichothecene Mycotoxins on Farm Animals. In Trichothecenes: Chemical, biological and toxicological aspects (Y. Ueno, ed., pp. 177-194. Elsevier, New York.

Van Damme, J., Opdenakker, G., Simpson, R. J., Rubira, M. R., Cayphas, S., Vink, A., Billiau, A., and Van Snick, J. (1987). Identification of the human 26-kD protein, interferon beta 2 (IFN-beta 2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. *J Exp Med* **165**, 914-919.

Vane, J. R., Bakhle, Y. S., and Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* **38**, 97-120.

Vogel, S. N., and Hogan, M. M. (1990). Role of cytokines in endotoxin-mediated host responses. In Immuno-physiology: the Role of Cells and Cytokines in Immunity and Inflammation (J. J. Oppenheim and E. M. Shevach, eds.), pp. 238-258. Oxford University Press, New York.

Walley, K. R., Lukacs, N. W., Standiford, T. J., Strieter, R. M., and Kunkel, S. L. (1997). Elevated levels of macrophage inflammatory protein 2 in severe murine peritonitis increase neutrophil recruitment and mortality. *Infect.Immun.* **65**, 3847-3851.

Wang, E., Ma, W. J., Aghajanian, C., and Spriggs, D. R. (1997). Posttranscriptional regulation of protein expression in human epithelial carcinoma cells by adenine-uridine-rich elements in the 3'- untranslated region of tumor necrosis factor-alpha messenger RNA. *Cancer Res* **57**, 5426-5433.

Wang, S. W., Pawlowski, J., Wathen, S. T., Kinney, S. D., Lichenstein, H. S., and Manthey, C. L. (1999). Cytokine mRNA decay is accelerated by an inhibitor of p38-mitogen-activated protein kinase. *Inflamm Res* **48**, 533-538.

Wang, Z. M., Liu, C., and Dziarski, R. (2000). Chemokines are the main proinflammatory mediators in human monocytes activated by Staphylococcus aureus, peptidoglycan, and endotoxin. *J Biol Chem* **275**, 20260-20267.

Warner, R. L., Brooks, K., and Pestka, J. J. (1994). In vitro effects of vomitoxin (deoxynivalenol) on T-cell interleukin production and IgA secretion. *Food Chem*

*Toxicol* **32**, 617-625.

Wetter, M. T., Trucksess, M. W., Roach, J. A., and Bean, G. A. (1999). Occurrence and distribution of Fusarium graminearum and deoxynivalenol in sweet corn ears. *Food Addit Contam* **16**, 119-124.

Winstall, E., Gamache, M., and Raymond, V. (1995). Rapid mRNA degradation mediated by the c-fos 3' AU-rich element and that mediated by the granulocyte-macrophage colony-stimulating factor 3' AU-rich element occur through similar polysome-associated mechanisms. *Mol Cell Biol* **15**, 3796-3804.

Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999). The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J* **18**, 4969-4980.

Wolf-Hall, C. E., Hanna, M. A., and Bullerman, L. B. (1999). Stability of deoxynivalenol in heat-treated foods. *J.Food Prot.* **62**, 962-964.

Wolpe, S. D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D. G., Nguyen, H. T., Moldawer, L. L., Nathan, C. F., Lowry, S. F., and Cerami, A. (1988). Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J Exp Med* **167**, 570-581.

Wolpe, S. D., Sherry, B., Juers, D., Davatelis, G., Yurt, R. W., and Cerami, A. (1989). Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci U S A* **86**, 612-616.

Wong, S. S., Zhou, H. R., Marin-Martinez, M. L., Brooks, K., and Pestka, J. J. (1998). Modulation of IL-1beta, IL-6 and TNF-alpha secretion and mRNA expression by the trichothecene vomitoxin in the RAW 264.7 murine macrophage cell line. *Food Chem Toxicol* **36**, 409-419.

Wong, S. S., Zhou, H. R., and Pestka, J. J. (2002). Effects of vomitoxin (deoxynivalenol) on the binding of transcription factors AP-1, NF-kB, and NF-IL6 in RAW 264.7 macrophage cells. *J Toxicol Environ Health, Part A* **65**, 101-120.

Wong, S., Schwartz, R. C., and Pestka, J. J. (2001). Superinduction of TNF-alpha and IL-6 in macrophages by vomitoxin (deoxynivalenol) modulated by mRNA stabilization. *Toxicology* **161**, 139-149.

Wong, S. S. (2000). Mechanisms for vomitoxin-induced cytokine superinduction in macrophages. dissertation. Michigan State University, East Lansing MI.

Xiao, Y. Q., Someya, K., Morita, H., Takahashi, K., and Ohuchi, K. (1999). Involvement of p38 MAPK and ERK/MAPK pathways in staurosporine-induced production of macrophage inflammatory protein-2 in rat peritoneal neutrophils. *Biochim.Biophys.Acta* **1450**, 155-163.

Yagen, B., and Joffe, A. Z. (1976). Screeing of toxic isolates of Fusarium poae and Fusarium sporotrichiodes involved in causing alimentary toxic aleukia. *Appl Environ Microbiol* **32**, 423-427.

Yamada, T., Yoshiyama, Y., and Kawaguchi, N. (1997). Expression of activating transcription factor-2 (ATF-2), one of the cyclic AMP response element (CRE) binding proteins, in Alzheimer disease and non-neurological brain tissues. *Brain Res* **749**, 329-334.

Yamagishi, S., Yamada, M., Ishikawa, Y., Matsumoto, T., Ikeuchi, T., and Hatanaka, H. (2001). p38 mitogen-activated protein kinase regulates low potassium-induced c-Jun phosphorylation and apoptosis in cultured cerebellar granule neurons. *J Biol Chem* **276**, 5129-5133.

Yan, D., Zhou, H. R., Brooks, K. H., and Pestka, J. J. (1997). Potential role for IL-5 and IL-6 in enhanced IgA secretion by Peyer's patch cells isolated from mice acutely exposed to vomitoxin. *Toxicology* **122**, 145-158.

Yan, D., Zhou, H. R., Brooks, K. H., and Pestka, J. J. (1998b). Role of macrophages in elevated IgA and IL-6 production by Peyer's patch cultures following acute oral vomitoxin exposure. *Toxicol Appl Pharmacol* **148**, 261-273.

Yan, D., Rumbeiha, W. K., and Pestka, J. J. (1998a). Experimental murine IgA nephropathy following passive administration of vomitoxin-induced IgA monoclonal antibodies. *Food Chem Toxicol* **36**, 1095-1106.

Yang, G. H., and Pestka, J. J. (2002). Vomitoxin (deoxynivalenol)-mediated inhibition of nuclear protein binding to NRE-A, an IL-2 promoter negative regulatory element, in EL-4 cells. *Toxicology* **172**, 169-179.

Yang, G. H., Jarvis, B. B., Chung, Y. J., and Pestka, J. J. (2000). Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharmacol* **164**, 149-160.

Yang, G. H., Li, S., and Pestka, J. J. (2000b). Down-regulation of the endoplasmic reticulum chaperone GRP78/BiP by vomitoxin (Deoxynivalenol). *Toxicol Appl Pharmacol* **162**, 207-217.

Ye, J., and Young, H. A. (1997). Negative regulation of cytokine gene transcription. *Faseb J* **11**, 825-833.

Yike, I., Allan, T., Sorenson, W. G., and Dearborn, D. G. (1999). Highly sensitive protein translation assay for trichothecene toxicity in airborne particulates: comparison with cytotoxicity assays. *Appl Environ Microbiol* **65**, 88-94.

Yoshidome, H., Lentsch, A. B., Cheadle, W. G., Miller, F. N., and Edwards, M. J. (1999). Enhanced pulmonary expression of CXC chemokines during hepatic ischemia/reperfusion-induced lung injury in mice. *J Surg Res* **81**, 33-37.

Yoshioka, K., Takemura, T., Murakami, K., Okada, M., Yagi, K., Miyazato, H., Matsushima, K., and Maki, S. (1993). In *In situ expression of cytokines in IgA nephritis*. (Yoshioka, K., Takemura, T., Murakami, K., Okada, M., Yagi, K., Miyazato, H., Matsushima, K., and Maki, S.), pp 825-833.

Yoshizawa, T., and Jin, Y. Z. (1995). Natural occurrence of acetylated derivatives of deoxynivalenol and nivalenol in wheat and barley in Japan. *Food Addit.Contam* **12**, 689-694.

Yuan, Q., Pestka, J. J., Hespenheide, B. M., Kuhn, L. A., Linz, J. E., and Hart, L. P. (1999). Identification of mimotope peptides which bind to the mycotoxin deoxynivalenol-specific monoclonal antibody. *Appl Environ Microbiol* **65**, 3279-3286.

Yuwai, K. E., Rao, K. S., Singh, K., Tanaka, T., and Ueno, Y. (1994). Occurrence of nivalenol, deoxynivalenol, and zearalenone in imported cereals in Papua, New Guinea. *Nat Toxins* **2**, 19-21.

Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993). Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. *Mol Cell Biol* **13**, 7652-7665.

Zhao, Q., and Lee, F. S. (1999). Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J Biol Chem* **274**, 8355-8358.

Zhou, H. R., Harkema, J. R., Hotchkiss, J. A., Yan, D., Roth, R. A., and Pestka, J. J. (2000). Lipopolysaccharide and the trichothecene vomitoxin (deoxynivalenol) synergistically induce apoptosis in murine lymphoid organs. *Toxicol Sci* **53**, 253-263.

Zhou, H. R., Harkema, J. R., Yan, D., and Pestka, J. J. (1999). Amplified proinflammatory cytokine expression and toxicity in mice coexposed to lipopolysaccharide and the

trichothecene vomitoxin (deoxynivalenol). *J Toxicol Environ Health* **57**, 115-136.

Zhou, H. R., Yan, D., and Pestka, J. J. (1997). Differential cytokine mRNA expression in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): dose response and time course. *Toxicol Appl Pharmacol* **144**, 294-305.

Zhou, H. R., Yan, D., and Pestka, J. J. (1998). Induction of cytokine gene expression in mice after repeated and subchronic oral exposure to vomitoxin (Deoxynivalenol): differential toxin-induced hyporesponsiveness and recovery. *Toxicol Appl Pharmacol* **151**, 347-358.

Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., and Smart, R. C. (2002). CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. *Proc Natl Acad Sci U S A* **99**, 207-212.

Zhu, T., and Lobie, P. E. (2000). Janus kinase 2-dependent activation of p38 mitogen-activated protein kinase by growth hormone. Resultant transcriptional activation of ATF-2 and CHOP, cytoskeletal re-organization and mitogenesis. *J Biol Chem* **275**, 2103-2114.

Zhu, W., Downey, J. S., Gu, J., Di Padova, F., Gram, H., and Han, J. (2000). Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J.Immunol.* **164**, 6349-6358.