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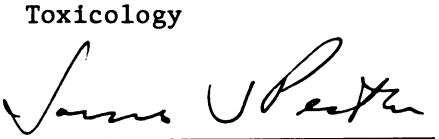

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MECHANISMS FOR VOMITOXIN-INDUCED CYTOKINE SUPERINDUCTION IN MACROPHAGES

By

Shu-shyan Wong

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ABSTRACT

MECHANISMS FOR VOMITOXIN-INDUCED CYTOKINE SUPERINDUCTION IN MACROPHAGES

By

Shu-shyan Wong

Potential mechanisms for superinduction of cytokine expression by the trichothecene vomitoxin (VT) were assessed in the cloned macrophage cell line RAW 264.7. First, the effects of VT on secretion of IL-1β, IL-6 and TNF-α protein and expression of their mRNA were measured with and without lipopolysacchride (LPS) activation. Secreted TNF-α was significantly increased after 2 days exposure to VT with and without LPS activation. While VT had no effect on IL-6 secretion in the absence of LPS, it significantly increased IL-6 secretion in LPS-exposed cells. Secreted IL-1β was not detected in cells treated with or without activation. Immunostaining of intracellular cytokines in conjunction with flow cytometric analysis suggested that the percentage of the cells producing intracellular TNF-α were significantly increased by VT with and without LPS; whereas, VT increased IL-6 output in LPS-activated cells. Elevated TNF-α mRNA was observed in RAW 264.7 cells incubated with VT. IL-1β and IL-6 mRNAs were increased in cells incubated with LPS. To assess the transcriptional effects of VT, binding activities of the transcription factors which enhance promoter functions of pro-inflammatory cytokine promoters AP-1, NF-κB and NF-IL6 (C/EBP) were studied by electrophorectic mobility gel-shift assay (EMSA). AP-1 binding was enhanced by VT and specifically targeted the phosporylated c-Jun, JunB, c-Fos, and Fra-2 subunits of AP-1. VT increased p50 and c-Rel of NF-κB/Rel complexes

in cells incubated with and without LPS. The binding activities of NF-IL6 (C/EBPα and C/EBPβ) were also increased by VT. However when the effects of VT on the activity of the *cis*-acting elements of the promoter were evaluated by in RAW 264.7 cells with transiently transfected a plasmid containing a TNF-α promoter linked to a CAT reporter gene, VT was found to induce CAT mRNA expression slightly. To assess potential post-transcriptional effects of VT, stability of cytokine mRNAs were assessed by Northern blot analysis in RAW cells treated with the transcriptional inhibitor DRB. TNF-α and IL-6 mRNA half-lives were prolonged from 2- to 10-fold. Taken together, these results suggested that (1) VT is able to superinduce cytokine protein and mRNA expression in macrophages; (2) VT can increase of the binding activities of transcription factors (3) increases in TNF-α transcription promoter activity were detectable but small; and (4) the ability of VT to stabilize TNF-α and IL-6 mRNA was marked. Therefore, elevated cytokine protein and mRNA expression may primarily attributable to post-transcriptional modulation by VT.

Dedicated to my deceased yet unborn child

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INTRODUCTION

Trichothecenes are secondary metabolites mainly produced by species of *Fusarium* found in wheat and corn supplies throughout the world (Vesonder and Hesseltine, 1980; Ueno, 1985; Rotter *et al.*, 1996). Trichothecenes have a tetracyclic epoxy-ring at their C12-C13 position and an unsaturated bond at the C9-C10 position (Bamburg, 1983; Betina, 1989). More than 100 different varieties of trichothecenes have been identified (Scott, 1990). Trichothecenes are classified into A, B, and D (macrocyclic) groups which include vomitoxin (VT, deoxynivalenol), T-2 toxin and nivalenol (NIV). Trichothecenes are very stable and do not degrade at high temperature during milling and processing (Scott, 1991). Therefore, they are common contaminants in crops and in food and feed supplies.

The trichothecene VT has been shown to cause a wide range of toxicological and immunological effects (Cundliffe *et al.*, 1974; Ueno, 1983; Bamburg, 1983; Forsell *et al.*, 1987; Fitzpatrick *et al.*, 1988; Thompson and Wannemacher, 1990; Bergmann *et al.*, 1992). VT can either be immunosuppressive or immunostimulatory depending on the dose and timing of exposure (Pestka and Bondy, 1994). VT has been shown to cause depression in the mitogen-induced proliferative responses of murine lymphocytes (Tryphonas *et al.*, 1986; Robbana-Barnat *et al.*, 1988). In contrast, VT also increases resistance to the mastitis pathogen *Mycobacterium* and to *Staphylococcus* in mice (Atroshi *et al.*, 1994). Increased serum IgA, IgM and IgG levels with mastitis mice gavaged with VT for a week was observed (Atroshi *et al.*, 1994). Dong and Pestka (1993) also showed that dietary exposure of VT in mice elevated serum IgA, circulating IgA immune complexes and mesangial IgA deposition in the kidney. Therefore it was suspected that these effects of VT might

contribute to the alteration of the function of immune cells including macrophage, T cells, or B cells.

Superinduction of cytokines by VT has been observed by several researchers. For example, Miller and Atkinson (1986) demonstrated the superinduction of inflammatory cytokine IL-1 in peritoneal macrophages treated with VT. Recently, increased mRNAs for interleukin-2, -4, -5 and/or -6 and their secreted proteins were observed upon exposure to VT and cycloheximide (CHX) *in vivo* (Warner *et al.*, 1994; Azcona-Olivera *et al.*, 1995a; Ouyang *et al.*, 1996). Transient increases of mRNAs for pro-inflammatory cytokines associated with macrophages, IL-1β, IL-6 and TNF-α, were also shown upon exposure to VT *in vivo* (Azcona-Olivera *et al.*, 1995b; Zhou *et al.*, 1997).

Macrophages are pivotal regulators of inflammation and immunity (Solbach *et al.*, 1991) and play an important role in regulating T cell function, proliferation, regulation of antibody and production of mediators for cellular immunity. The aforementioned macrophage cytokines may be related to the pathogenic manifestations associated with trichothecene exposure. For example, IL-1β, IL-6 and TNF-α are cytokines observed to mediate septicemia and fatal circulatory shock-like syndrome by LPS (Vogel and Hogan, 1990). IL-1β, IL-6 and TNF-α also have been associated with anorexia and weight loss, or cachexia (Abbas *et al.*, 1994; Schobitz *et al.*, 1994). Thus, feed refusal and reduced weight gain observed in the trichothecene-exposed animals (Rotter *et al.*, 1996) might be mediated by superinduction of these pro-inflammatory cytokines. Also, IL-6 has been shown to be involved in the development of IgA responses (VanCott *et al.*, 1996) and is able to drive IgA production when added to the Peyer's patch B cell cultures (Yan *et al.*, 1997). Taken

together, these results suggest that superinduction of those cytokines in macrophage by VT might contribute to hyperelevation of the IgA production. However, the superinduction of cytokines by VT in macrophage cells has not yet been studied. Therefore, it was important to assess the effects of VT on the cytokine protein and mRNA expression in the macrophage cells. The RAW 264.7 murine cell line was employed for this purpose in my research.

Gene expression is regulated at the transcriptional, and post-transcriptional level. Transcriptional control has been suggested to be a main regulatory mechanism of macrophage activation (Yu *et al.*, 1990; Paulnock, 1994). Previous study (Ouyang *et al.*, 1996) revealed that the activity of transcription factor NF-κB increased in IL-2 gene expression in EL-4 cells treated with VT. NF-κB also plays an important role in induction of various gene expression in macrophages, such as M-CSF, G-CSF, TNF-α, IL-1β, and IL-6 (Akahane *et al.*, 1994; Baeuerle and Henkel, 1994). NF-IL6 and AP-1 have also been cited as important transcription factors in myeloid specific genes (Clark and Gordon, 1998). Thus, it is possible that VT can superinduce IL-1β, TNF-α and IL-6 in macrophage cells by altering the activity of these transcriptional factors. Therefore, it is useful to study the effects of VT on the binding activity of those transcription factors on the promoters in macrophage RAW 264.7 cells.

cis-acting elements also play important roles in the transcriptional control of the cytokine mRNA expression. Transcriptional activity of the TNF-α gene was enhanced by CHX in human monocytes (Osipovich *et al.*, 1993) and the macrophage cell line, J774 (Akahane *et al.*, 1994). Transcription of the IL-2 gene also increased 2-3 fold after exposure to CHX in previously activated EL-4 cells (Zubiaga *et al.*, 1991). The extent of the

transcription in mammalian cells can be studied by using a plasmid containing the desired promoter linked to a reporter gene; promoter activity is measured based on expression of the reporter protein. Therefore, it was desirable to employ a promoter construct to fully understand the effects of VT on the promoter activity of these cyokines.

In addition to the transcriptional control, post-transcriptional control also plays a key role in the regulation of the cytokine superinduction. Protein synthesis inhibitors, such as CHX, have been shown to increase the half-life of TNF-α mRNA in macrophages (Celada *et al.*, 1989; Taffet *et al.*, 1989) in the presence of the transcriptional inhibitor actinomycin D (Act-D). Fort *et al.* (1987) also demonstrated that cytokine mRNA degradation seems to resume quickly after the removal of protein synthesis inhibitors such as CHX. VT may also superinduce expression of cytokines in macrophages by stabilizing their mRNAs. Thus, it was important to evaluate the stabilization effects of VT on the cytokine mRNA expression in the trichothecene-mediated cytokine superinduction.

The four aforementioned areas were studied in this dissertation, which is comprised of seven parts. Chapter I reviews literature on trichothecenes, macrophages, lipopolysaccharides, and the regulation of the IL-6, TNF-α and IL-1β gene expression. Chapter II describes the effects of VT on the cytokine protein and mRNA expression in murine macrophage RAW 264.7 cells. Chapter III describes the effects of VT on the binding activity of transcription factors AP-1, NF-IL6 and NF-κB in RAW 264.7 cells.. Chapter IV describes the effects of VT on the transcriptional activity of TNF-α promoter in RAW 264.7 cells. Chapter V describes the stabilizing effects of VT on cytokine mRNA half-life in RAW 264.7 cells. Chapter VI summarizes these inter-related studies and makes suggestions

for future research. In addition, the appendix contains three reprints of published work to which I have contributed either as a primary author or as a collaborator.

CHAPTER I

LITERATURE REVIEW

A. Trichothecenes

A.1. Introduction

Trichothecenes are toxic secondary metabolites produced by genera of fungi such as *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma* and *Trichothecium* that can be found in food grains or environmentally (Schoental, 1983; Ueno, 1985; Vesonder and Hesseltine, 1980; Rotter *et al.*, 1996). They have a sesquiterpenoid structure which is characterized by the presence of an unsaturated bond at the C9-C10 position and a tetracyclic 12,13-epoxy ring (Ueno *et al.*, 1973; Schoental, 1983; Betina, 1989, Rotter *et al.*, 1996). Trichothecenes are often produced during temperature cycling under high humidity (Gu *et al.*, 1991; Sherwood and Peberdy, 1972). Trichothecenes have been classified into A, B, and D (macrocyclic) groups according to their functional groups.

Trichothecenes have been to induce acute shock-like toxicity as well as chronic effects. They can cause gastrointestinal disturbances such as vomiting, diarrhea, and inflammation, feed refusal, abortion, dermal irritation and hematological sequelae (anemia and leukopenia) (Coulombe, 1993). Ingestion of thrichothecenes has been shown to cause outbreaks of alimentary toxic aleukia (ATA) disease in the former Soviet Union, India, China and Japan (Joffe, 1978; Bhat *et al.*, 1989; Ueno, 1987; Luo *et al.*, 1990). Dietary exposure to trichothecenes has been associated with high esophageal cancer incidence in South Africa and China (Marasus *et al.*, 1977; Luo *et al.*, 1990). The underlying mechanism for trichothecene toxicity relates to their ability to be potent protein synthesis inhibitors in eukaryotic cells (Ueno *et al.*, 1968; Rosenstein and Lafarge-Frayssinet, 1983; Suneja *et al.*, 1983). They inhibit all steps of protein synthesis: initiation, elongation and termination by binding to the 60 S ribosomal subunit (Ueno, 1983). They also inhibit DNA synthesis *in*

vitro.

T-2 (3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutryloxy)-12,13-epoxytrichothec-9-ene) toxin, one of the most potent trichothecenes, is also a protein synthesis inhibitor. It is found as a natural contaminant in foods and feeds (Ueno, 1986) as is VT. It can be produced by different species of *Fusarium, Myrothecium, Trichothecium, Trichoderma* and *Stachybotrys* (Schoental, 1985). T-2 was first identified in moldy corn that causes a fatal hemorrhagic disorder in lactating cows (Hsu *et al.*, 1972). As a protein synthesis inhibitor, it inhibits protein synthesis in mammalian cells (Ueno, 1983; Cannon *et al.*, 1976) by binding to ribosomes (McLaughlin *et al.*, 1977). T-2 toxin reduces weight gain, and causes emesis, inflammation, internal hemorrhage, and damage in the bone marrow and thymus (Saito and Ohtsubo, 1974; Hayes and Schiefer, 1980).

A.2. Vomitoxin (Deoxynivalenol)

Vomitoxin (VT) was first isolated from barley and characterized by Yoshizawa and Morooka (1973) in Japan. It was given the name because of its observed emetic effect on swine.It is also known as 'Rd toxin' in barley and corn infected with *Fusarium sp.*(Vesonder *et al.*, 1973; Vesonder and Ciegler, 1979) from the mid-western United States and Canada. VT is mainly produced by strains of *Fusarium graminearum* and *F. culmarum*, which are pathogens in wheat and corn. When *Fusarium* grows on wheat, it produces scabbed wheat, which is soft and shriveled, often with pink discoloration on the kernels and flowering head (Vesonder and Hesseltine, 1980). Environmental conditions such as low temperature and high humidity encourage the growth of mold on crops and VT production, thereby increasing the chances of VT contamination. VT is a very stable compound and does not degrade at high temperature during milling and processing and readily enters into finished food products

(Scott, 1991). Therefore, VT is a common contaminant of corn and wheat worldwide and widely found in food and feed.

VT has been shown to cause a wide range of toxicological and immunological effects. Low and acute doses of VT are known to cause anorexia and emesis in animals. A significant increase of serotonin was observed in the cerebrospinal fluid following oral administration of VT (Fitzpatrick *et al.*, 1988). VT is also reported to cause necrosis of the intestinal tract, bone marrow and lymphoid tissues, and kidney and heart lesions at very high doses (≥100mg/kg) (Forsell *et al.*, 1987). VT can either be immunosuppresive or immunostimulatory depending on the dose and timing of exposure (Pestka and Bondy, 1994). *In vivo* exposure to VT depresses mitogen-induced proliferative responses of murine lymphocytes (Robbana-Barnat *et al.*, 1988; Tryphonas *et al.*, 1986). It has also been shown that both single and multiple gavage of mice with VT increase resistance to the mastitis pathogens *Mycobacterium* and to *Staphylococcus* (Atroshi *et al.*, 1994).

Elevated serum IgA, IgM and IgG levels have been found in mastitis mice gavaged with VT for a week (Atroshi *et al.*, 1994). Similarly, Forsell *et al.* (1987) detected a dramatic increase in serum IgA, but found decreases in serum IgM and IgG in mice fed with VT. Dong and Pestka (1993) also demonstrated that dietary exposure of mice to VT can elevate serum IgA, circulating IgA immune complexes and mesangial IgA deposition in the kidney. These features are highly analogous to the human glomerulonephritis, IgA nephropathy (D'Amico, 1987).

VT is able to stimulate *in vitro* IgA production. Prior exposure to VT *in vitro* enhances $CD4^+$ cell mediated help for IgA production by B cells through elevated cytokine production (Warner *et al.*, 1994). VT can also increase the percentage of total T cells, $CD4^+$

cells and the $CD4^+/CD8^+$ cell ratio in Peyer's patches (PP) (Pestka *et al.*, 1990a, b) and spleen. Increased mRNAs of interleukin-2, -4, -5 and/or IL-6 (T-helper cytokines) and their secreted proteins have been observed upon exposure to VT or cycloheximide (CHX) *in vivo* (Warner *et al.*, 1994; Azcona-Olivera *et al.*, 1995a; Ouyang *et al.*, 1996). Thus, VT-induced elevation of IgA production might be related to the superinduction of various T helper cytokines. Superinduction of inflammatory cytokines such as IL-1 has been observed in peritoneal macrophages treated with VT (Miller and Atkinson, 1986). Oral exposure of mice to VT causes transient increases in mRNAs for cytokines IL-1β, IL-6 and TNF-α within 2 hr (Azcona-Olivera *et al.*, 1995b; Zhou *et al.*, 1997).

A.3. Cycloheximide

Cycloheximide (CHX), a glutarimide antibiotic (Pestka, 1971) is the most well-studied protein synthesis inhibitor. It binds to 80S ribosomes in eukaryotic cells (Cannon *et al.*, 1994). CHX inhibits protein synthesis in yeast and mammalian cells (Kerridge, 1958; Young *et al.*, 1963; Ennis and Lubin, 1964; Gorski and Axman, 1964). It has been shown to inhibit translocation of peptidyl-tRNA into the the ribosomes. CHX is thought to be involved in the inhibition of the initiation, elongation and termination steps of protein synthesis. CHX superinduces cytokines such as TNF-α and IFN-γ (Efrat and Kaempfer, 1984; Cockfield *et al.*, 1993; Faggioli *et al.*, 1997). CHX also superinduces IL-2, IL-6, IFN-γ and TNF-α mRNA both *in vivo* and *in vitro* (Efrat and Kaempfer, 1984; Zubiaga *et al.*, 1991; Cockfield *et al.*, 1993; Osipovich *et al.*, 1993; Akahane *et al.*, 1994; Biragyn and Nedospasov, 1995; Faggioli *et al.*, 1997). Increased cytokine mRNA half-life has also been observed in macrophages treated with CHX (Celada *et al.*, 1989; Taffet *et al.*, 1989; and

Zuckerman *et al.*, 1991). CHX also induces the activity of the transcription factor NF-κB (Menegazzi *et al.*, 1996). The induction may be related to cytokine superinduction observed in CHX-treated cells or animals.

B. Macrophages

B.1. Introduction

Macrophages are multi-functional cells involved in the regulation of both innate and acquired immunity (Van Rooijen *et al.*, 1996). Mononuclear phagocytes originate from pluripotent stem cells in bone marrow (Auger and Ross, 1992; van Furth *et al.*, 1986a). A monoblast, the most immature cell, can divide into two pro-monocytes. The pro-monocytes then divide into two monocytes each. Monocytes migrate from the bone marrow and into the blood within 24 hr after they are formed. The peripheral monocytes then migrate into tissues in which they differentiate and become macrophages (van Furth *et al.*, 1986b). The constant influx of the monocytes into the tissues requires a constant efflux from the tissue and/or cell death in the tissues. Macrophages are important regulators in inflammation. They are able to perform phagocytosis, intracellular killing, chemotaxis, antigen processing and presentation, as well as secretion of cytokines and prostaglandins (Solbach *et al.*, 1991; Augus and Ross, 1992).

B.2. Macrophage activation

Upon stimulation, macrophages can be activated and involved in inflammation. There are two different kinds of extracellular stimulation (Schneider and Dy, 1985). The components that present in the immune system such as cytokines, immunoglobulins and

complement are the major and most effective stimuli (Schneider and Dy, 1985; Paulnock, 1994). Non-immunological components, such as endotoxin, pyron polymer and polyanions, are additional sources of stimulation. The binding of these components by receptor molecules on macrophages lead to activation. Several different receptors on the macrophage have been identified. These include CD14, CD33, CD18, Mac-1, and a variety of cytokine receptors (Augus and Ross, 1992). Activation of macrophages involves at least two different steps (Schneider and Dy, 1985; Ohmori and Hamilton, 1994). During the priming step, resident macrophages are stimulated with a cytokine such as interferon-γ (IFN-γ). Further encounter with endotoxin or other signals will trigger their differentiation into fully activated, cytotoxic macrophages. Activation of macrophages can increase their secretory protein products (>100 varieties of protein) (Adams and Hamilton, 1984; Nathan, 1987) including monokines and prostaglandins that alter morphology (causing an increase in size, spreading and membrane ruffling), modify receptor expression, and increase oxidative metabolism (Schneider and Dy, 1985; Auger and Ross, 1992). These reactions will in turn enhance chemotaxis, phagocytosis and cytotoxicity in the host inflammatory system.

B.3. Macrophage and cytokines

Macrophages can release many different cytokines including interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-6, IL-8 and IFN-γ (Ohmori and Hamilton, 1994).

IL-1 is a low molecular weight protein that can be produced by many cells, but macrophages are major producers (Cavaillon and Haeffner-Cavaillon, 1990). IL-1 can increase proliferation and activation of T and B lymphocytes, macrophages and other cells. It also increases postaglandin production by macrophages and enhances their cytotoxicity. IL-1 also promotes B cell maturation and division and induces acute phase proteins in

hepatocytes (Dayer and Burger, 1994). This cytokine is also known as " endogeneous pyrogen" because it can cause fever (Dinarello, 1994).

TNF-α, also known as "cachectin", is primarily produced by activated macrophages. It is named for its ability to kill tumor cells (Laskin and Pendino, 1995). TNF-α has been found to be involved in septic shock and inflammation (Dayer and Burger, 1994). It regulates acute phase protein gene expression and the P450 activity during cellular proliferation and apoptosis. TNF-α also stimulates release of other cytokines such as IL-1 and IL-6. It can cause severe weight loss and also death. Like IL-1, it can also induce fever.

Another important cytokine produced by macrophages is IL-6. Both IL-1 and TNF-α upregulate IL-6 production while IL-4 is known to inhibit IL-6 production. IL-6 enhances proliferation of both immature and mature T cells and an important differentiation factor for B cells and can induce immunoglobulin (Ig) secretion.

IL-8 is a chemokine that attracts neutrophils and T-cells (Wuyts *et al.*, 1998). It inhibits IFN-γ production by natural killer (NK) cells. Three different kinds of TGF-β (type 1,2,3) are produced by macrophages (Derynck and Choy, 1998). It is immunosuppresive by inhibiting IL-2 production. TGF-β is found to be involved in wound healing and also plays a role in fibrosis.

B.4. Regulation of macrophage gene expression

Gene expression is controlled at different levels which include transcriptional, and post-transcriptional mechanisms (Manthey and Vogel, 1994).

B.4.1. Transcriptional control

Relative to transcriptional control of macrophage cytokine gene expression, several

cis elements have been found in the cytokine genes. For TNF-α gene, the region 5' of the transcription start site and the 3' untranslated region (UTR) are the important regulatory regions (Collart *et al.*, 1990; Drouet *et al.*, 1991). Different motifs such as a κB site, cytokine-1 (CK-1) site, and activator protein-1 (AP-1) site have been found (Ohmori and Hamilton, 1994). There are at least three κB motifs in the human TNF-α gene (Ohmori and Hamilton, 1994). In the IL-1β gene, several motifs have also been found in the promoter region. These include κB, cyclic AMP(cAMP), AP-1 and the nuclear factor (NF) -IL6 sites. Deletion of the NF-IL6 site reduces IL-1β gene expression (Shirakawa *et al.*, 1993). In the IL-6 promoter, NF-κB, NF-IL6 and the cAMP sites appear to be important.

Several *trans* acting proteins are also involved in cytokine transcription regulation. These are NF-κB/Rel proteins, NF-IL6 (or C/EBP) proteins and AP-1 proteins (Ohmori and Hamilton, 1994; Valledor *et al.*, 1998). When macrophages are activated, these proteins are induced and bind to the related motifs on the cytokine promoters.

B.4.2. Post-transcriptional control

Post-transcriptional control has been suggested to be important in the regulation of cytokine genes in macrophages (Paulnock, 1994; Ohmori and Hamilton, 1994). Notably, AU-rich sequences in the 3'-untranslated region (UTR) of many cytokines and oncogenes contribute to the mRNA stability of those genes (Caput *et al.*, 1986; Shaw and Kamen, 1986). Examples of where such sequences are found include TNF-α, IL-1β, IL-6 and c-*fos* genes. These sequences can confer instability in the resultant mRNAs and decrease the formation of mature transcripts in the cytoplasm. Different proteins that recognize the AU-rich sequences have been identified: AU-A, AU-B, AU-C and hnRNP (heterogeneous

nuclear ribo-nucleoprotein) or known as AUF1 (Bohjanen *et al.*, 1991; Chen and Shyu, 1995; Ross, 1995). AU-A, a 34-kd protein, is abundant in nuclear extracts and regulates mRNA metabolism. AU-A was found to induce stabilization of c-*myc* mRNA *in vitro* by binding to the AU-rich response elements (ARE) (Ross, 1996). The 30-kd AU-B is another cytoplasmic factor that binds ARE in the 3' UTR of lymphokine mRNAs and promotes degradation of their mRNAs (Bohjanen *et al.*, 1991). The induction of this protein requires protein synthesis. Similarly, the protein AUF1 enhances mRNA degradation by binding to the ARE site *in vitro* assay (DeMaria and Brewer, 1996; Kiledjian *et al.*, 1997).

C. Lipopolysaccharide

C.1. Structure of lipopolysaccharide

Lipopolysaccharide (LPS), a component found on the outer membrane of Gram negative bacteria like the Enterobacteriaceae (*Escherichia coli* and *Salmonella typhimurium*) (Mayeux, 1997), consists of a polysaccharide region and a lipid A region (Fig. 1.1). The polysaccharide region is comprised of "O-Antigen", a polymer of oligosaccharides and a "core" region of oligosaccharides (Schletter *et al.*, 1995; Henderson *et al.*, 1996). The "O-Antigen" is a variable region which confers serologic specificity to the Gram negative microbes. The core oligosaccharide consists of an inner structural region and an outer region (hexose region). The core region has less diversity than the "O-Antigen" (Schletter *et al.*, 1995). The lipid A region of LPS is the least variable portion and is responsible for the biological properties induced by endotoxin (Vogel and Hogan, 1990; Watson *et al.*, 1994). The lipid A component consists of a phosphorylated glucosamine disaccharide that is substituted with ester- and amide-linked fatty acids, including hydroxylated fatty acids.

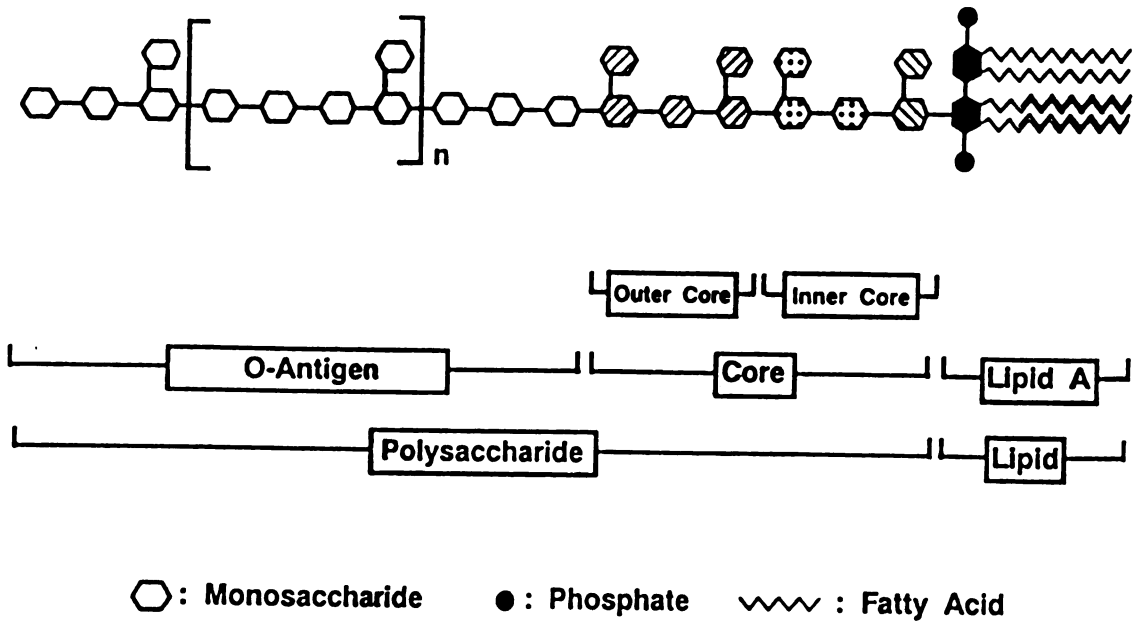


Figure 1.1. General chemical structure of bacterial lipopolysaccharides. (Adapted from Ulevitch and Tobias, 1995)

It has been shown that any changes in the hydrophilic backbone or in the hydrophobic region will lead to a partial or total loss of the LPS activity (Rietschel *et al.*, 1994).

C.2. Proteins that bind lipopolysaccharides

C.2.1. Lipopolysaccharide binding proteins (LBP)

LBP, (lipopolysaccaride-binding protein), is a 60-kd glycoprotein synthesized in the liver and released into the circulation (Schumann, 1992; Ulevitch and Tobias, 1995; Henderson *et al.*, 1996; Fenton and Golenbock, 1998). It is constitutively expressed in liver, but can be further induced by LPS and other stimuli. It serves as a transport protein mainly presenting LPS monomers from bacteria to CD14 (Fenton and Golenbock, 1998). It recognizes LPS by interaction with the lipid A moiety of LPS. The lipid A moiety has a high affinity binding to the LBP protein with a k_d of 10^{-9} (Su *et al.*, 1995). The amino terminal of the LBP is the binding site for LPS and the carboxyl terminal is the site for CD14 interactions. It also serves as an opsonin for bearing particles such as the Gram negative bacteria. Normally, 5-10 μg/ml of LBP is expressed in human serum. During the acute phase response, the concentration of LBP is induced up to 200 μg/ml (Tobias *et al.*, 1992). Anti-LBP antibodies can suppress low LPS dose-induced endotoxemia shock in mice (Gallay *et al.*, 1994). When LPS is bound to LBP, 1000-fold less LPS (0.1 ng/ml) is required to stimulate monocyte secretion of TNF-α (Su *et al.*, 1995). IL-6 can induce expression of LBP in the serum and TNF-α, IL-1 and dexamethasone can synergize with IL-6 in this induction. LBP plays a different role in directing or transporting LPS to bind to the high density lipoprotein (HDL) particles. This binding will lead to a marked reduction in the clearance rate of LPS but decreases the toxicity of LPS. Therefore, binding to HDL is a detoxification process of LPS (Ulevitch *et al.*, 1990).

C.2.2. Cluster differentiation antigen 14 (CD14)

Cluster differentiation antigen 14 (CD14), a myeloid differentiation antigen, is mainly expressed on the cell surface of monocytes, basophils and granulocytes (Wright *et al.*, 1990; Chaby and Girard, 1993; Kielian and Blecha, 1995). The CD14 gene is found on the long arm of chromosome 5 which carries genes also encoding for IL-3, GM-CSF, CSF-1, CSF-1R and PDGF-R (Goyert *et al.*, 1988). There are two types of CD14, the membrane bound CD14 (mCD14) and the soluble CD14 (sCD14) found in the serum (Bazil *et al.*, 1986). mCD14 is a 55-kd glycoprotein which is anchored to the membrane by a glycophosphatdylinositol (GPI) glycan moeity (Kielian and Blecha, 1995). sCD14 lacks the GPI which penetrates into the membrane. Binding of the LPS to mCD14 induces the production of IL-1, IL-6 and TNF-α by monocytic cells. The binding of LPS to CD14 can induce the secretion of free radicals such as O_2^-, H_2O_2, OH and singlet oxygen (Watson *et al.*, 1994), monokines such as IL-6, IL-8, and TNF-α, and arachidonic acid metabolites. An increase of the concentration of O_2^- enhances phagocytosis of macrophages which in turn increases the ability to kill pathogenic microorganisms. Stable transfection of CD14 has been shown to increase the cell's sensitivity to LPS by 10,000-fold (Lee *et al.*, 1992). Anti-CD14 antibodies cannot block the high LPS dose-induced stimulation and binding to the protein (Beaty *et al.*, 1994).

C.2.3. Bactericidal/permeability increasing protein (BPI)

BPI binds specifically to the lipid A moiety of LPS. The BPI gene is located on the chromosome 20 as is the LBP gene (Su *et al.*, 1995). BPI is a 55-kd membrane associated protein found in neutorphil granules and is released upon activation by LPS and cytokines (Marra *et al.*, 1992). Two domains have been found in this protein: N-terminal and C-

terminal domains (Gray *et al.*, 1989). The N-terminal domain binds LPS and the C-terminal domain is a transmembrane region that anchors the holoprotein in the granule membranes. It has 44% identity with LBP protein and it possess LPS neutralizing ability (Fenton and Golenbock, 1998). BPI concentration is induced from 5 to 20 μg/ml upon activation by LPS (Horwitz *et al.*, 1995).

C.3. Signal transduction by lipopolysaccharides

C.3.1 CD14 dependent pathway

LBP catalyzes the binding of monomeric LPS to mCD14. After binding to CD14, the LPS-LBP-CD14 complex activates two different protein kinase system: protein tyrosine kinase (PTK) (Mayeux, 1997) and mitogen activated protein kinase (MAP kinase) (Weinstein *et al.*, 1992). Activation of PTK leads directly to the activation of the IκBα kinase and phosphrylation of the IκBα. This phosphorylation results in the dissociation of the IκBα from the NF-κB. NF-κB then translocates from the cytoplasm to the nucleus and binds to the κB sites on the related genes, which in turn induces transcription of the targeted genes (Mukaida *et al.*, 1996). PTK is required for induction of IL-1, IL-6 and TNF-α expression. For the MAP kinase pathway, dual phosphorylation of threonine and tyrosine residues of MAP kinases is required. This is mediated by the MAP kinase kinases (MEKs). When cells are stimulated with LPS, the LPS-LBP-CD14 complex causes activation of MEKs and consequently phosporylates the MAP kinases including p38, ERK1 and ERK2 (the 42 and 44-kd isoforms of MAP kinases), and c-JUN-N-terminal kinase (JNK)-1 (Schletter *et al.*, 1995).

In cell types which lack membrane-bound CD14 (mCD14), the sCD14 in the serum

replaces the mCD14 for the binding of the LPS-LBP complex (Mayeux , 1997). It is postulated that a putative receptor that recognizes the complexes existed on the CD-14 negative cells (Schletter *et al.*, 1995). Cell types which are deficient in CD14 can also respond to the LPS stimulation but require higher concentrations of LPS (>100 μg/ml LPS) (Mayeux, 1997).

C.3.2. CD14 independent pathway

In the CD14 independent pathway, a transient increase of intracellular Ca^{2+} concentration is found in many cell types including macrophages. LPS cause a rapid increase of $[Ca^{2+}]$ which is generated from the activation of myoinositol 1,4,5-triphosphates (InsP3) (Mayeux, 1997). The released $[Ca^{2+}]$ activates nitric oxide synthase (NOS) resulting in the synthesis of nitric oxide (NO) and cGMP. This is a critical step in the pathway which ultimately leads to cell injury because the generated NO causes formation of oxidizers peroxynitrite anions ($ONOO^-$) and hydroxyl radical ($HO^\cdot$) (Mayeux, 1997). This kind of pathway requires a high concentration of LPS or lipid A of the LPS.

C.4. LPS and cytokine induction

LPS is also known as "endotoxin", because of its role in pathophysiological phenomenon associated with Gram negative infections and the induction of responses that are toxic in nature. Different cytokines can be induced by LPS and they are able to mediate various aspects of endotoxicities such as fever, which is an indicator of inflammation. Several cytokines that are involved in LPS-induced fever are IL-1, TNF-α and IFN-α (Watson *et al.*, 1994). They are also known as "endogenous pyrogens" and are mainly produced by macrophages (Vogel and Hogan, 1990). LPS is also involved in septicemia.

This disease is one of the leading causes of death in the United States (Stone, 1994; Dal Nogare, 1991). Sepsis is the clinical term for the presence of bacteria or their toxins in the blood or tissues.

D. Regulation of interleukin-6 (IL-6)

D.1. Introduction

Interleukin 6 (IL-6) is a multifunctional cytokine which has a molecular weight of 21 to 28-kd (Hirano, 1994). This glycoprotein produced both by lymphoid and non-lymphoid cells, can regulate immune responses, acute phase responses and hematopoiesis. It is also known as B cell stimulatory factor 2 (Hirano *et al.*, 1985). There is 65% homology between human and mouse IL-6 DNA. IL-6 can be produced by a variety of cells such as macrophages, T and B cells, and endothelial cells. Diverse stimuli can induce production of IL-6 and these include LPS, IL-1, TNF-α, IFN-γ, viruses (human immunodeficiency virus), and others (Hirano, 1994). However, agents such as IL-4, IL-13, and glucocorticoids inhibit IL-6 production. In a negative regulatory role, IL-6 can inhibit the production of both IL-1 and TNF-α

There are two IL-6 receptor components: the 80-kd α chain which binds IL-6 and the 130-kd β chain (gp130) for which transduces the signal (Yamasaki *et al.*, 1988; Taga *et al.*, 1989). Binding of the IL-6 to its receptor leads to the dimerization of gp130, and activates Janus tyrosine kinases (JAK1, JAK2, JAK3, Tyk-2) associated with the receptor. These kinases phosphorylate STATs (signal transducers and activators of transcription). Upon phosphorylation, STATs translocate to the nucleus as homodimers or heterodimers. They then bind to promoters and activate transcription of genes associated with acute phase

responses, differentiation and growth arrest. STATs have also been shown to activate junB transcription.

IL-6 plays an important role in the immune response. IL-6 is the key factor for antibody production in B cells. Increases of 3-10 fold in IgG, IgM and IgA antibody production have been seen in mononuclear cells treated with recombinant human IL-6 (Cox and Gauldie, 1997). IL-6 and IL-1 synergistically enhance the growth and differentiation of the murine B cells (Vink *et al.*, 1988). Also, IL-6 increases IgA production in murine Peyer's Patch B cells (Beagley *et al.*, 1989) and involved in T cell function. IL-6 stimulates proliferation of thymocytes and T cells (Cox and Gauldie, 1997) and enhances delayed-type hypersensitivity in mice (Jayaraman *et al.*, 1990). IL-6 is suggested to be involved in glomerulonenephritis as it acts as an autocrine factor for the mesangial cells (Horii *et al.*, 1989; Ruef *et al.*, 1990). It also acts as a hemopioetic factor and is involved in the acute phase response (Cox and Gauldie, 1997).

D. 2. IL-6 and its regulation

Key *cis* elements in the IL-6 promoter include a c-fos serum responsive element (c-fos SRE) site and binding sites for activator protein-1 (AP-1), NF-κB, C/EBP (NF-IL6), cAMP responsive element binding protein (CREB) (Tanabe *et al.*, 1988; Ray *et al.*, 1988; Sehgal, 1992). Glucocorticoids (GCs) have also been identified as the important transcription factors in regulation of IL-6.

D.2.1. Activator protein-1 (AP-1)

AP-1 is a family of transcription factors consist of Fos (c-Fos, Fra-1, Fra-2, and Fos B) and Jun (c-Jun, Jun B, Jun D) proteins. The Fos/Jun heterodimers or homodimers are known to bind to a DNA consensus sequence (5'-TGAC/GTCA-3') (TPA response element

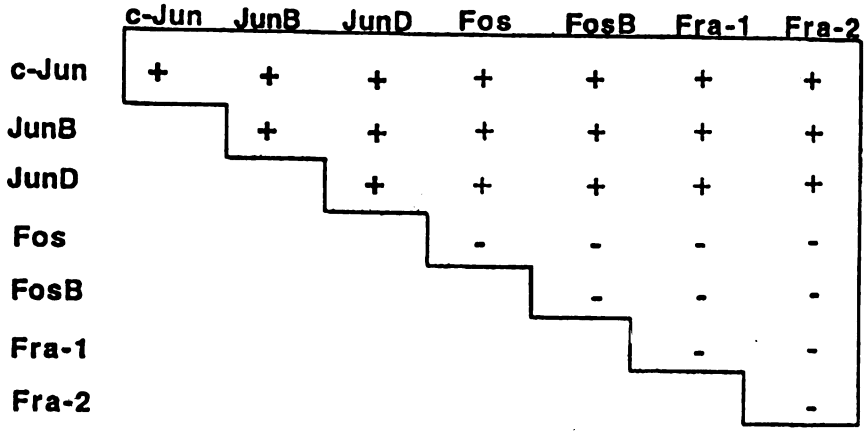


Figure 1.2. Dimer combinations of Fos and Jun proteins capable of forming stable AP-1 transcription factor complexes. (+) means Jun and/or Fos proteins able to form stable homo or hetero-dimers. (-) means Fos proteins are not able to form stable proteins by associating with each other (Adapted from Liebermann *et al.*, 1998).

(TRE)) (Adcock, 1997) (Fig. 1.2). AP-1 is related to the members of other transcription factor family such as activating transcription factor (ATF) and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). AP-1 sites can be found in the genes encoding pro-inflammatory cytokines, cell growth, hematopoietic differentiation (Liebermann *et al.*, 1998), T and B cell activations (Foletta *et al.*, 1998), and apoptosis (Liebermann *et al.*, 1998). The expression of AP-1 is regulated by both transcriptional and post-transcriptional modifications via phosphorylation and dephosphorylation of the proteins (Pennypacker, 1998). The genes encoding Fos/Jun proteins are immediate early genes which do not require *de novo* protein synthesis (Angel and Karin, 1991). Extracellular stimuli such as UV light, inflammatory cytokines and stress induce AP-1 protein production (Liebermann *et al.*, 1998). Recently, it has been shown that LPS can induce activation of JNK thus resulting in the AP-1 in macrophage RAW 264.7 cells (Hambleton *et al.*, 1996). Cytokine expression in Th1 or Th2 cells can also be regulated by AP-1 (Foletta *et al.*, 1998). The regulated cytokines include IL-2, IFN-γ, TNF-β, IL-4, IL-5, IL-6 and IL-10. AP-1 sites have also been found in the IL-6 promoter region at -61 bp to -55 bp and -280 bp to -274 bp (Ray *et al.*, 1990). It is reported that these elements responsed to several agents such as serum, forsokolin and phorbol esters (Ray *et al.*, 1989). However, they are not significant in the induction of IL-6 but might be important in down regulation of IL-6 gene expression.

D.2.2. Nuclear factor kappa B (NF-κB)

NF-κB was first identified as a B cell nuclear factor that bound to the immunoglobulin κ light chain enhancer (Sen and Baltimore, 1986). It is now known that NF-κB binding sites can be found in promoters of many genes such as those encoding IL-2,

IL-6, GM-CSF, ICAM-1, and class I MHC (Baldwin, 1996). NF-κB is activated by a variety of stimuli, including LPS, exotoxin, TNF, IL-1, viruses, parasites, T and B-cell mitogens, stress, and phorbol esters (Baeuerle and Henkel, 1994; Baldwin, 1996). NF-κB is a heterodimer protein belonging to the Rel homology family. The Rel family includes proteins such as NF-κB1(p50/p105), NF-κB2(p52/p100), p65 (Rel A), c-Rel, Rel B and dorsal (Baeuerle and Henkel, 1994; Adcock, 1997) (Fig. 1.3). The Rel homology domain (RHD) can be found at the N-terminus in all of these proteins. This domain is capable of DNA binding, dimerization and interaction with IκB (Baldwin, 1996; Adcock, 1997). The p105 and p100 proteins contain copies of the ankyrin repeat at their c-terminus. These repeats function as protein-protein interaction motifs which are required for the interaction with DNA binding subunits on the NF-κB. RelA, c-Rel and Rel B have potent transactivation domains which are important in transcriptional activation. p50 and p52 are poor transactivators but are the main DNA binding subunits (Adcock, 1997). Heterodimers of NF-κB can bind to the DNA consensus sequence 5'-GGGRNNYYCC-3' with high affinity (R:purine; Y:pyrimdine) (Baeuerle and Henkel, 1994; Baldwin, 1996). NF-κB is regulated as an inactive complexes by binding to an inhibitory molecule (IκB) in the cytoplasm (Fig. 1.4). When cells are activated with agents such as LPS or PMA, the IκB molecule is phosphorylated and degraded, resulted in its dissociation from NF-κB and translocation of NF-κB into the nucleus and binding to the targeted genes (Baldwin, 1996). There are at least five different IκB proteins found in cells: IκBα, IκBβ, IκBγ, Bcl3, and IκB R(Baldwin, 1996; Adcock, 1997). They contain the ankyrin repeats as in the c-terminal domain of p105 or p100 (Baldwin, 1996). NF-κB plays a key role in immune responses

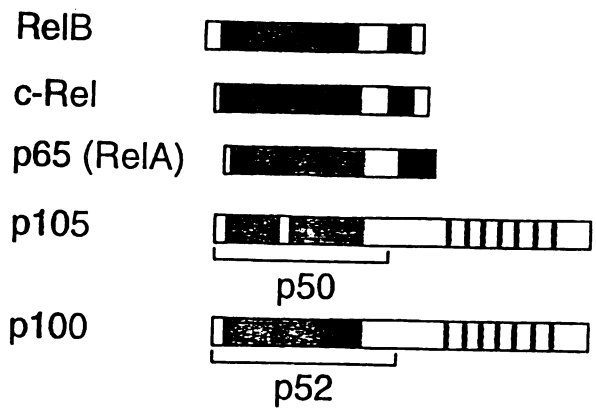


Figure 1.3. Members of the NF-κB family of proteins (Adapted from Dumont *et al.*, 1998).

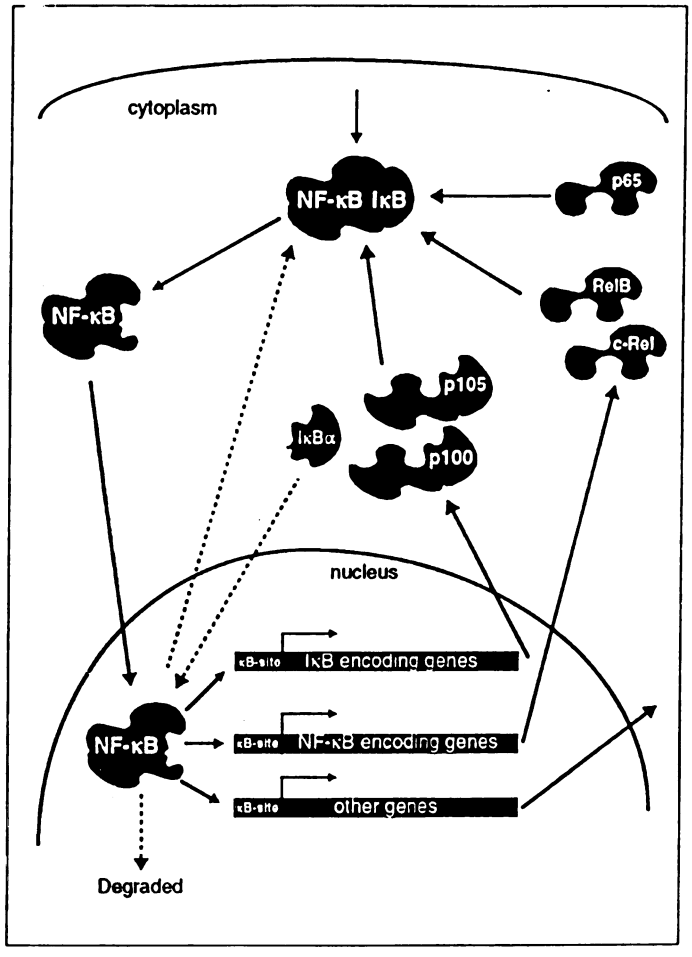


Figure 1.4. Activation for the mechanism of NF-κB activation (adapted from Finco and Baldwin, 1995)

due to its ability to turn on various inflammatory genes. NF-κB sites are present in IL-2, IL-2α receptor, IL-3, IL-4, IL-6 and c-*fos* gene. NF-κB can be found in B and T cells, and macrophages (Lenardo and Baltimore, 1989; Baeuerle and Henkel, 1994). An NF-κB site has been found at position -73 to -64 bp in the IL-6 promoter and is required for induction of IL-6 (Shimizu *et al.*, 1990). When cells are stimulated by IL-1, NF-κB binds to its specific site and activates transcription of the IL-6 mRNA. Glucocorticoids (GCs)seems to inhibit IL-6 expression by binding the NF-κB p65 to its GC receptor.

D.2.3. Nuclear factor (NF)-IL6 or CCAAT/enhancer binding proteins (C/EBP)

NF-IL6 (C/EBP) was first isolated from a rat liver cDNA library (Landschulz *et al.*, 1988). Six different kinds of C/EBP proteins have been identified, C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε and C/EBPζ(Yamanka *et al.*, 1998). They all have a DNA binding domain close to their c-terminal regions and a transacting domain in their N-terminal regions (Wedel and Ziegler-Heitbrock, 1995). The leucine zipper and basic region are capable of forming dimers (Fig. 1.5). C/EBPα mostly is expressed in liver and adipose tissue. It transactivates genes such as insulin-responsive glucose transporter (GLUT4) and regulates adipocyte differentiation. C/EBPβ is also known as NF-IL6, a nuclear factor that mediates the IL-6 response in human glioblastoma cells. It is expressed in almost every tissue. C/EBP β is responsible for the regulation of genes encoding for the acute phase response and cyokines. It is induced by stimuli such as IL-1, LPS, TNF or IL-6. It can serve as both an activator and repressor. C/EBPγ is a protein that binds to the immunoglobulin heavy chain enhancer. It is expressed in early B-cells. C/EBPδ is also called NF-IL6β. It is highly expressed in lung and has no intron (Lekstrom-Himes and Xanthopoulos, 1998).

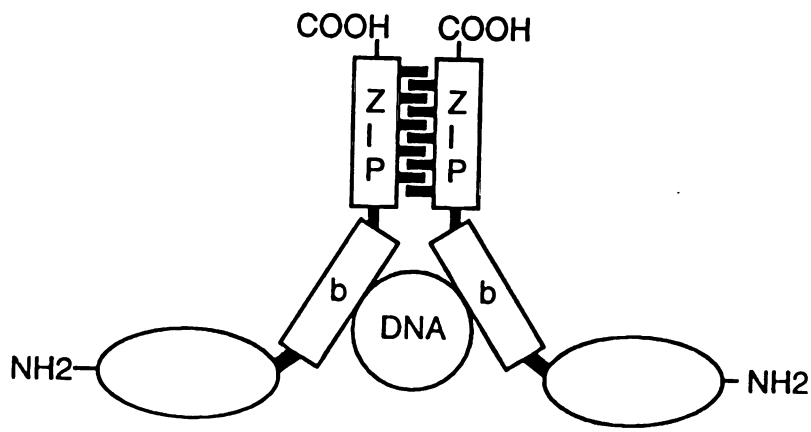


Figure 1.5. Model of the C/EBP structure (adapted form Wedel and Ziegler-Heitbrock, 1995)

It is induced by LPS, IL-1, or IL-6 and is a stronger activator than C/EBPβ and mostly regulates genes involved in the immune responses and inflammation. C/EBPε is normally expressed in the organs or cells involved in the immune system such as myeloid and lymphoid lineages (Lekstrom-Himes and Xanthopoulos, 1998). There are four isoforms of C/EBPε mRNA after splicing. These four isoforms encode three different proteins of the C/EBPε with molecular weight of 32.2-kd, 27.8-kd and 14.3-kd. C/EBP binds to the DNA consensus sequence of 5'-T(T/G) NNGNAA(T/G)-3' (Akira *et al.*, 1992; Yamanaka *et al.*, 1998).C/EBP (NF-IL6) site is located at -158 bp of IL-6 promoter with a 14-bp palindromic sequence 5'-ACATTGCACAATCT-3' (Isshiki *et al.*, 1990). The C/EBP can be induced upon stimulation with LPS, IL-1 or IL-6. C/EBP can bind to the respective site on the IL- 6 promoter and induces expression of IL-6 as heterodimers or homodimers. C/EBP can also interact with NF-κB for maximal expression of IL-6.

D.2.4. Cyclic AMP response element binding protein (CREB)

CREB is the transcription factor that regulates cyclic AMP (cAMP) and calcium-dependent genes. This protein can bind to conserved regions of the 8-nucleotide sequence of 5'-TGACGTCA-3' which is located within 100 bp from the TATA box of the targeted genes (Montminy, 1997; Short *et al.*, 1986). CREB has been identified as a 43-kd protein which is phosphorylated by protein kinase A (PKA) at its consensus site of RRPSY (Gonzalez and Montminy, 1989). The phosphorylation at one serine (ser-133) leads to the dimerization of the CREB proteins on the CRE sites of the promoter of the targeted genes. Increases in cAMP levels or intracellular calcium levels activate CREB. The activation leads to the activation of the calmodulin-dependent kinase IV (CaMKIV), which in turn phosphorylates CREB and consequently stimulates its binding to the CRE site (Fig. 1.6)

(Lamprecht, 1999). There are different forms of CREB that have been identified, α, β and δ. They bind to the CRE site with different affinities (Montminy, 1997). cAMP inducing agents such as PGE_2 seems to enhance transcription of IL-6.

D.2.5. Glucocorticoids (GCs) as negative regulators

GCs can act as both activators or repressors of gene transcription. GCs are steroids that exert effects on immunoregulation by binding to their GCs receptor (GR). This receptor usually binds to the 90-kd heat shock protein (Hsp90) in the cytoplasm (Munck *et al.*, 1990). Upon activation with GCs, the GR dissociates from the Hsp90, translocates to the nucleus and binds to the targeted DNA (Berg, 1989). The DNA binding domain has a zinc finger structure which can bind to DNA (Berg, 1989). The GC response element (GRE) has a 15 bp consensus sequence of 5'- GGTACANNNTGTTCT-3' palindrome (Drouin *et al.*, 1992). GREs are located in the 5' flanking region of GRE responsive genes. Binding of GR on this element leads to the alteration of the transcriptional activity (Schmidt *et al.*, 1994). GR can inhibit cytokine gene expression by binding on the GREs and thus prevent the binding of the transcription factors to their putative binding sites. The GR can also bind directly to transcription factors and abrogate their binding to the DNA and thus inhibit transcription (Paliogiani *et al.*, 1993; Caldenhoven *et al.*, 1995). The repressive effects of the GR have been studied widely in the NF-κB transcription by binding to NF-κB directly or by binding to the GRE site to enhance IκBα transcription (Dumont *et al.*, 1998). GREs can be found in the IL-6 promoter. It has been shown that GCs inhibit IL-6 expression by binding to the AP-1 and the NF-κB p65 (Ray *et al.*, 1990; Ray and Prefontaine, 1994).

D.3. Post-transcriptional control

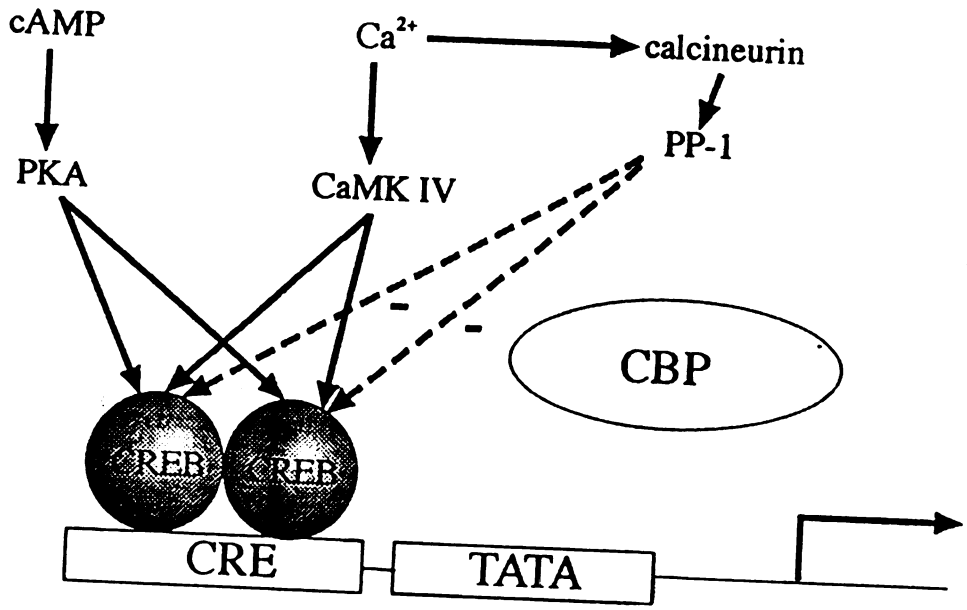


Figure 1.6. Regulation of gene expression by CREB. Phosphorylation of CREB on Ser-133 is the critical step in CREB activation (adapted from Lamprecht, 1999).

The AU-rich regions in the 3'-untranslated region play a key role stabilizing IL-6 mRNA expression. It is known that the IL-1- and TNF-α-modulated IL-6 mRNA half-lives are different (Ng *et al.*, 1994). Stabilization of mRNA plays a major role in IL-6 mRNA superinduction (Elias and Lentz, 1990; Roger *et al.*, 1998a). Ultraviolet (UV) B upregulates IL-6 production by enhancing IL-6 mRNA stability (de Vos *et al.*, 1994). IL-10 has been reported to inhibit IL-6 mRNA expression by increasing degradation of the IL-6 transcripts (Takeshita *et al.*, 1996). GCs such as Dex can inhibit IL-6 production by decreasing mRNA stability of IL-6 (Amano *et al.*, 1993). Therefore, post-transcriptional control is an important regulation in IL-6 gene expression.

D.4. Summary

In the regulation of IL-6 gene expression, several factors are involved. First, the positive acting transcription factors NF-κB, NF-IL6 (C/EBP), AP-1, and CREB contribute to IL-6 transcription. It is suggested that cooperative effects of NF-κB and NF-IL6 are the most important factors in transcriptional regulation of IL-6. Also, the effects of negative regulators such as GCs are important in the transcription of IL-6 expression. There are multiple regulatory sites for some of these factors which might indicate a role of these factors in the regulation of IL-6 gene expression. Recently, the regulation of mRNA decay was also recognized as an important mechanism in regulating cytokine gene expression. As with other cytokines, the AU repeats in the 3' non coding region of IL-6 mRNA confer instability and thus may play a part in IL-6 protein and mRNA expression. In the future, more work should focus on both transcription and post-transcriptional control of this gene.

E. Tumor necrosis factor-alpha (TNF-α) and its regulation

E.1. Introduction

TNF-α is a 26-kd protein that synthesized and anchored in the cell membrane. When it is released from the cell surface, it is cleaved to a 17-kd protein by enzymes (Dayer and Burger, 1994; Beyaert and Fiers, 1998). The secreted protein folds back onto itself into two β-pleated sheets (Jones *et al.*, 1989). Secreted TNF-α was found to cause necrosis in some mouse tumors and was the mediator causing cachexia associated with hyperglyceridema in rabbits chronically infected with *Trypsanoma brucei* (Goodwind and Guy, 1973; Rouzer and Cerami, 1980). Monocytic cells are the main source of TNF-α *in vivo*. Other cells such as basophils, eosinophils, NK cells, and polymorphonuclear leukocytes can also produce TNF (Vilcek and Lee , 1991; Tracey, 1994). The TNF family includes two related proteins, TNF-α (cahcetin) and TNF-β (lymphotoxin) which bind to the same cell surface receptors (Vilcek and Lee, 1991). TNF-α plays a major role in beneficial effects as an immunostimulant and also mediate detrimental effects such as wasting during chronic infections. TNF-α is the main mediator in the development of septic shock caused by Gram negative bacteria (Tracey *et al.*, 1987).

TNF-α can activate neutrophils, mast cells, macrophages and endothelial cells *in vitro* and is considered to be a pro-inflammatory cytokine. *In vitro*, it can cause selective cytolysis of tumor cells and induces differentiation of monocytes and secretion of IL-1 and IL-6, and induces B cell proliferation and increased antibody production. *In vivo*, TNF-α also enhances lipolysis and muscle wasting. It induces shock and is a major mediator in endotoxin-mediated shock. TNF-α induces fever and is regarded as an endogenous pyrogen. It also induces acute phase responses and stimulates the production of the

procoagulant factor. TNF-α is involved in the pathogenesis of lupus nephritis. It is angiogenic and promotes proliferation of fibroblasts. It also stimulates production of synovial and macrophages (resting) collagenase and PGE_2 which contributes to the inflammation and tissue destruction (Dofferhoff *et al.*, 1991; Pauli, 1994; Dayer and Burger, 1994; Beyaert and Fiers, 1998).

E.2. Receptors of TNF and signal transduction pathways

There are two types of murine TNF-α receptors which are involved in signal transduction, mediated by TNF-α: the 55-kd (TNFR1) and the 75-kd (TNFR2) (Beutler and van Huffel, 1994) (Fig. 1.7). These glycoproteins are of similar length but differ in their carbohydrate content. The extracellular domains of these two receptors are similar in sequence (Beyaert and Fiers, 1998). Both receptors bind TNF-α and TNF-β equally well and are members of the nerve growth factor receptor (NGFR)/TNFR superfamily (Dayer and Burger, 1994). The extracellular domain of each of the two receptors contains four cysteine-rich repeats (Dembic *et al.*, 1990). The intracellular domains (or cytoplasmic signaling components) of both receptors are distinct from each other, therefore a different signaling apparatus may connect with each of the two receptors (Beyaert and Fiers, 1998). It has been suggested that the TNFR1 mediates cytotoxic signals (apoptosis) and most of the TNF-α responses (Beyaert and Fiers, 1998), while the TNFR2 is involved in T cell proliferation and inhibition of the early hematopoisis (Beyaert and Fiers, 1998).

It has been shown that binding of TNF-α to the TNF receptor leads to the formation of the disulfide-linked receptor aggregates. Each TNF trimer contains three cognate receptor binding sites and the change in receptor conformation that results from engagement of the

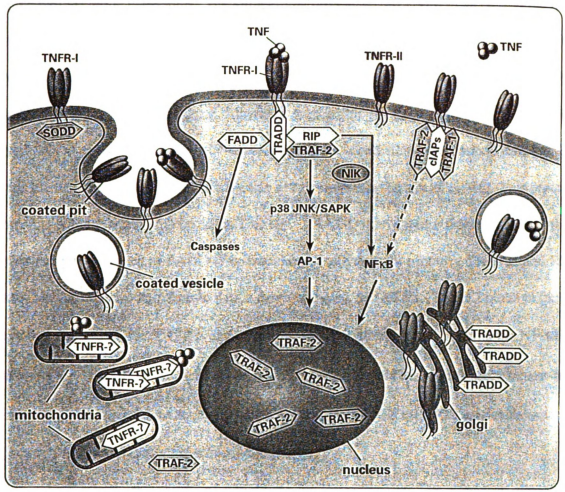


Figure 1.7. Schematic representation of the TNF receptors, TNF and associated signaling molecules (adapted from Ledgerwood *et al.*, 1999).

TNF trimer is sufficient to initiate signal transduction (Beutler and van Huffel, 1994). The multimerization of the receptors with the ligands leads to the internalization and degradation of the ligands in the lysosomes. The intracellular domain of the TNFR1 can be divided into a membrane distal domain and membrane proximal domains (Beyaert and Fiers, 1998). The membrane distal domain is known as the "death domain" (Tartaglia *et al.*, 1991; Ledgerwoood, 1999). The death domain is responsible for the cytotoxicity. A 34-kd cytoplasmic protein known as TNF receptor-associated death domain protein (TRADD) has been identified and contained death domain at its c-terminus (Hsu *et al.*, 1996). This protein interacts with the death domain of the TNFR1. TRADD is an adaptor that can recruit other signaling proteins such as FADD (Fas-associated death domain protein), TRAF2, and RIP (a serine/threonine protein kinase with a death domain), to TNFR1. TRAF2 binds to the c-terminal domain of the TNFR1 and cause NF-κB activation. It also recruits the NF-κB - inducing kinase (NIK) to the membrane, which in turn activates the IκB kinase, a kinase that phosphorylates the IκB thus releasing active NF-κB. Both FADD and RIP are death domains and mediate TNFR1 receptor-induced apoptosis.

TNF-α induces a variety of signal transduction pathways. The activation of protein kinase C (PKC) (Brenner *et al.*, 1989), the release of Ca^{2+} and increases of inositol 1,4,5-triphosphate have all been demonstrated following stimulation with TNF-α (Kronkel *et al.*, 1991). Activation of the phospholipase A2 and the release of arachidonic acid by TNF-α has also been observed. TNF-α also activates protein kinase A (PKA) and causes increased levels of cAMP (Zhang *et al.*, 1988). Tyrosine kinases might also be involved in the activation by TNF-α (Donato *et al.*, 1989). TNF-α can induce a variety of proteins

including the induction of IL-6. Other transcription factors activated by TNF-α are NF-κB, AP-1(c-fos and c-jun), NF-IL6 , CREB and interferon regulatory factors 1 and 2 (IRF-1 and -2) (Vilcek and Lee, 1991).

E. 3. Regulation of TNF-α gene expression

Different stimuli can induce the production of TNF-α including LPS, synthetic lipid A, enterotoxin, IL-1, GM-CSF, phorbol esters, oxygen radicals, and TNF itself (Dofferhoff *et al.*, 1991; Pauli, 1994). LPS is the most studied stimulant that induces TNF-α production in monocyte/macrophage cells. Many agents can inhibit TNF-α mRNA expression such as dexamethasone (DEX), cAMP, protein kinase inhibitors (staurosporine or H7), cyclosporin A, and prostaglandin E2 (Pauli, 1994). TNF-α expression is controlled at different levels: transcriptional, post-transcriptional and translational (Beutler *et al.*, 1986).

TNF-α is encoded by a single copy gene that is approximately 3 kb pairs long. It consists of four exons and three introns. In human, several regulatory sites had been found in the TNF-α promoter region. These include the AP-1 sites (-108/-101 bp), the three κB sites (-600/-576 bp, - 242/ -199 bp, -118/-89 bp), the cyclic AMP-responsive element (CRE) (-95/-36 bp), SP-1 sites, a TATA box and a Y box (Pauli, 1994). In mice, TNF-α consists of four κB sites on its promoter. LPS-responsiveness is abrogated with the mutation of all four κB sites. Deletion of the κB site from a promoter construct leads to reduction of the reporter activity. This suggests that these κB sites are important in the regulation of transcription of TNF-α. It has been shown that TNF-α gene transcription is enhanced 3-fold upon LPS stimulation (Beutler *et al.*, 1986). The deletion of the κB sites from the TNF-α promoter will reduce its LPS responsiveness (Goldfeld *et al.*, 1990, 1991). An AP-1 site

has been found at -108 to -101 bp of palindrome sequence of TGAGCTA, and is PMA-responsive. Deletion of this site markedly reduces the transcription of TNF–α (Rhoades *et al.*, 1992; Pauli, 1994). An NF-IL6 binding site is also found in the promoter of TNF-α with a consensus sequence of 5'-GGATTTGGAAAGTT-3' (Akira *et al.*, 1992). The role of dexamethasone as an inhibitor for TNF-α expression suggests that GCs might be involved the transcription of this gene (Pauli , 1994). cAMP has been shown to be involved in inhibiting TNF-α mRNA expression via protein kinases. Apart from transcriptional activation, the regulation of the TNF-α gene is also found at the post-transcriptional level (Pauli, 1994; Vilcek and Lee, 1991). TNF-α gene consists of the repeating units (TTATTAT) in the 3' untranslated region of its mRNA. The AU rich region has been proved to cause instability and superinducibility upon exposure to protein synthesis inhibitors. GCs such as Dex inhibit TNF-α production by reducing the amount of the TNF-α mRNA and by blocking its translation (Beutler *et al.*, 1986).

F. IL-1β and its regulation

F.1. Introduction

Interleukin (IL)-1 has been implicated as a major inflammatory mediator in a variety of pathological situations. There are two different forms of IL-1, IL-1α and IL-1β. They are prototypic multi-functional cytokines. IL-1 can mediate different biological activities including pyrogenicity, stimulation of the immune response and release of pro-inflammatory mediators (Cavaillon and Haeffner-Cavaillon, 1990). It is found on the long arm of the band q17 of chromosome 17. The precursors of both IL-1 are 31-kd and lack a signal

peptide that enables the protein to insert into the Golgi (Dinarello, 1994). The IL-1α precursor is more active and IL-1β precursor is inactive and needs to be cleaved for its activity (March *et al.*, 1985). Mature IL-1α and IL-1β are both 17-kd. Though they have different amino acid sequences (<26% similarity), the structure of the two isoforms are closely related at three-dimension level and have similar activities (Dayer and Burger, 1994). Expression of IL-1 can be induced by variety of stimuli such as direct contact with activated T cells, cytokines, thrombin, steroid hormones, bacterial toxin (LPS, lipid A), viruses, mitogens, specific forms of immune complexes, fragments of complements, and crystals (Dayer and Burger, 1994; Tocci and Schmidt, 1997). When stimulated with LPS, there are 5 to 20 times more pro-IL-1α production than the pro-IL-1β (Demczuk *et al.*, 1987; Cavaillon and Haeffner-Cavaillon, 1990).

IL-1β can induce a wide variety of biological effects. Its action is similar to those induced by TNF-α. IL-1β is a potent pyrogen and causes fever, sleepiness, lack of appetite, muscular fatigue (Dayer and Burger, 1994; Tocci and Schmidt, 1997). This suggests that IL-1β may affect structures in the central nervous system. IL-1β also induces symptoms of shock and is a key mediator in acute phase responses and induces the release of the acute phase proteins (Dinarello, 1984). These include C-reactive protein, serum amyloids, fibrinogen, and various protease inhibitors. Injection of IL-1β also leads to the release of corticotropin releasing factor (ACTH) and cortisone, which is anti-inflammatory in nature and inhibits cytokine gene expression (Dayer and Burger, 1994). This might be a biological feedback loop. IL-1β also stimulates both B and T cells, causes chemotaxis of neutrophils and monocytes, and release of prostaglandins. It also induces modification of metabolism

as it reduces food intake in animals, decreases body weight and exhibits anorectic properties (Dinarello, 1994). IL-1β can induce expression of multiple genes including IL-1β itself , TNF, IL-2, IL-6, IL-8, IL-12, GM-CSF and also cytokine receptor genes such as IL-2, IL-3, and IL-5 receptor. The induction of these genes might be responsible for the biological effects caused by IL-1β.

F.2. Receptors of IL-1β and signal transduction

When monocytes are stimulated, both proIL-1α and IL-1β are produced and released from the nucleus (Cavaillon and Haeffner-Cavaillon, 1990). The proIL-1α is active as a precursor and remains intracellular. The synthesis of the proIL–1α is associated with the microtubules of the cytoskeleton structure. The proIL–1α remains in the cytosol after translation and is then myristoylated. Myristoylated IL-1α is transported to the cell membrane (Kurt-Jones *et al.*, 1985). Membrane IL-1α is biologically active and accounts for only 5% of the total proIL-1α (Dinarello, 1997). The myristoylated pro IL-1α can also be cleaved by calpain and released into the extracellular compartment or it can bind to nuclear DNA (Kobayashi *et al.*, 1990). Following synthesis, proIL-1β remains in the cytosol until cleavage by protease. Cleaving is required for its optimal biological activity before transported out of the cells. This specific protease for cleaving IL-1β precursor is known as the IL-1β converting enzyme (ICE). It belongs to the family of the cysteine proteases known as caspases with a molecular weight of 20-kd and it does not cleave the IL-1α precursor (Dinarello, 1994).

After binding to cells, IL-1β immediately induces several biochemical events. IL-1β binds to the extracellular domain of either type I or type II IL-1 receptor (IL-1R)

(Dinarello, 1997). Following binding of the IL-1β to the receptor, a structural change of the IgG-like domain of the receptor is observed. The binding of IL-1β to this receptor leads into dimerization of the two cytosolic domains of the two IL-1R. Then activation of the putative GTP binding sites on the cytosolic domains, binding of a G-protein, hydrolysis of the GTP and activation of phospholipase are involved (Dinarello, 1997). Diacylglycerol or phosphatidic acids are generated following the hydrolysis of this phospholipid. Phosphorylation of the JNK and the p38 MAP kinase and downstream phosphorylation of the MAPKAP-kinase-2 and phosphorylation of the heat shock protein 27 (hsp27) occur. Following binding of the IL-1β to the IL-1RI, inhibitory κB (I-κB) is phosphorylated and translocation of the NF-κB to the nucleus is observed (DiDonato *et al.*, 1995).

F.3. IL-1β and its regulation

The IL-1β promoter contains sites for AP-1(*c-jun*), the octamer binding protein (Octa-1), NF-κB, Pu.1(transcription factor Spi-1), NF-IL6, and a TATA box and CAAT box. AP-1 sites have been identified in the upstream region of the IL-1β promoter as TCCGTCA (-610/-604) and ATGTGTCAT (-208/-200) (Cavaillon and Haeffner-Cavaillon, 1990). AP-1 helps sustain IL-1β gene transcription in response to PMA . However, no major role has been indicated for AP-1 in IL-1β transcription. The NF-κB site is found on the transcription promoter (-286 to -296) (Cavaillon and Haeffner-Cavaillon, 1990) and further upstream (-2757 bp) (Tocci and Schmidt, 1997) of IL-1β gene with a sequence of 5'-GGGAAATCC-3'. Both sites are required for maximal IL-1β transcription. Agents that inhibit NF-κB activation also block IL-1β transcription. The sequence between -2896 and -2846 bp of IL-1β promoter is shown to bind the heterodimer of cAMP response element

binding protein (CREB) (Dinarello, 1996). Increased cAMP levels induced by agents such as histamine or PGE_2 augment IL-1β gene expression and synthesis. However, increased cAMP levels deplete LPS-induced IL-1β gene expression. IL-1 has the octamer binding site in its promoter and a single base difference will affect the binding of Oct (Lakin *et al.*, 1995). Octamer binding protein can bind to its specific sequence ATGCAAAT or its complement ATTTGCAT (Falkner *et al.*, 1986). The octamer motif is found in various eukaryotic regulatory elements and is responsible for the transcription of variety of genes which include immunoglobulin genes in B cells and cytokine genes in the immune and nervous systems (Schreiber *et al.*, 1989b). Ubiquitous Oct-1 is expressed in a wide variety of mammalian cells and controls the general octamer site-dependent transcription, Oct-2 is only expressed in lymphoid cells and neuronal cells, and regulates B cell-specific expression (He *et al.*, 1989; Schreiber *et al.*, 1990). Some other octamer proteins also have been found in the embryonic cells and testis. Apart from its role as a transcription factor, Oct-1 has also been shown to stimulate DNA replication (Verrijzer *et al.*, 1990). The octamer motif is located next to the AP-1 site on IL-1β promoter. Transcription of the IL-1β does not require *de novo* protein synthesis, suggesting that the activation of pre-existing transcription factors occurs. Cytokines such as IFN-γ and GM-CSF synergize with LPS in inducing IL-1 gene transcription.

Post-transcriptional control of IL-1β is also implied as the addition of CHX increases the stability of IL-1β mRNA. IL-1β contains AU-rich regions in its 3'-untranslated region (UTR). IL-1α transcripts are much more stable than those for IL-1β (Turner *et al.*, 1988). It has been shown that IFN-γ is able to increase IL-1β production by enhancing both IL-1β

transcription and to increase mRNA stability (Arend *et al.*, 1989). Different mRNA half-lifes of IL-1β were found in monocytes and macrophages. Both IL-4 and GC hormone dexamethasone (Dex) can serve as the negative regulators of IL-1β by increasing mRNA degradation (Fenton, 1992; Lee *et al.*, 1988). The destabilization effect can be blocked by the addition of protein synthesis inhibitors, suggesting that *de novo* protein synthesis is required for the mRNA degradation (Lee *et al.*, 1988). It has been indicated that the NF-IL6 sites in IL-1β are sensitive to Dex.

In summary, transcription factors such as AP-1, NF-κB, NF-IL6 and Oct are involved in the transcription of IL-1β gene expression. It has been mentioned that these transcription factors work cooperatively for its maximal gene expression (Adcock, 1997). Post-transcription control also plays another important role in the IL-1β gene expression as increased mRNA stability can be observed with modulators. However, negative regulators such as IL-4 and GC can increase mRNA degradation of IL-1β. Therefore, the regulation of IL-1β is complicated, and future work on the its regulation is needed for better understanding.

G. Rationale for this research

VT can superinduce cytokine production both *in vivo* and *in vitro* systems (Miller and Atkinson, 1986; Azcona-olivera *et al.*, 1995a,b; Ouyang *et al.*, 1996). Therefore it is reasonable to suggest that VT can also superinduce cytokine expression in macrophage cells. Viriyakosol and Kirkland (1995) suggested a model of LPS activation of macrophages (Fig. 1.10) where in a complex of LPS:LBP binds to the CD14 receptor and an unidentified second receptor. The binding of this LPS:LBP:CD14 complex to the hypothetical second receptor

then leads to signal transduction. Since CD14 is incapable conducting the signal transduction itself, other protein kinases such as tyrosine kinase plays a key role in transducing the signal for activation (Weinstein *et al.*, 1992). When macrophages are activated by LPS, proinflammatory cytokines such as TNF-α, IL-6, IL-1β and IL-8 are upregulated (Manthey *et al.*, 1994; Salkowski *et al.*, 1995; Su *et al.*, 1995). It is hypothesized that VT increases the LPS-induced cytokine production by increasing transcription of the cytokines and/or prolonging the mRNA half-life of the cytokines. These interactions of VT with LPS may further enhance the effects of LPS such as endotoxic septicemia, the shock-like syndrome, organ damage and apoptotic cell death. The synergistic effects of LPS and VT probably contribute to the pathogenic manifestations associated with trichothecene exposure. The exact mechanisms of the superinduction of macrophage cytokine gene expression by VT remain unclear. Protein synthesis inhibitors, such as CHX, have been shown to enhance and prolong the normally transient expression of specific gene transcripts via impaired synthesis of high turn-over proteins that down regulate transcription, or via decreased mRNA half-life of those proteins (Sun *et al.*, 1993; Cockfield *et al.*, 1993; Efrat and Kaempfer, 1984). Previous studies have also revealed increases in activity of transcription factors such as NF-κB in IL-2 gene regulation in the EL-4 cells with VT (Ouyang *et al.*, 1996). Therefore, it is reasonable to suggest that VT can also superinduce cytokine expression in macrophage utilizing similar mechanisms. The main goal of this dissertation is to better understand the cellular and molecular mechanisms by which VT superinduces cytokines in macrophages and monocytic cells.

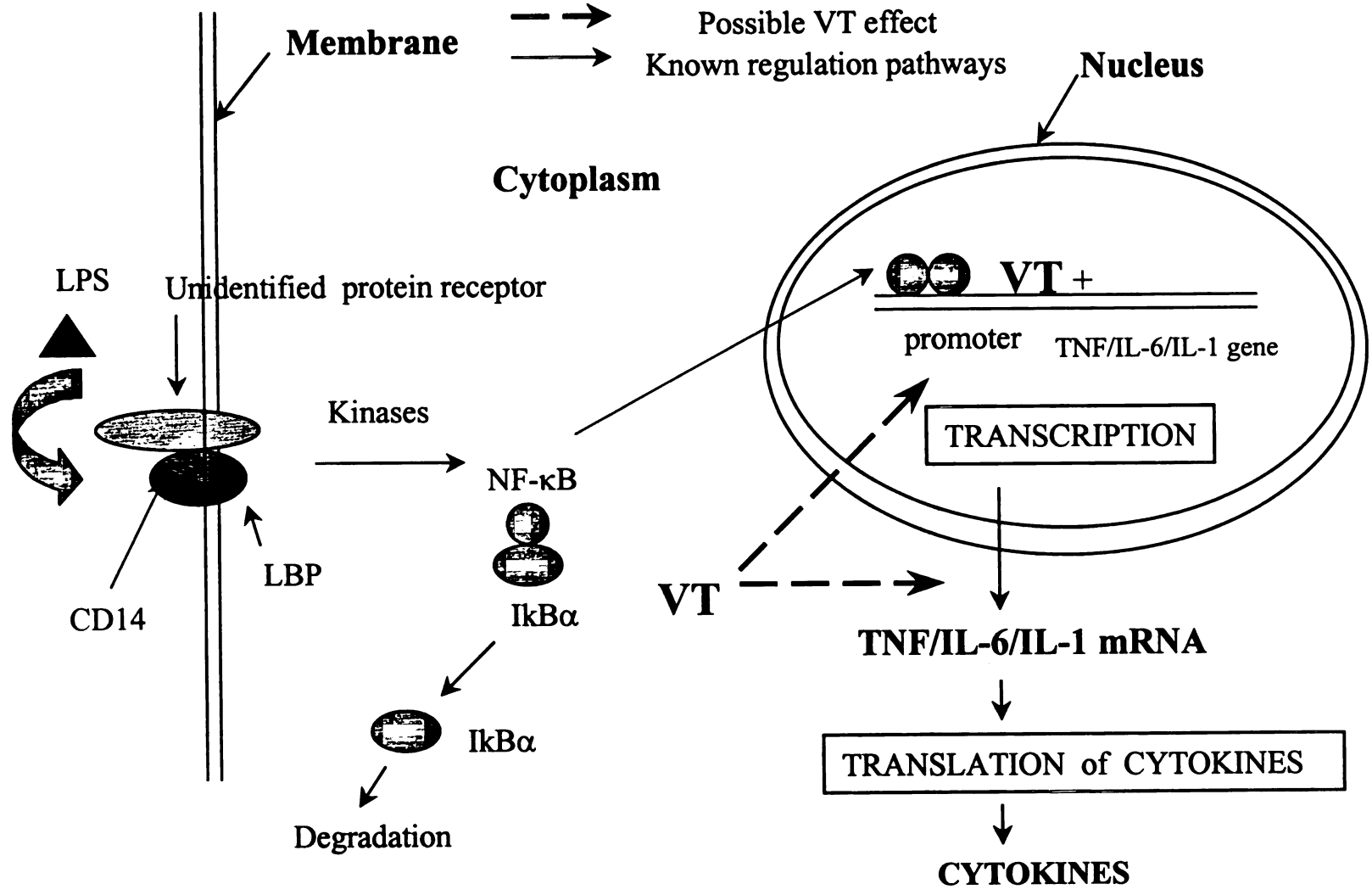


Fig.1.8. Interaction of VT with LPS activation pathway

CHAPTER II

MODULATION OF IL-1β, IL-6, TNF-α SECRETION AND MRNA EXPRESSION BY THE TRICHOTHECENE VOMITOXIN IN THE RAW 264.7 MURINE MACROPHAGE CELL LINE

ABSTRACT

Oral exposure of mice to vomitoxin (VT) has been previously shown to enhance gene expression of several cytokines associated with macrophage activation. Here, the effects of exposure to VT *in vitro* on cytokine secretion and mRNA expression were determined in the murine macrophage cell line RAW 264.7. Enzyme-linked immunosorbent assay (ELISA) of supernatants revealed that significant increases in secreted tumor necrosis factor alpha (TNF-α) were observed 2 days after exposure to VT at 100 ng/ml and 250 ng/ml both with and without Lipopolysaccharide (LPS) activation. While VT did not affect IL-6 secretion in the absence of LPS, significantly increased IL-6 production was observed in culture supernatants after 1, 2 and 5 days exposure to VT at 250 ng/ml in the presence of LPS. Soluble IL-1β was not detected in control or VT-treated cell cultures with or without LPS activation. Immunochemical staining of intracellular cytokines in conjunction with flow cytometric analysis was used to detect the effects of VT on the percentage of positive cells and output per cell. The percentage of cells that produced intracellular TNF-α were significantly increased at 100 and 250 ng/ml VT with and without LPS whereas increased IL-6 output per cell was observed at 100 and 250 ng/ml VT with LPS. To assess the effects of VT on cytokine mRNA expression, RAW 264.7 cells were analyzed semi-quantitatively using reverse transcription-polymerase chain reaction(RT-PCR) in conjunction with Southern hybridization analysis. Elevated TNF-α mRNA was observed at 100 and 250 ng/ml VT at 6 and 24 hr in the absence of LPS. With the addition of LPS, superinduction of TNF-α was not observed in the presence of VT. Increased IL-1β and IL-6 mRNAs were observed at 100 and 250 ng/ml VT at 24 hr in the presence of LPS. These results demonstrated that

VT could superinduce both cytokine secretion and mRNA levels in macrophage cultures.

INTRODUCTION

The trichothecene vomitoxin (VT or deoxynivalenol) is produced by several *Fusarium* species and has been identified as a common contaminant in world wheat and corn supplies (Rotter *et al*, 1996). VT and other trichothecenes are potent protein synthesis inhibitors that can cause acute shock-like toxicity or chronic effects such as feed refusal in experimental animals (Cundliffe *et al*, 1974; Bamburg, 1983). Notably, exposure to trichothecenes *in vivo* inhibits DNA and protein synthesis in bone marrow, lymph nodes, spleen and thymus (Rosenstein and Lafarge-Frayssinet, 1983). Trichothecenes can either be immunosuppressive or immunostimulatory depending on the dose and timing of exposure (Pestka and Bondy, 1994). For example, trichothecenes increase susceptibility to bacterial and viral infections, and can impair cell-mediated and humoral immunity (Arnold *et al*, 1986; Tryphonas *et al*, 1986). Paradoxically upon dietary exposure to VT, mice exhibit elevated serum. Immunoglobulin A (IgA), circulating IgA immune complexes and mesangial IgA deposition in the kidney (Dong and Pestka, 1993). These features are highly analogous to the human glomerulonephritis, IgA nephropathy (D'Amico, 1987).

Several cytokines are potentially involved in differentiation of B cells to IgA secretion (McGhee and Kiyono, 1993). Prior exposure to VT *in vitro* enhances $CD4^+$ cell mediated help for IgA production by B cells through elevated cytokine production (Warner *et al*, 1994). Ingestion of vomitoxin can also increase the percentage of total T cells, $CD4^+$ cells and $CD4^+/CD8^+$ cell ratio in Peyer's patches (PP) (Pestka *et al*, 1990a,b). Increased mRNAs for and secreted forms of interleukin- (IL-) -2, -4, -5 and/or IL-6 are observed upon

exposure to VT or cycloheximide (CHX) (Warner *et al*, 1994; Azcona-Olivera *et al.*, 1995a; Ouyang *et al*, 1996). Thus, VT-induced elevation of IgA production might be related to the superinduction of various T helper cytokines.

Macrophages are also pivotal regulators of inflammation and immunity (Solbach *et al*, 1991). Besides being involved in microbial clearance, they play an important role in regulating T cell function, proliferation, regulation of antibody production and production of mediators for cellular immunity. Many of these activities are mediated through the proinflammatory cytokines (Nathan *et al*, 1987). Since these cytokines may directly or indirectly affect IgA production by modulation of B and T cell function, respectively, macrophages may potentially be to be involved in the VT-induced hyperelevation of IgA. Superinduction of inflammatory cytokines occurs upon addition of other protein synthesis inhibitors such as CHX and has been observed in several macrophage cell lines (Bendtzen, 1983). VT has also been reported to enhance the release of IL-1 in the peritoneal macrophages (Miller and Atkinson, 1986). Recently, we have determined that oral exposure of mice to VT causes transient elevation of mRNAs for cytokines associated with macrophage (IL-1β, IL-6, and TNF-α) as well as T helper cell activation (Azcona-Olivera *et al.*, 1995b; Zhou *et al.*, 1997) within 2 hrs. As yet, the mechanistic basis for VT-stimulated macrophage gene expression *in vivo* remains unknown. The objective of the study was therefore to investigate the toxic effects of VT on cytokine secretion and mRNA using murine RAW 264.7 cells as a cloned macrophage cell model. The results suggested that VT could superinduce both cytokine secretion and mRNA levels in macrophage cell cultures.

MATERIALS AND METHODS

Experimental design. The RAW 264.7 murine macrophage cell line (TIB 17) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's tissue culture medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), NCTC-135 medium (Gibco BRL, Chagrin Falls, IL) and 10 % (v/v) fetal bovine serum (FBS; Gibco Laboratories, Chagrin Falls, IL) at a temperature of 37 °C in 5 % CO2.

For toxin exposure, cells (2.5 x 10^5 per ml) were seeded in 6- or 12-well flat-bottomed tissue culture plates (Corning, NY) in the absence or presence of 1 μg/ml *Salmonella typhimurium* lipopolysaccharide (LPS; Sigma) and 0 to 500 ng/ml of VT (Romer Labs, St. Louis, MO) for various time intervals. Cultures were centrifuged at 300 xg for 10 min to separate supernatant from cells. Macrophage cell numbers and viabilities were assessed by trypan blue (Sigma) dye exclusion using a hemacytometer (American Optical, Buffalo, NY) as described by Strober (1991).

Cytokine assay. IL-6 and TNF-α concentrations were measured by ELISA as described by Dong *et al* (1994) using rat anti-mouse interleukin monoclonal antibodies (Pharmingen, San Diego, CA) and streptavidin horseradish peroxidase conjugate (Sigma). The IL-1β assay was performed by a modified ELISA in which Immunolon IV Removawell microtitre strips (Dynatech Laboratories., Chantilly, VA) were coated with 50 μl/well of monoclonal hamster anti-IL-1β antibody (Genzyme, Cambridge, MA) diluted 1: 1000 in phosphate buffered saline (PBS)and incubated at 4°C by overnight. Plates were washed three

times with PBS containing 0.05% (v/v) Tween 20 (Sigma) (PBS-T). To reduce non-specific protein binding, 300 µl PBS containing 1 % (w/v) bovine serum albumin (BSA) was added to each well, incubated for 30 min at 37°C and then washed four times with PBS-T. Reference IL-1β or samples were diluted in DMEM with 10 % (v/v) FBS, and 50 µl was added to appropriate wells. Plates were incubated for 60 min at 37°C and then washed four more times with PBS-T. Rabbit polyclonal anti-IL-1β antibody (Biosource, Camarillo, CA), diluted 1:1000 in PBS containing 1 % (w/v) BSA, was added to each well and the plate incubated at room temperature for 60 min. The plates were then washed six times with PBS-T, and 50 µl of goat anti rabbit IgG antibody conjugated with horse-radish peroxidase (Cappel Laboratories, Durham, NC) diluted 1 : 5000 in PBS with 1% (w/v) BSA was added to each well and incubated for room temperature for 60 min. ELISA plates were washed eight times with PBS-T. Substrate (100 µl) consisting of 3', 3', 5', 5'- tetramethyl benzidine (0.1 mg/ml; Fluka Chemical Corp., Ronkonkoma, NY) in 0.1 M citric-phosphate buffer (pH 5.5) and 0.003 % (w/v) hydrogen peroxide (H_2O_2) was added to each well and incubated 20 minutes at room temperature for color development. The reaction was terminated with 100 µl of 6 N sulfuric acid (H_2SO_4). Absorbance was measured at 450 nm with Vmax kinetic microplate Reader (Molecular devices, Menlo Park, CA) and interleukins production quantified using the Softmax software (Molecular Devices).

Intracellular cytokine and flow cytometry staining of TNF-α and IL-6. Monoclonal anti-mouse IL-6 (Biotinylated. rat IgG2a, MP5-32c11), biotinylated rat IgG2a (κ isotype, R35-95), anti-mouse TNF-α (PE conjugated; mouse IgG1, MP6-XT22), mouse IgG1 (PE conjugated, κ isotype, R3-34), and anti-mouse CD32/CD16 (Fcγ II/III receptors)

(rat IgG2b, 2.4G2) were purchased from Pharmingen (San Diego, CA). Cells were cultured as described above and stained for intracellular cytokines by a modification of the method of Sander *et al.* (1991). Briefly, cells were harvested with cold Dulbecco's phosphate buffered saline (DPBS, Sigma) containing 1 %(v/v) heat inactivated FBS (Gibco) and centrifuged at 300xg for 10 min. The cell pellet was washed with DPBS and blocked with anti- mouse CD32/CD16 Fc Block antibodies for 30 min at 4°C for prevention of nonspecific binding. Cells were fixed on ice using cold DPBS containing 4 % (w/v) paraformaldehyde (Sigma) for 40 min. After washing with DPBS, cells were stained for 30 min on ice with fluorochrome conjugated cytokine specific antibodies or biotinylated Monoclonal antibodies (Mab) (Pharmingen) diluted in DPBS containing 0.1 % saponin (Sigma) and 0.1 % sodium azide (Sigma). Isotype controls were incubated at this step too. Cells treated with biotinylated Mab were washed in saponin buffer and subsequently incubated with streptavidin conjugates (Pharmingen) for 20 min on ice. Cells were resuspended in DPBS and stored on ice before analyzed.

Samples were analyzed with a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) equipped with Argon ion laser, and Consort 32 computer using Lysis II Software. The 488 nm Line of the Argon laser was used to excite fluorescein isothiocyanate (FITC) and phycoerythrin (PE) fluorochromes which were quantified at 530 ± 15 and 575 ± 13 nm respectively. Electronic color compensation was accomplished using single color controls. Data collection was limited to lymphocytes selected sequentially via gates in cell scatter and 1 color fluorescent cytograms. List mode data files of 15,000 - 20,000 cells were gathered.

Measurement of Cytokine mRNA abundance. Total cellular RNA was extracted

from RAW 264.7 cells by a modification of the single step method (Chomczynski and Sacchi, 1987) using RNA-Stat-60 isolation reagent (Tel test " B" inc., Friendswood, TX). Reverse transcription (RT) for first strand cDNA synthesis was carried out by the method of Kawasaki (1991). For RT, RNA (up to 2 μg) in a maximum of 18.7 μl of Diethyl pyrocarbonate (DEPC) water was mixed with 1.1 μl of 45 mM oligo dT15 mer (Promega Co., Madison, WI) and incubated for 10 min at 70°C. Immediately following incubation, 10.2 μl of RT master mix [comprised of 6 μl 5X RT buffer, 1.5 μl 10 mM of four deoxynucleotides mix (dNTP mix; Boehringer Mannheim, Indianapolis, IN), 0.7 μl of RNase inhibitor (40 U/ml; BM) and 2 μl of M-MLV reverse transcriptase (200 U/μl; Gibco BRL)], was added, mixed and then incubated at 42°C for 1 hr. The reaction was terminated by incubation at 70°C for 10 min and cooling on ice. The RT products were then stored at -80°C. cDNA products were amplified by PCR according as described by Svetic *et al* (1991) employing the modifications of Azcona-Olivera *et al* (1995a). Primer and internal oligonucleotide probes (Table 2.1) were synthesized at Macromolecular Structure Facility (Michigan State University). β_2-microglobulin (β_2MG) was used as a " house keeping gene to verify initial quantities of RNA and the integrity of RNA preparation. In brief, 3 μl of RT reaction product was added to a PCR master mix comprised of 6 μl of 25 mM $MgCl_2$ (Perkin Elmer Gene Amp PCR System, Norwalk, CT), 5 μl of 10X PCR buffer (Perkin Elmer), 4 μl of 10 mM dNTP mix, 2 μl of sense primer (0.2 μg/μl), 2 μl antisense primer (0.2 μg/μl), 0.5 μl of Taq polymerase (5 U/μl; Boehringer Mannheim) and variable amounts of DEPC-treated water to make up the volume to 50 μl. Following mixing, 75 μl of mineral oil was added and PCR was performed in a 9600 Perkin Elmer thermocycler. The program entailed

Table 2.1. Primer Sequences for Cytokine cDNA Amplification and Probe Sequences for Southern Blot Detection of Amplified cDNA Product

	Sense and antisense primer	Probe
Cytokine	5' 3'	5' 3'
IL-1β	TTGACGGACCCCAAAAGATG[a]	ATTGTGGCTGTGGAGAA[c]
	AGAAGGTGCTCATGTCCTCA[a]	
IL-6	GTTCTCTGGGAAATCGTGGA[a]	TGTGCAATGGCAATTCTGAT[d]
	TGTACTCCAGGTAGCTATGG[a]	
TNF-α	TCTCATCAGTTCTATGGCCC[b]	CCTGTAGCCCACGTCGTAG[c]
	GGGAGTAGACAAGGTACAAC[b]	
β2MG	TGACCGGCTTGTATGCTATC[a]	CTCACGCCACCCACCGGAGA[d]
	CAGTGTGAGCCAGGATATAG[a]	

References for oligonucleotides are designated by letter as follows: [a]Personal communication from Dr. David Shire, Sanofi Elf Bio Recherches; [b]Svetic *et al.*(1991); [c]Montgomery and Dallman (1991); [d]Designed in Biochemistry Macromolecular Facility Laboratory with Computer Program OLIGO(National Biosceinces); [e]Wesselingh *et al.*(1994).

an initial denaturation step for 5 min at 95°C, followed by three temperature cycling steps: denaturation for 1 min at 95°C, primer annealing for 1 min at 52°C, and extension for 3 min at 72°C. This was repeated for an optimized number of cycles for each transcript(15, 18, 20 and 15 cycles for β_2MG, IL-1β, IL-6 and TNF-α , respectively). The final cycle concluded with a 10 min incubation at 72°C.

RT-PCR products (10 µl) were electrophoresed on a 2 % agarose gel at 75 V for 6 hr. Following staining with 1 mg/ml of ethidium bromide, the bands were visualized by UV light and photographed using Polaroid Type 55 film (Polaroid Co., Cambridge, MA). Gel denaturation and neutralization as well as DNA transfer on to nylon membranes (Nytran, Schleicher & Schuell Inc., Keene, NH) were carried out by the procedures according to Sambrook *et al.* (1989). ^{32}P-labelled internal oligonucleotides(specific for each transcript) were prepared using a DNA 3'- end labeling system (Promega Co., Madison, WI). Following cross-linking and prehybridization at 42°C for 2 hr in a buffer containing 6X SSPE, 10X Denhart's, 1% sodium dodecyl sulfate (SDS) and 50 µg/ml of denatured herring sperm DNA, blots were hybridized in a solution (6X SSPE, 10% SDS) containing ^{32}P-labeled probe at 49°C for 18 hr in a hybridization incubator (Robbins Scientific, Sunnyvale, CA). The hybridized membranes were washed once in 6X SSPE containing 0.1 % SDS at 49°C for 20 min and once more in 2X SSPE at room temperature for 10 min with shaking. All oligoprobes had a minimum specific activity of 1 x 10^9 cpm/µg and all hybridizations were performed with no less than 10^7 cpm/ml. Blots were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) with an intensifying screen at -80°C for variable length periods depending on signal strength.

Following autoradiography, resultant gel bands were analyzed using a Scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) and GS365W Densitometer Program (Hoefer). Each composite band (consisting of three separate samples lanes) was divided to its corresponding β_2MG band and the resulting ratio was compared to all other timepoints and doses. This facilitated the comparison of relative cytokine mRNA expression across all the experimental groups.

Statistical analysis. A one way analysis of variance (ANOVA) using Student-Newman-Keuls tests and Dunnett's test or Kruskal Wallis ANOVA on ranks were applied to parametric data and non-parametric data with application of Sigma-Stat Analysis System (Jandel Scientific, San Rafael, CA). A p value of less than 0.05 was considered statistically significant.

RESULTS

Effects of VT on cytokine secretion. To determine the potential effects of VT on IL-1β, IL-6, and TNF-α release, RAW 264.7 cells were incubated with 0, 50, 100, 250 and 500 ng/ml of VT in the absence or presence (1 μg/ml) of LPS for 1, 2 and 5 days. In the absence of LPS stimulation, IL-1β and IL-6 supernatant levels were negligible at all time and toxin treatments (data not shown). In contrast, TNF-α levels were significantly increased by VT at 100 and 250 ng/ml without LPS stimulation at days 1 and days 2 (Table 2.2). TNF-α was not detectable in the supernatant after 5 days. When RAW cells were activated with 1μg/ml LPS, significant increases of IL-6 were also detected in the presence of 250 ng/ml of VT at day 1, 2 and 5 incubation time whereas IL-1β was undetectable. TNF-α was significantly increased by 50 ng/ml VT on day 1, 100 ng/ml VT on day 1 and 2, and 250 VT ng/ml on

Table 2. 2. Effect of VT on Proinflammatory Cytokine Secretion by RAW 264.7 cells.

Day	Vomitoxin (ng/ml)	TNF-α		IL-6
		LPS -	LPS +	LPS +
1	0	0.89 ± 0.18^{a}	3.44 ± 0.48^{a}	10.11 ± 2.39^{a}
	50	0.77 ± 0.09^{a}	4.30 ± 0.18^{b}	15.32 ± 2.56^{a}
	100	1.30 ± 0.12^{a}	4.92 ± 0.42^{b}	19.98 ± 4.02^{a}
	250	2.95 ± 0.22^{b}	3.06 ± 0.37^{a}	47.54 ± 4.48^{b}
2	0	1.62 ± 0.03^{a}	11.48 ± 1.85^{a}	$< 1^{a}$
	50	2.46 ± 0.30^{a}	$15.56 \pm 4.10^{a,b}$	6.01 ± 0.93^{a}
	100	5.02 ± 0.23^{b}	20.14 ± 6.83^{b}	11.48 ± 9.75^{a}
	250	14.00 ± 0.93^{c}	21.66 ± 1.73^{b}	42.59 ± 22.13^{b}
5	0	$< 1^{a}$	$< 1^{a}$	12.37 ± 4.41^{a}
	50	$< 1^{a}$	$< 1^{a}$	13.22 ± 0.72^{a}
	100	$< 1^{a}$	$< 1^{a}$	18.61 ± 3.39^{a}
	250	$< 1^{a}$	$< 1^{a}$	26.08 ± 2.97^{b}

RAW 264.7 cells (5×10^5 cells/ml) were cultured with VT and with and without LPS. Values are means ± S.D. (n=3). In the column on the same day, values with different superscripts differ significantly ($P < 0.05$). IL-6 production of cells treated with 0 µg/ml LPS are all < 1ng/ml.

day 2. At higher concentrations of VT (500 ng/ml), cell proliferation was markedly inhibited and thus cytokine levels showed a pronounced depression (data not shown). Based on these results, 100 ng/ml and 250 ng/ml of VT were selected for further examination of cytokine superinduction in subsequent experiments.

Effects of VT on cytoplasmic cytokine levels. To characterize the effects of VT on the intracellular cytokine expression, RAW cells were cultured with 0, 100 and 250 ng/ml VT and 1 μg/ml LPS for 24 hr and then intracellular IL-6 and TNF-α were measured by flow cytometry. Fig. 2.1A shows typical fluorescence profile of IL-6 positive-cells with and without VT treatment. As shown in Fig. 2.1B, the percentages of VT-treated cells containing cytoplasmic IL-6 in RAW cells were not significantly different than controls. However, VT at both 100 and 250 ng/ml significantly increased the level of cytoplasmic IL-6 in cells as indicated by elevated average fluorescence intensities as compared to the control (0 ng/ml) (Fig. 2.1C).

Figures 2.2A and 2B contain the fluorescence profiles for TNF-α positive cells. Here, percentages of cells containing TNF-α were significantly increased at 100 and 250 ng/ml VT as compared both to control with and without LPS (Fig. 2.2C). In contrast to IL-6, the average level of cytoplasmic TNF-α positive cells was not significantly affected by VT treatment (Fig. 2.2D).

Effects of VT on cytokine gene expression. To relate VT-induced superinduction of cytokine secretion to cytokine mRNA expression, RAW 264.7 cells were treated with combinations of VT and LPS for 2, 6 and 24 hr and cytokine mRNA abundance was measured by using RT-PCR in conjunction with Southern analysis. IL-1β and IL-6 mRNAs

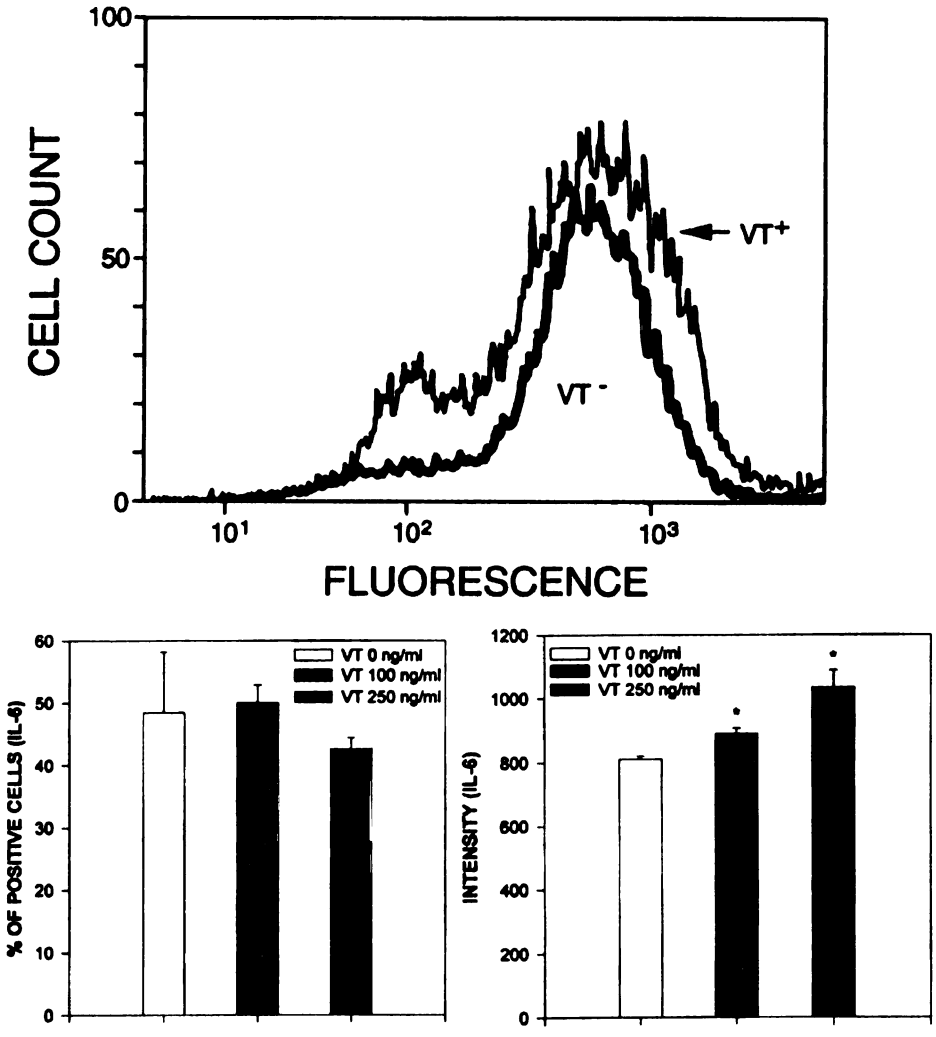


Fig.2.1. Flow cytometric analysis of cytoplasmic IL-6 in RAW 264.7 cells after incubation with VT for 24 hr in the presence of LPS. Cells were fixed and stained with specific antibody and subjected to by flow cytometry. Histograms contain data for cells treated with VT are compared with the control non-VT treated cells in the presence (A) of LPS. The data are representative of three experiments. Each histogram represents cell frequency per 15, 000 cells (ordinate) *v.* immunofluorescent (abscissa, log scale). (B) represents percentage of cytoplasmic IL-6^{+} cells following stimulation with LPS and VT for 24 hr. Data in (C) represents mean fluorescence intensity or average channel number for cytoplasmic IL-6 cells population samples. Results in (B) and (C) are mean ± SEM taken from three replicates cultures of each doses of LPS and VT. Data were analyzed by using the one way ANOVA ($P<0.05$; Student's-Newman-Keul's test).

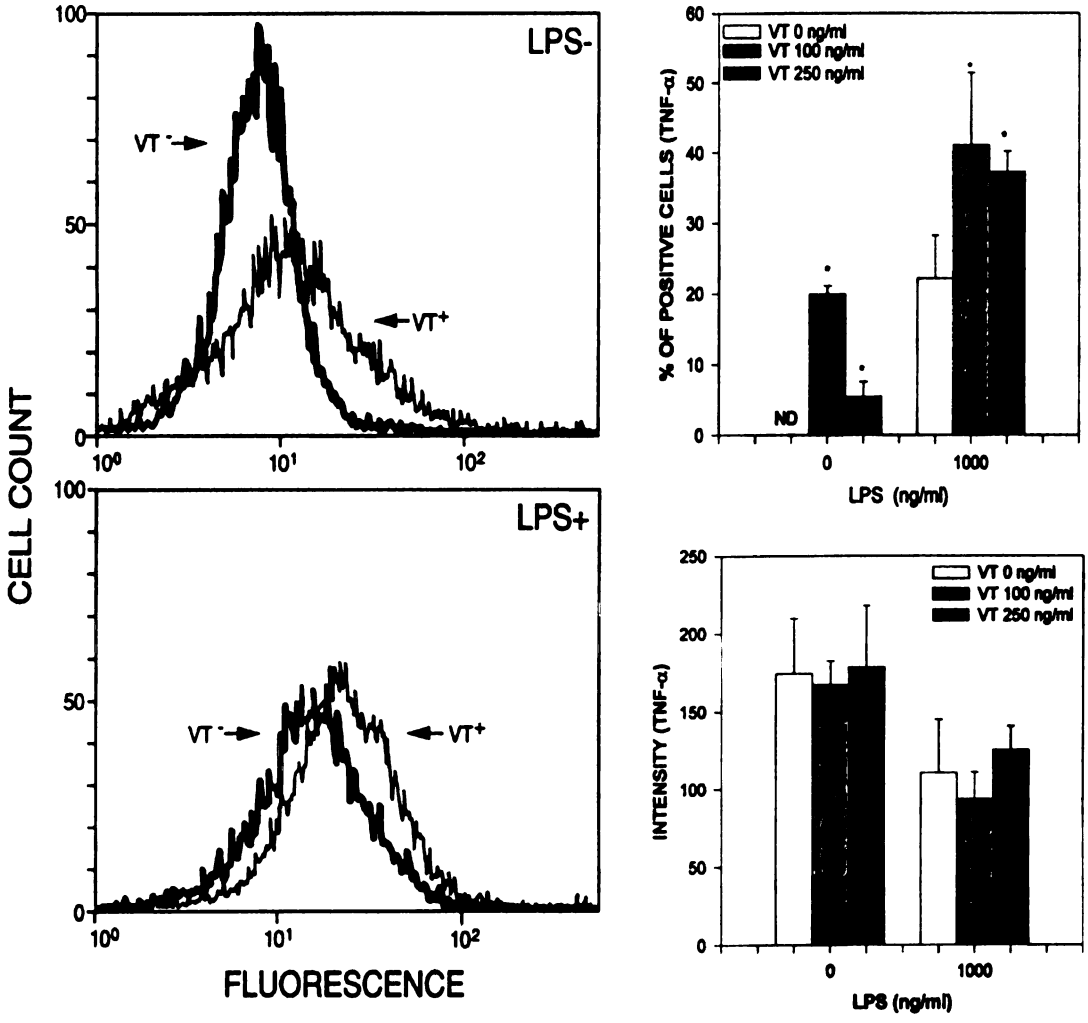


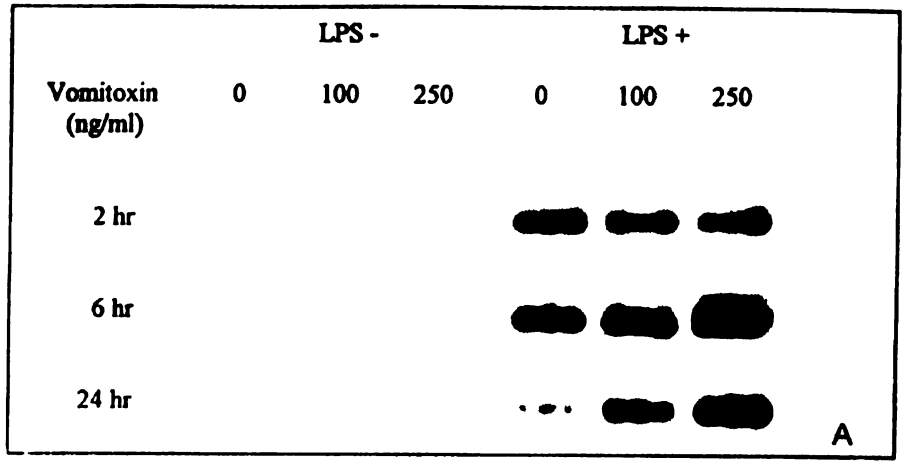
Fig.2.2. Flow cytometric analysis of cytoplasmic TNF-α in RAW 264.7 cells after incubation with VT for 24 hr in the absence (A) or presence (B) of LPS. The experiments was reported and analyzed as in Fig.2.1. legend. (A) and (B) indicate histograms of cytoplasmic TNF-α^+ cells following stimulation with VT for 24 hr in the absence or presence of LPS, respectively. (C) and (D) represent percentage cytoplasmic TNF-α^+cells and mean fluorescence intensity, respectively.

were not affected by VT in the absence of LPS (Fig. 2.3A and 2.4A). However, in LPS-stimulated cultures, IL-1β mRNA abundance, as revealed by cDNA hybridization signal after RT-PCR, was 3- and 4-fold higher than controls for cells treated with VT at 100 and 250 ng/ml, respectively after 24 hr (Fig. 2.3B). Similarly, relative IL-6 mRNA abundance was increased 2- and 8-fold at 100 and 250 ng/ml VT, respectively in LPS-stimulated cultures at 24 hr (Fig. 2.4B). Consistent with supernatant concentrations, TNF-α mRNA abundance was increased 2-4 fold at 6 and 24 hr with 100 and 250 ng/ml VT-exposure in the absence of LPS (Fig. 2.5 A, B). With LPS stimulation, TNF-α mRNA expression increased very early in LPS-treated culture (Fig. 2.5A, C) and diminished rapidly at all three VT doses.

DISCUSSION

RAW 264.7 cells were selected for study of VT-induced cytokine effects because this clonal macrophage model produces high levels of IL-6 and TNF-α in culture upon activation with LPS. The results of this study demonstrated that exposure of those cells to VT could enhance mRNA expression of proinflammatory cytokines, IL-1β, IL-6 and TNF-α and increase secretion of IL-6 and TNF-α. These data are consistent with earlier findings that VT exposure elevates expression of macrophage cytokine mRNAs *in vivo* (Azcona-Olivera *et al.*, 1995a,b; Zhou *et al.*, 1997).

The three cytokines studied herein exhibited markedly different patterns of mRNA expression and protein secretion following VT exposure. For example, IL-1β gene expression increased within 2 hrs of LPS stimulation but remained elevated in the presence of VT. This is consistent with the findings *in vivo* that IL-1β mRNA abundance increased



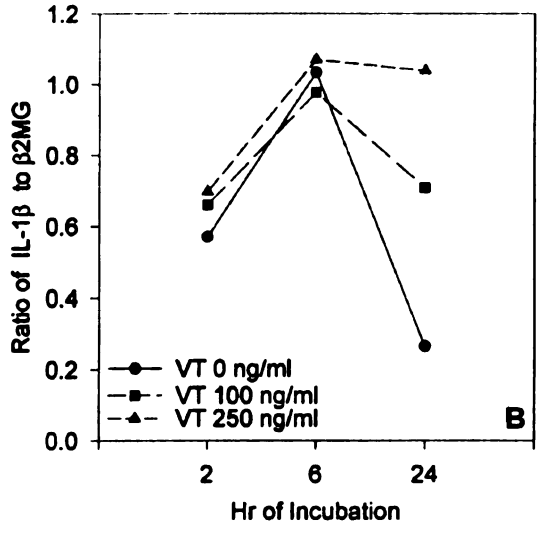
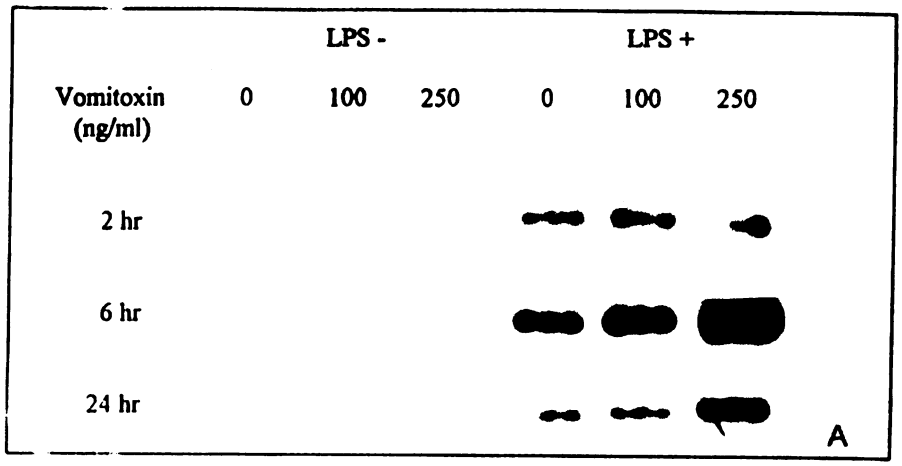


Fig. 2.3 Relative abundance of IL-1β mRNA in RAW 264.7 cells after *in vitro* exposure to VT with and without LPS. (A) indicates Southern analysis of RT-PCR products form RAW 264.7 cells. Each dose and time point represents three separate lanes prepared from three different replicates; (B) indicates image analysis data for above autoradiographs. Data are percentage of maximal signal based on pooled measurement of three lanes for each dose and time point.



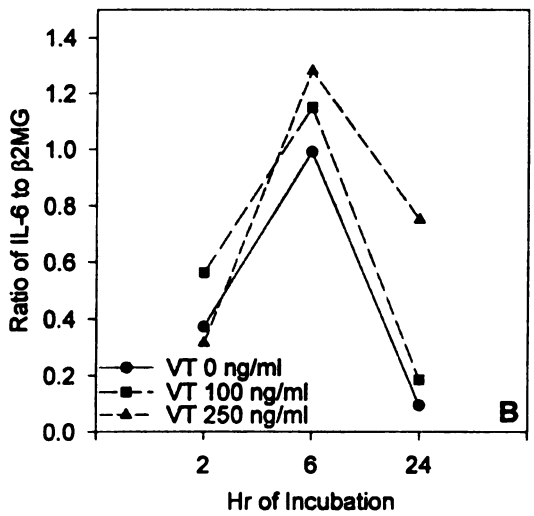


Fig. 2.4. Relative abundance of IL-6 mRNA in RAW 264.7 cells after *in vitro* exposure to VT with and without LPS. Experiment was conducted and analyzed as in Fig.2.3.

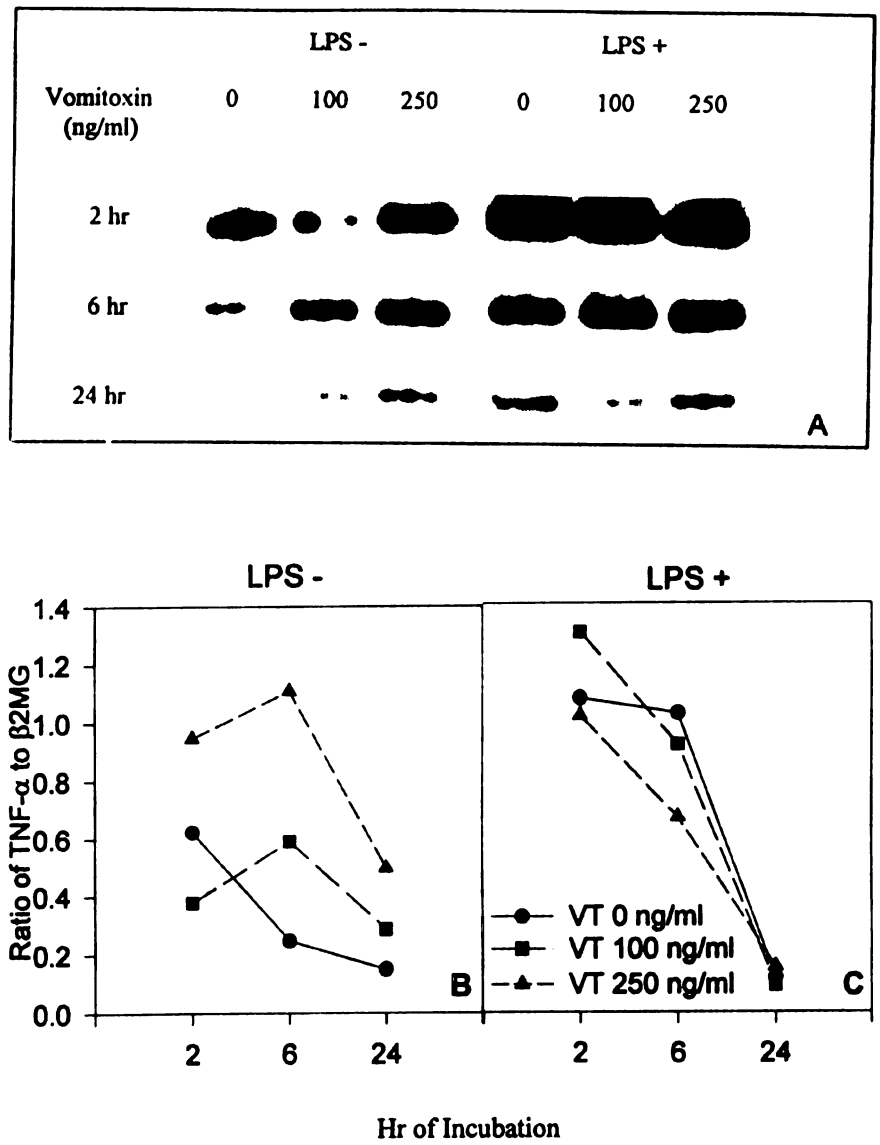


Fig.2.5 Relative abundance of TNF-α mRNA in RAW 264.7 cells after *in vitro* exposure to VT with and without LPS. Experiment was conducted and analyzed as in Fig. 2.3. (A) indicates the Southern analysis of RT-PCR product form RAW cells; (B) and (C) indicate image analysis data for above autoradiographs in the absence and presence of LPS, respectively.

in spleens and Peyer Patches after gavage with VT for 2 hrs (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997). The inability to observe IL-1β protein secretion *in vitro* along with the increases in gene activity might be due to the fact that IL-1β was present as a cell-associated molecule, it bound to receptors or it remained intracellularly located (Bayne *et al*, 1986; Cavaillon and Haeffner-Cavaillon, 1990). IL-6 mRNA expression *in vitro* also appeared within 2 hrs of LPS stimulation and the mRNA rapidly disappeared in the absence of VT. With VT, mRNA expression was prolonged after 2 and 6 hrs. Similarly, mRNA expression is increased *in vivo* at 2 hrs and disappeared at 4 hrs after gavage with VT (Azcona-Olivera *et al.*, 1995a). Supernatant IL-6 protein was increased at 24 hrs although the mRNA declined at this time point. In contrast, TNF-α mRNA appeared to be expressed earlier than other cytokines (IL-1β and IL-6) with and without LPS stimulation and its levels diminished more rapidly. The addition of VT in the absence of LPS may have prolonged TNF-α mRNA production. In mice, TNF-α mRNA remained elevated after VT treatment at 2 and 4 hrs (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997).

Altered cytokine expression patterns were further verified by flow cytometric measurement of intracellular changes in IL-6 and TNF-α. The results suggested that the increases of IL-6 in culture following treatment with VT appeared to be due to increased production of IL-6 on a per cell basis and this corresponded to the ELISA results of the culture supernatants. In contrast, increases of TNF-α in cells treated with VT appeared to be due to an increase in TNF-α secreting cell populations. These results suggested that VT could differentially superinduce cytokines both by increasing numbers of cytokine secreting cells or increasing cytokine output per cell.

VT's effects on cytokine production may be generally relevant to pathogenic manifestations associated with trichothecene exposure. For example, because the expressed cytokines (IL-1β , IL-6 and TNF-α) are identical to those observed to mediate septicemia and fatal circulatory shock induced by LPS (Vogel and Hogan, 1990), these same cytokines may also play a role in shock-like syndrome caused by acute high dose trichothecene poisoning. Also, IL-1, IL-6 and TNF-α administration have also been associated with anorexia and weight loss (Abbas *et al.*, 1994; Schobitz *et al.*, 1994). Thus, feed refusal and reduced weight gain observed in the trichothecene-exposed animals (Rotter *et al.*, 1996) might be mediated by superinduction of proinflammatory cytokines.

Of the several known cytokines that mediate IgA response (McGhee and Kiyono, 1993), IL-6 is one of the most important (Akira *et al.*, 1990; Bao *et al.*, 1993). Macrophages are a primary producer of IL-6 (Bauer *et al.*, 1988) and VanCott *et al.* (1996) demonstrated that IL-6 secreted by macrophages contributes critically to development of mucosal IgA responses. IL-6 drives IgA production when it is added to Peyer's patch B cell cultures (Beagley *et al.*, 1989; Yan *et al.*, 1997) and mucosal IgA-producing cells are dramatically decreased in IL-6 knock-out mice (Ramsay *et al.*, 1994). In previous studies, we found that acute oral VT exposure in mice can increase serum IL-6 (Zhou *et al.*, 1997) as well as the capacity of PP and spleen cell cultures to secrete IL-6. Furthermore, specific neutralizing anti-IL-6 Ab inhibits IgA production *in vitro* in Peyer's patch cultures obtained from mice exposed to single oral dose of VT (Yan *et al.*, 1997). Thus, it is very likely that, by superinducing IL-6 expression during VT exposure, macrophages provide excessive help for B cell production of IgA, and contribute to the manifestations of IgA nephropathy.

The exact mechanisms for superinduction of specific cytokines in macrophages by VT are unclear. Gene expression is regulated at several levels, including transcriptional, translational and post-translational control. Protein synthesis inhibitors enhance and prolong the normally transient expression of specific gene transcripts via impaired synthesis of high turn-over proteins that downregulate transcription or decrease half-life mRNA (Sun *et al.*, 1993; Cockfield *et al*, 1993; Efrat and Kaempfer, 1984). Transcriptional control mechanisms have been suggested to be primary in regulation of macrophage activation (Yu *et al,* 1990). Previous studies have revealed that transcriptional control of genes in macrophages appears to involve several transcriptional factors such as those in the Rel homology family. For example, nuclear factor -κB (NF-κB) plays an important role in induction of various genes expression in macrophages, such as Macrophage colony stimulating factor (M-CSF), G-CSF (Granulocyte colony-stimulating factor), TNF-α, IL-1, IL-6 and IL-2 Receptor alpha (Rα) chain and Nitric Oxide synthase (Akahane *et al.*, 1994; Baeuerle and Henkel, 1994). An inhibitory protein, IκB–α, is known to be able to regulate the gene expression by binding to the NF-κB dimer as an inactive complex in the cytoplasm (Baldwin, 1996). Upon stimulation with mitogen, IκB–α is degraded rapidly and NF-κB is activated and translocated into the nucleus for gene expression. Relatedly, the translational inhibitor CHX inhibits the resynthesis of degraded IκB–α in phorbol 12-myristate 13-acetate (PMA)-stimulated Jurkat T cells thereby increasing nuclear bound NF-κB and IL-2 mRNA expression (Sun *et al.*, 1993). It has similarly been demonstrated that VT inhibits resynthesis of degraded IκB-α in EL-4 and $CD4^+$ cells stimulated with PMA and ionomycin (Ouyang *et al.*, 1996) and this increases nuclear-bound NF-κB thus leading to increased IL-2

transcription. Therefore it is reasonable to suggest that NF-κB activation might similarly be involved in VT toxicity and contribute to cytokine superinduction and dysregulation in macrophages.

We can conclude from the present study that VT is capable of superinducing cytokine production and mRNA expression in a clonal model of the murine macrophage. Increases in these cytokines might contribute to the toxicity and immunopathological effects associated with foodborne VT. Further studies of the mechanisms of cytokine superinduction in the RAW 264.7 model should facilitate our understanding of the effects of VT on regulation of the immune system *in vivo* and their contribution to the diverse toxic effects of trichothecenes.

CHAPTER III

EFFECTS OF VOMITOXIN ON TRANSCRIPTION FACTOR BINDING IN MACROPHAGE RAW 264.7 CELLS

ABSTRACT

Vomitoxin (VT, deoxynivalenol), a trichothecene mycotoxin, superinduces IL-1β, IL-6 and TNF-α gene expression in macrophage RAW 264.7 cells. Cytokine gene regulation in macrophages may be affected by VT at the transcriptional level. In this study, the effects of VT on the binding activity of AP-1, NF-κB and NF-IL6, transcription factors important to pro-inflammatory cytokine regulation, were assessed using electrophoretic mobility shift assay (EMSA) with RAW 264.7 cells. When cells were treated with VT in the absence of LPS (synchronous model), DNA binding activity of AP-1 was increased at 8 hr with 250 ng/ml of VT. When cells were incubated in medium alone for 2 hr prior to the addition of VT (delayed synchronous model), binding activity of AP-1 was enhanced at 2 and 8 hr with VT treatment. Binding activities of AP-1 were not enhanced when cells were treated with VT in the presence of LPS. Using specific antibodies in supershift EMSA, VT was found to preferentially induce phosphorylated c-Jun and JunB, c-Fos and Fra-2 binding activities of the AP-1 family. VT was also found to alter binding activity of NF-κB/Rel complexes and this was verified by competition assays. A faster migrating band (lower complex) was constitutively expressed whereas a slower migrating band (upper complex) was induced by the stimulation of LPS. The binding activities of the upper complex were increased at 2 and 8 hr when cells were subjected to synchronous and delayed synchronous VT-exposure in the presence of LPS. The p-50 and c-Rel subunits of the NF-κB/Rel were found to be specifically affected by a supershift EMSA. NF-IL6 binding was increased at 2 hr with or without LPS in synchronous and delayed synchronous VT-exposure models, respectively. Here, C/EBPα and β subunits were involved in the enhancement of

NF-IL6 binding. Binding specificity for all three transcription factors was confirmed in EMSA competition studies with unlabeled and mutant probes. The capacity of VT to elevate binding of AP-1, NF-κB, and NF-IL6 may contribute to the VT-mediated cytokine superinduction in macrophages as well as other toxic manifestations of this mycotoxin.

INTRODUCTION

Vomitoxin (VT, deoxynivalenol), a trichothecene mycotoxin produced by *Fusarium graminearum*, is frequently found in feed and grain supplies (Ueno, 1985; Vesonder and Hesseltine, 1980; Rotter *et al.*, 1996) . Trichothecenes can inhibit protein synthesis and cause a wide range of toxicological and immunological effects (Ueno, 1983; Cundliffe *et al.*, 1974; Bamburg, 1983; Forsell *et al.*, 1987; Fitzpatrick *et al.*, 1988; Thompson and Wannemacher, 1990; Bergmann *et al.*, 1992). Trichothecenes are also able to elevate serum IgA, IgM and IgG levels in mice exposed with VT (Forsell *et al.*, 1987; Atroshi *et al.*, 1994). It was demonstrated that dietary exposure of mice to VT increases serum IgA and IgA complexes as well as accumulation in the mesangial kidney (Dong *et al.*, 1991; Dong and Pestka, 1993). These features are highly analogous to the glomerulonephritis human IgA nephropathy (D'Amico, 1987).

IL-6 plays an important role in VT-induced IgA nephropathy based on *ex vivo* culture studies with Peyer's patches (Yan *et al.*, 1997). Recently, Yan *et al.* (1998) have also demonstrated that increased IgA and IL-6 production was observed in macrophage-depleted Peyer's patches and spleen cells from VT-treated mice when cocultured with VT-treated peritoneal macrophages. Therefore, macrophages may be involved in the regulation of

superinduction of IL-6 and other cytokines by VT.

Transient increases of mRNAs for macrophage-associated cytokines, IL-1β, IL-6 and TNF-α, have been shown upon exposure to VT *in vivo* (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997). Miller and Atkinson (1986) demonstrated the superinduction of inflammatory cytokine IL-1 in peritoneal macrophages by VT. Recently, Wong *et al.*(1998) observed elevated expression of IL-6 and TNF-α mRNA and proteins in macrophage RAW 264.7 cells. The superinductive effects by VT are quite consistent with the effects observed for protein synthesis inhibitors such as CHX (Efrat and Kaempfer, 1984; Cockfield *et al.*, 1993; Faggioli *et al.*, 1997). The aforementioned macrophage cytokines may also be related to pathogenic manifestations associated with trichothecene exposure. Gene expression for these cytokines is regulated at the transcriptional, post-transcriptional and post-translational levels in macrophages. Transcriptional control has been suggested to be important in the regulation of macrophage activation (Yu *et al.*, 1990; Paulnock, 1994). Proinflammatory cytokine promoters contain various binding sites for functional transcription proteins. Therefore, it is highly possible that VT can superinduce cytokines in macrophage cells by altering the transcriptional activity via their promoters.

One important transcription factor for cytokine expression is the activator protein-1 (AP-1), which is a complex of proteins made up from members of the Jun and Fos families. The AP-1 family contains four Fos proteins (c-Fos, Fos B, Fra-1, and Fra-2) and three Jun proteins (c-Jun, Jun B and Jun-D) (Pennypacker, 1998). The Fos and Jun proteins can form dimeric complexes with one another and these complexes can bind to the AP-1 consensus sequence. The Fos-Jun heterodimers form the most stable complexes

(Halazonetis, 1988; Liebermann *et al.*, 1998). AP-1 has been found to be involved in B- and T-cell activation, differentiation of Th1 and Th2 cytokines, regulation of immunoglobulin production, and monocytic differentiation and apoptosis (Pennypacker, 1998; Foletta *et al,* 1998). Notably, AP-1 regulates IL-4, -5, -6, -10, IFN-γ, TNF-α, IL-8 and CM-CSF expression (Foletta *et al.*, 1998). Also, AP-1 binding sites have been identified in the promoters of IL-3 and IL-9 genes. Recently, it has been shown that VT can enhance the binding activity of AP-1 in the EL-4 cells (Li *et al.*, 2000). Therefore, it is reasonable to suggest that VT similarly enhances AP-1 binding activity in macrophages.

A second important transcription factor in monocytes is the nuclear factor kappa B (NF-κB). The NF-κB/Rel family is known to regulate a variety of signal pathways (Lenardo and Baltimore, 1989; Legrand-Poels *et al.*, 1997). It is an inducible transcriptional activator which can respond to different stimulation agents, such as lipopolysaccharide (LPS), phorbol esters, IL-1, -2, TNF-α and protein synthesis inhibitors (Grilli *et al.*, 1993). NF-κB/Rel can also regulate a wide variety of genes involved in acute phase responses and inflammation, such as M-CSF, G-CSF, TNF-α, IL-1β, IL-6, IL-2, IL-8, IL-2Rα chain and NO synthase (Baeuerle and Henkel, 1994; Akahane *et al.*, 1994). Five members of the NF-κB/Rel family have been identified : c-Rel, NF-κB_1(p50/p105), NF-κB_2(p52/p100), Rel A (p65), and Rel-B (Baeuerle, 1991; Baeuerle and Henkel, 1994). In the resting phase, dimers of these proteins are inactive in the cytoplasm, and are associated with an inhibitory factor, IκB. Upon stimulation, IκB is phosphorylated, and dissociates from NF-κB complexes. The NF-κB dimer translocates into the nucleus in an active form and activates different responsive genes (Li *et al*, 1994; Finco and Baldwin, 1995; Baldwin, 1996). Previous

studies of Ouyang *et al.*(1996) revealed that the activities of the transcription factor NF-κB were increased concurrently with IL-2 gene expression in EL-4 cells treated with VT. Therefore, VT might also be able to increase the NF-κB activity in the macrophage cells.

NF-IL6, another important monocytic transcription factor, is also known as CCAAT enhancer-binding proteins or C/EBP. It can be induced by various stimuli such as LPS, IL-1, TNF-α and IL-6 (Ohmori and Hamilton, 1994). It is also able to stimulate different target genes in macrophages such as IL-1β, IL-6, IL-8, TNF-α, M-CSF and IP-10 (Ohmori and Hamilton, 1994; Koj, 1996). Expression of C/EBP protein is limited to the myeloid lineage (Scott *et al.*, 1992; Haas *et al.*, 1992; Valledor *et al.*, 1998). C/EBPα is mainly expressed in undifferentiated myeloid cells. Expression of C/EBPβ and C/EBPδ is observed during macrophage maturation. C/EBPβ and δ are phosphoproteins that translocate into the nucleus following phosphorylation by MAP kinase at their threonine residues (Yamanka *et al.*, 1998) . Defects in C/EBPβ(–/–)genes have been shown to increase the susceptibility of mice to infection by *Listeria monocytogenes* and impair tumor cytotoxicity of macrophages (Tanaka *et al.*, 1995). Disruption of C/EBPβ genes also results in an increased production of IL-6 levels *in vivo* (Screpanti *et al.*, 1995).

All three of the aforementioned transcription factors have been implicated in the regulation of macrophage cytokine gene expression. The specific objective of this study was to test the hypothesis that VT alters binding activities of AP-1, NF-κB and NF-IL6, in macrophage RAW 264.7 cells.

MATERIALS AND METHODS

RAW 264.7 cell cultures. Raw 264.7 cells (TIB 77, American Type Culture Collection, Rockville, MD) (2.5 x 10^5 cells/ml) were cultured in 100-mm tissue culture dishes (Corning, NY) in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 1% (v/v) NCTC 135, 1 mM sodium pyruvate (Gibco BRL), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in a 5-8% CO_2 incubator at 37°C. For both temporal and dose-response studies, cells were stimulated with and without LPS (*Salmonella typhimurium*; Sigma, stimulation index 110.7) (0, 100, or 1000 ng/ml) and VT (Sigma) (0, 100 and 250 ng/ml) for 0, 2, 8, and 24 hr in both synchronous and delayed synchronous modes. In the synchronous model, cells were treated simultaneously with VT and either LPS or vehicle. In the delayed synchronous model, cells were pre-stimulated with LPS or vehicle for 2 hr prior to the addition of the VT.

Nuclear Extract Preparation Cells were collected from cultures at time intervals and sub-cellular protein extracts were prepared according to Schreiber *et al.* (1989a). Briefly, supernatant was removed and Dulbecco's Phosphate Buffer Saline (DPBS; Sigma) was added (10 ml/dish) to each dish. Using a cell lifter (Fisher Scientific, Springfield, NJ), cells were detached from the surface of dishes and transferred to a 50 ml conical centrifuge tube and centrifuged at 240 xg for 5 min. The pellet was resuspended in 400 μl of ice-cold buffer A [10 mM HEPES (Sigma) (pH 7.9) containing 10 mM KCl (Sigma), 0.1 mM EDTA (Boehringer Manheim, Indianapolis, IN), 0.1 mM EGTA (Sigma), 1 mM dithiothreitol (DTT; Sigma), 0.5 mM phenylmethysulfonyl fluoride (PMSF; Sigma), 1 %(v/v) aprotinin (Boehringer Manheim) , 1 μg/ml pepstatin A (Sigma), 2 μg/ml leupeptin (Sigma), 10 mM

sodium fluoride (Sigma) and 1 mM sodium orthovanadate (Sigma)]. The resuspended cells were held on ice for 15 min and gently lysed by adjusting concentration to 0.7% IGEPAL (v/v) (Sigma Co., St. Louis, Mo) detergent. After a centrifugation at 4 °C at 420 xg for 10 min, pelleted nuclei were resuspended in 100 µl of ice-cold buffer B [20 mM HEPES (pH 7.9) containing 0.4 M KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 %(v/v) aprotinin, 10% (v/v) glycerol (Sigma), 1 µg/ml pepstatin A, 2 µg/ml leupeptin, 10 mM sodium fluoride and 1 mM sodium orthovanadate] and lysed by gently rocking for 1 hr at 4 °C. After centrifuging at 10,000 rpm for 10 min at 4°C, the supernatant was dialyzed in dialysis cassettes (10, 000 MW; Pierce Co.) for 2 hr in dialysis buffer contains 20 mM HEPES (pH7.9), 60 mM KCl, 1 mM EDTA, 0.5 mM DTT and 10% (v/v) glycerol, supplemented with 0.5 ng/ml leupeptin, 1% (v/v) aprotinin and 10 mM sodium fluoride, and stored at -80° C until analysis. Protein concentrations of the extracts were analyzed by using Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc., Melville, NY) according to the method of Bradford (1976).

Electrophoretic mobility gel shift assay (EMSA) Kinetics of the AP-1, NF-κB/Rel, and NF-IL6 binding activity in nuclear extracts were assessed by EMSA (Baeuerle and Baltimore, 1988). Nuclear extracts (5 µg) were added to DNA binding reaction buffer [20 mM HEPES (pH7.9) containing 60 mM KCl, 1 mM EDTA, 0.5 mM DTT and 10% (v/v) glycerol, and 2 µg poly (I-C)] to a total volume of 30 µl and then pre-incubated on ice for at least 1 hour to block the non-specific binding. ^{32}P-labeled consensus sequence of AP-1, NF-κB and NF-IL6 (Santa Cruz Biotechnology, Santa Cruz, CA) (Table 3.1) containing 30,000 cpm was added in a volume of 1 µl and incubated at room temperature for 20 min to

Table 3.1. Oligonucleotides for Transcription factors in EMSA

Transcription Factors	Sequence for the oligonucleotides
AP-1 consensus sequence	5'-CGC TTG ATG ACT CAG CCG GAA-3'
mutant sequence	5'-CGC TTG ATG ACT TGG CCG GAA-3'
NF-κB consensus sequence	5'-AGT TGA GGG GAC TTT CCC AGG C-3'
mutant sequence	5'-AGT TGA GGC GAC TTT CCC AGG C-3'
C/EBP consensus sequence	5'-TGC AGA TTG CGC AAT CTG CA-3'
mutant sequence	5'-TGC AGA GAC TAG TCT CTG CA-3'

allow the formation of nucleoprotein. ^{32}P-labeled probe was prepared using Ready To-Go™ T4 Polynucleotide Kinase (Pharmacia Biotech Inc., Piscataway, NJ) according to the method of Tabor (1987). For competition assays, an excess of unlabeled probes of AP-1, NF-κB and NF-IL6 or mutants of those consensus sequences (Table 1) were added prior to the addition of labeled probe. For super-shift assays, 1- 2 μg of antibodies specific for NF-κB subunits (p65, p50, c-rel and Rel-B) (Santa Cruz), AP-1 subunits(c-jun, JunB, JunD, c-Fos, Fra-1, and Fra-2) (Santa Cruz) , and NF-IL6 subunits (C/EBPα, C/EBPβ, and C/EBPδ) (Santa Cruz) or 2 μg rabbit IgG (Santa Cruz) control antibody were added and mixed after the addition of ^{32}P-labeled probe and pre-incubated overnight at 4°C. Resultant nucleoprotein complexes were resolved on native 4 % (w/v) polyacrylamide gels, dried and visualized by autoradiography.

RESULTS

AP-1 Binding activity The effects of VT on binding activities of AP-1 was studied by EMSA in RAW 264.7 macrophage cells. Cells were incubated with different concentrations of VT with and without LPS for different time periods in both synchronous and delayed synchronous models. When cells were treated with VT alone in the synchronous model, effects of VT on AP-1 binding activity were negligible at 2 hr (Fig. 3.1A). Whereas AP-1 binding was increased in cells treated with 100 and 250 ng/ml of VT at 8 hr (Fig. 3.1A) . Upon simultaneous incubation with LPS (100 and 1000 ng/ml), the activity of AP-1 binding was increased at 100 and 250 ng/ml of VT at 2 hr but not at 8 hr (Fig. 3.1 B and Fig. 3.1C). In the delayed synchronous model, cells were treated with and

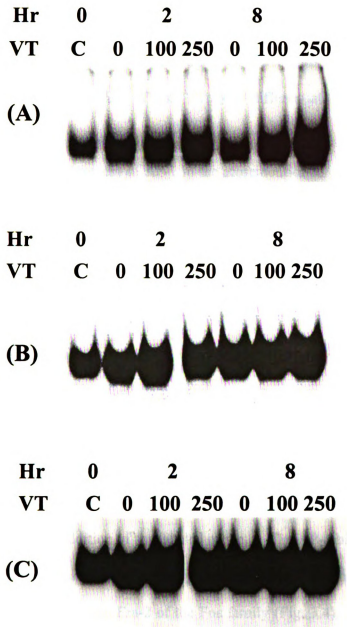


Fig. 3.1. Dose and temporal effects, of VT on AP-1 binding activity in a synchronous exposure model. RAW 264.7 cells were treated with VT in the absence (A) or presence of of (B) 100 ng/ml and (C) 1000 ng/ml of LPS for different time periods simultaneously. Nuclear protein extracts were prepared at the time indicated and 5 μg of nuclear protein of each sample was subjected to EMSA as described in the Materials and Methods. Data are representative of two separate experiments.

without LPS for 2 hr prior to addition of VT. AP-1 binding activity was markedly increased with 100 and 250 ng/ml of VT in the absence of LPS after 2 and 8 hr (Fig. 3.2A). Upon addition of 1000 ng/ml of LPS, binding activities were inhibited by VT after 2 hr but unaffected after 8 hr (Fig. 3.2B). Thus, VT could differentially affect AP-1 binding in RAW 264.7 cells.

The specific involvement of AP-1 in the aforementioned protein-DNA complexes was assessed by binding competition studies with unlabeled wild type oligonucleotides. A decrease in the amount of bound complex was observed as the concentration of unlabeled AP-1 consensus sequences increased in both synchronous and delayed synchronous models (Fig. 3.3A and 3.3B). Here, 100X excess of mutant AP-1 oligonucleotide did not inhibit the DNA-binding to the same extent as 100X excess of wild type. These results confirmed the specificity of the AP-1 DNA-binding activity.

Both c-Jun and c-Fos subunits are involved in AP-1 transactivation effects. Supershift EMSA was performed with nuclear extracts from control and VT-treated RAW cells to identify subunits altered by VT. Antibodies against the Jun and Fos family subunits were added prior to the addition of the labeled probe. The AP-1 binding band was most markedly supershifted by antibodies against phosphorylated c-Jun, and JunB subunits of the Jun family, and by c-Fos, and Fra-2 of the Fos family (Fig. 3.4). A supershift was not observed when IgG was added (data not shown). These results suggest that both homo- and hetero-dimers of *c-jun* and *c-fos* family might be involved in altered AP-1 binding.

NF-κB Binding activity. κB-binding of nuclear extracts was visualized in EMSA as multiple bands which is consistent with the existence of multiple monomers that form

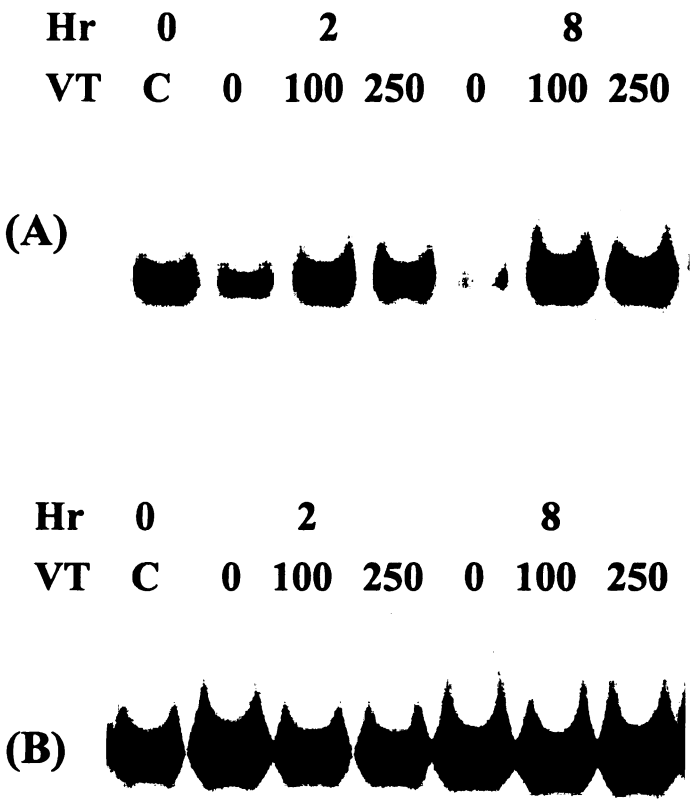
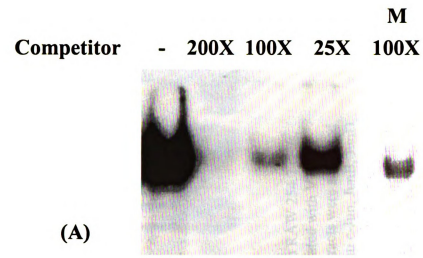


Fig. 3.2. Dose and temporal effects of VT on AP-1 binding activity in a delayed synchronous model. RAW 264.7 cells were stimulated in the absence (A) and (B) presence of 1000 ng/ml LPS for 2 hr prior to the addition of VT and incubated for another time periods. Nuclear protein extracts were prepared at the time indicated and 5 μg of nuclear protein of each samples were subjected to EMSA as described in Materials and Methods. Data are representative of two separate experiments.



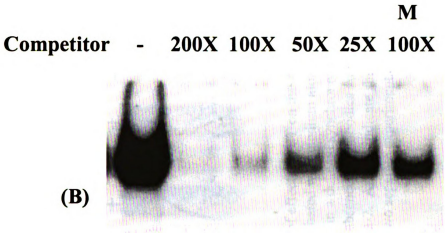


Fig.3.3. Specificity of AP-1 DNA binding activity in RAW 264.7 cells in (A) synchronous model and (B) delayed synchronous model. Cells were treated with LPS (1000 ng/ml) and VT (0, 100 ng/ml) for 2 hr in synchronous model. Cells were pre-stimulated with medium alone for 2 hr and then treated with VT (0, 100 ng/ml) for another 8 hr in the delayed model. EMSAs were performed by using protein nuclear extracts (5 μg/lane) from the RAW cell culture in the presence of ^{32}P-labeled AP-1 probe alone or with 200, 100, 50 or 25 molar excess of unlabeled AP-1,and 100 molar excess of mutant AP-1 probes. Data are representative of two separate experiments.

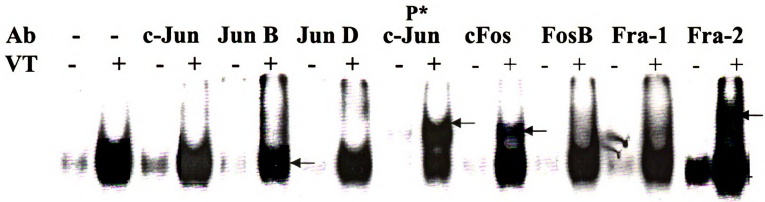


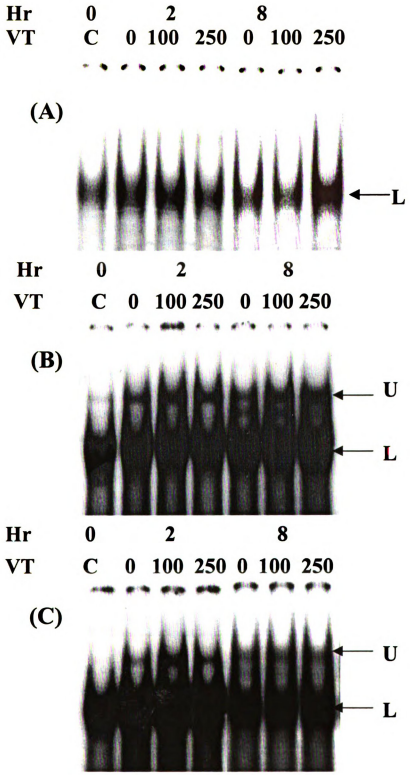
Fig.3.4. Supershift EMSA of effects of VT on the AP-1 subunit binding in RAW 254.7 cells. RAW 264.7 cells were incubated with medium alone for 2 hr and then treated with VT (0, 250 ng/ml) for another 8 hr in a delayed synchronous model. Nuclear extracts were subjected to supershift EMSA in the absence of antibodies and in the presence of anti c-Jun, JunB, JunD, c-Fos, Fos B, Fra-1, and Fra-2. Arrows indicate shifted bands or the reduction of the binding. Data are representative of two separate experiments.

dimeric NF-κB binding proteins (Baeuerle, 1991; Grilli *et al*, 1993). In the synchronous model, binding was not altered in a lower complex with VT in the absence of LPS at 2 hr, but increased with 250 ng/ml of VT at 8 hr (Fig. 3.5A). In the presence of 100 ng/ml of LPS, VT at 100 and 250 ng/ml enhanced binding of the upper band as early as 2 hr (Fig. 3.5B) whereas the effect at 1000 ng/ml LPS was less remarkable (Fig. 3.5C). In the delayed synchronous model, binding of the lower band was not enhanced by VT in the absence of LPS at 2 hr (Fig. 3.6A) but was at 8 hr. When cells were stimulated with 1000 ng/ml of LPS prior to the addition of VT, the upper band was increased slightly upon incubation with 100 and 250 ng/ml of VT for 2 and 8 hr (Fig.3.6 B).

The specific involvement of NF-κB/Rel in the protein DNA complexes was determined by inhibition studies with unlabeled probe. When a nucleotide corresponding to the κB consensus site was added to the nuclear extracts of VT-treated RAW cells, two complexes (the upper and lower complexes) were found to be elevated. In the delayed synchronous model, 500X fold molar excess of cold κB oligonucleotide decreased the binding of the lower band (Fig. 3.7A). A 200X concentration of the mutant inhibited the binding slightly in the delayed synchronous model. The slower migrating complex (upper complex) was decreased as the concentration of cold κB consensus sequences increased in the synchronous model (Fig.3.7B). Mutated κB sequence (100 X) failed to inhibit the binding of the upper complex in the synchronous model. These results suggested that the binding of the upper complex of NF-κB to DNA was specific.

For the studies of the subunits of NF-κB/Rel family, antibodies against c-Rel, Rel-B, p65 and p50 subunits were used in a supershift EMSA (Fig. 3.8). The upper complexes of

Fig. 3.5. Dose and temporal effects of VT on NF-κB binding activity in a synchronous exposure model. Cells were treated with VT in the absence (A) or presence of (B) 100 ng/ml and (C) 1000 ng/ml of LPS for different time periods. Experiments were performed as described in the legend of Fig.3.1. Arrows indicate the upper complex (U) and lower complex (L). Data are representative of two separate experiments.


Hr 0 2 8
VT C 0 100 250 0 100 250
(A)
L
Hr 0 2 8
VT C 0 100 250 0 100 250
(B)
U
L
Hr 0 2 8
VT C 0 100 250 0 100 250
(C)
U
L

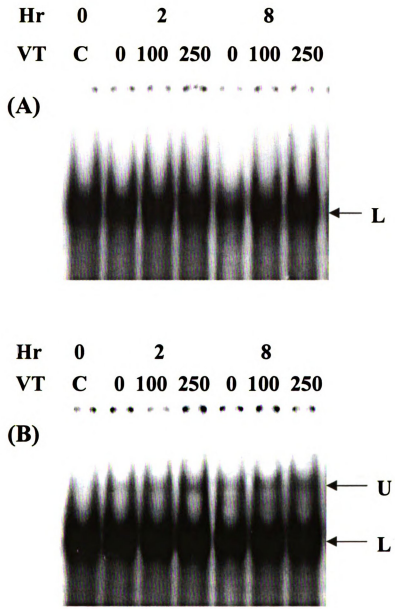


Fig.3.6. Dose and temporal effects of VT on NF-κB binding activity in a delayed synchronous model. Cells were stimulated with medium alone (A) and 1000 ng/ml of LPS (B) for 2 hr prior to the addition of VT and incubated for another time periods. Experiments were performed as described in the legend of Fig.3.2. Arrows indicate the upper complex (U) and lower complex (L). Data are representative of two separate experiments.

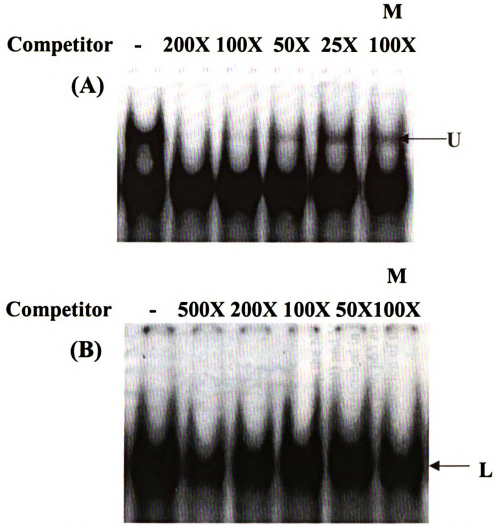


Fig.3.7. Specificity of NF-κB DNA binding activity in RAW 264.7 cells in (A) delayed synchronous model and (B) synchronous model. Cells were incubated with medium alone for 2 hr and then treated with VT (0, 250 ng/ml) for another 8 hr in the delayed model. Cells were treated with LPS (100 ng/ml) and VT (0, 100 ng/ml) for 2 hr in synchronous model. Experiments were performed as described in the legend of Fig.3.3, except 200 molar excess of mutant NF-κB was used in the delayed synchronous model. Arrows indicated upper complex (U) and lower complex (L). Data are representative of two separate experiments.

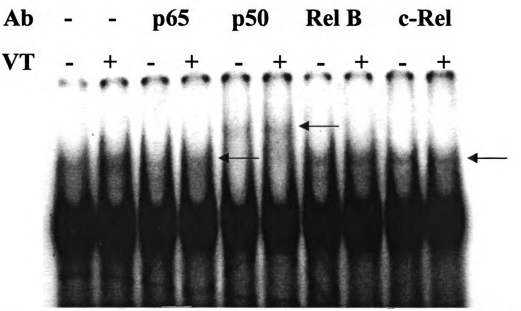


Fig.3.8. Supershift EMSA of effects of VT on the NF-κB/Rel subunit binding in RAW 264.7 cells . Cells were treated with LPS (100 ng/ml) and VT (0, 100 ng/ml) for hr in a synchronous model. Experiments were performed as described in the legend of Fig.3.4. In the the absence and presence of antibodies against Rel-A, p50, p65 and c-Rel. Data are representative of two separate experiments. Arrows indicate reduction of the intensities of the binding or shifted bands.

the control and VT-treated group were supershifted by anti-p50 and c-Rel. No detectable supershifted bands were observed with p65 and Rel-B antibodies. The lower complex was not supershifted by any of the antibodies. The results suggested that both p50 and c-Rel subunits might be involved in the VT-enhanced NF-κB binding.

NF-IL6 Binding activity. The binding activity of NF-IL6 was also assessed in these studies with RAW 264.7 macrophage cells. In the synchronous model, the binding activity of NF-IL6 was increased with 250 ng/ml of VT at 8 hr in the absence of LPS (Fig. 3.9A). When cells were treated with 100 ng/ml of LPS, VT enhanced NF-IL6 binding at the concentrations of 100 and 250 ng/ml at 2 hr, but not at 8 hr (Fig. 3.9B). At a higher dose of LPS (1000 ng/ml), enhanced binding was found in cells treated with 100 and 250 ng/ml of VT at 2 hr (Fig. 3.9C). At 8 hr, no difference in the binding activity was found in either doses of VT. In the delayed synchronous model, 100 ng/ml of VT was able to enhance the binding at 8 hr; and 250 ng/ml at 2 and 8 hr in the absence of LPS (Fig.3.10A). A decrease in the binding activities were observed in LPS-stimulated cells treated with VT and 1000 ng/ml of LPS for 2 hr but no effect was seen at 8 hr (Fig. 3.10B).

Nuclear extracts were further assessed for binding specificity. In both synchronous and delayed synchronous models, the increase in the concentration of cold C/EBP consensus sequences showed a dose-dependent inhibition in the DNA-binding (Fig. 3.11 A and B). Mutated C/EBP sequences (100 fold excess) were not effective in inhibiting binding in both models. These results suggested that a specific DNA-binding activity of NF-IL6.

To identify NF-IL6 subunits affected by VT, antibodies against C/EBP-α, -β , and -δ, were used in supershift studies. The C/EBP binding band was supershifted by anti-

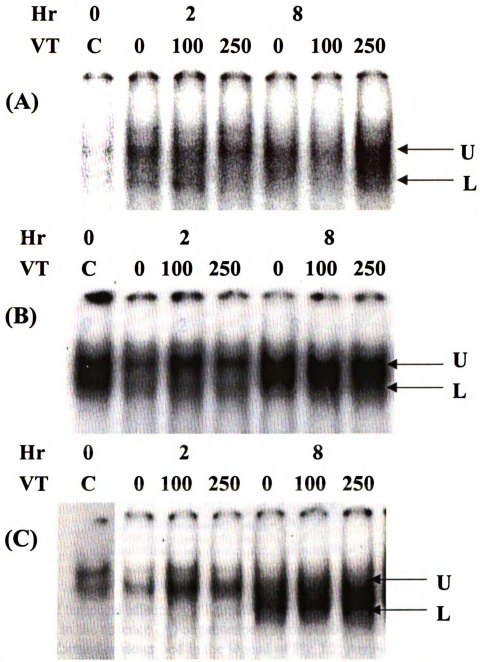


Fig.3.9. Dose and temporal effects of VT on NF-IL6 binding activity in a synchronous model. RAW cells were treated with VT in the absence (A) or presence of (B) 100 ng/ml and (C) 1000 ng/ml of LPS for different time periods simultaneously. Experiments were performed as described in the legend of Fig.3.1. Arrows indicate upper complex (U) and lower complex (L). Data are representative of two separate experiments.

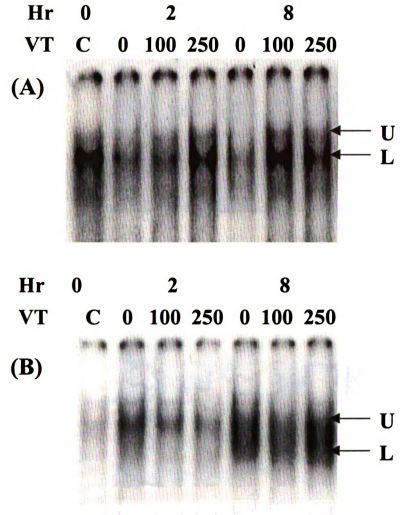


Fig. 3.10. Dose and temporal effects of VT on NF-Il6 binding activity in a delayed synchronous model in the absence (A) or presence (B) of 1000 ng/ml of LPS. Experiments were performed as described in the legend of Fig.3.2. Arrows indicate upper complex (U) and lower complex (L). Data are representative of two separate experiments.

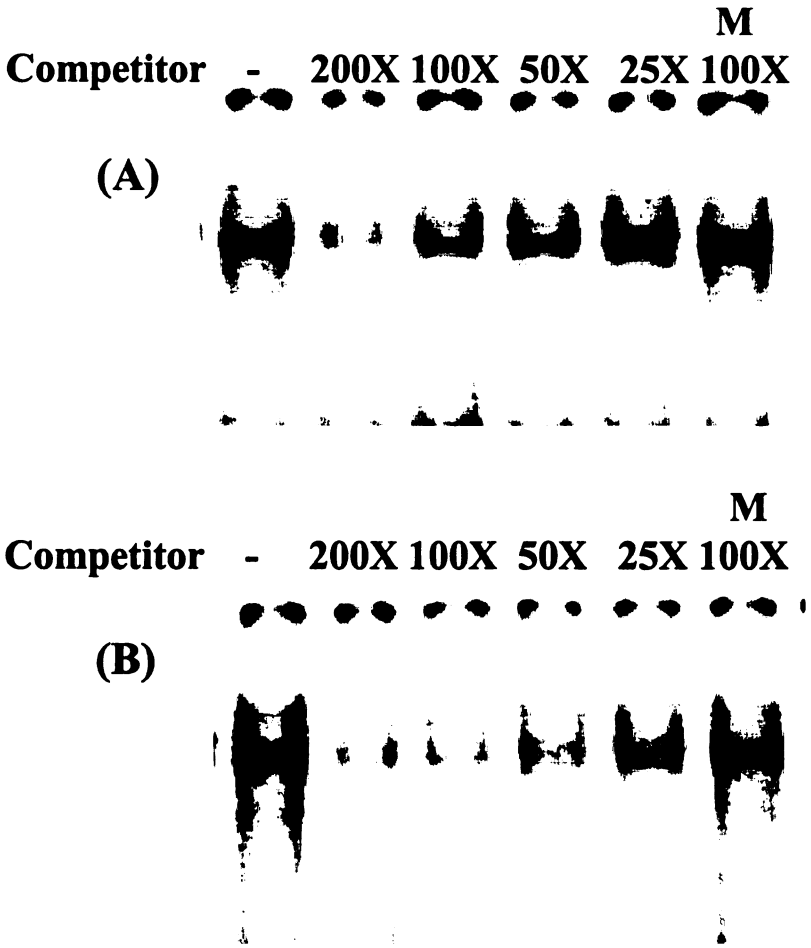


Fig.3.11. Specificity of NF-IL6 DNA binding activity in RAW 264.7 cells in a (A) synchronous model and (B) delayed synchronous model. Cells were treated with LPS (1000 ng/ml) and VT (0, 100 ng/ml) for 2 hr in the synchronous model. Cells were incubated with medium alone for 2 hr and then treated with VT (0, 250 ng/ml) for another 2 hr in the delayed model. Experiments were performed as described in the legend of Fig.3.3. Data are representative of two separate experiments.

C/EBPβ in both control cells and 250 ng/ml VT-treated cells (Fig. 3.12). Anti- C/EBPα also had effects on reducing the binding of both control and treatment groups. This indicates that both C/EBPα and C/EBPβ might be involved in increased NF-IL6 binding observed in cultures incubated with VT.

DISCUSSION

AP-1, NF-κB, and NF-IL6 have been implicated as important transcription factors in immune and inflammatory responses mediated by macrophages. Notably, the binding sites for these three factors have been found on the promoters of proinflammatory cytokines IL-1β, IL-6 and TNF-α (Fig. 3.13). In this study, we demonstrated that VT was able to alter binding activities of these transcription factors differentially in macrophage RAW 264.7 cells. Several major findings have been observed. First, VT alone induced the AP-1 binding in both synchronous and delayed synchronous models. Secondly, the binding activity of upper complex of NF-κB was enhanced by VT in the presence of LPS of both synchronous and delayed synchronous models. Thirdly, NF-IL6 binding activity was enhanced by VT in the absence (synchronous and delayed synchronous models) and presence (synchronous model) of LPS. Finally, the results showed that VT specifically targeted the phosporylated c-Jun and JunB, c-Fos, and Fra-2 subunits of the AP-1 components; p50 and c-Rel of NF-κB; and C/EBPα and C/EBPβ of NF-IL6.

AP-1 regulates transcription of genes through its ability to bind specifically to the recognition site, 5'-TGANTCA-3', which is also known as the TPA(12-O-tetradecanoyl-phorbol-13-acetate) response element (TRE) (Lee *et al.*, 1987; Risse *et al.*, 1989). AP-1

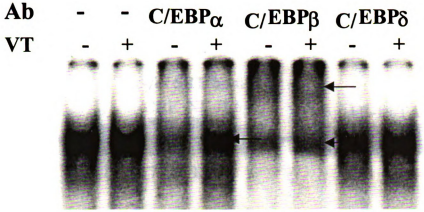


Fig. 3.12. Supershift EMSA of effects of VT on the NF-IL6 subunit binding in RAW 264.7 cells were treated with LPS (1000 ng/ml) and VT (0, 250 ng/ml) for 2 hr in a synchronous model. Experiments were performed as described in the legend of Fig.3.4. In the absence and presence of antibodies against C/EBPα, C/EBPβ, and C/EBPδ. Arrows indicate the reduction of the binding of the original complex or shifted bands. Data are representative of two separate experiments.

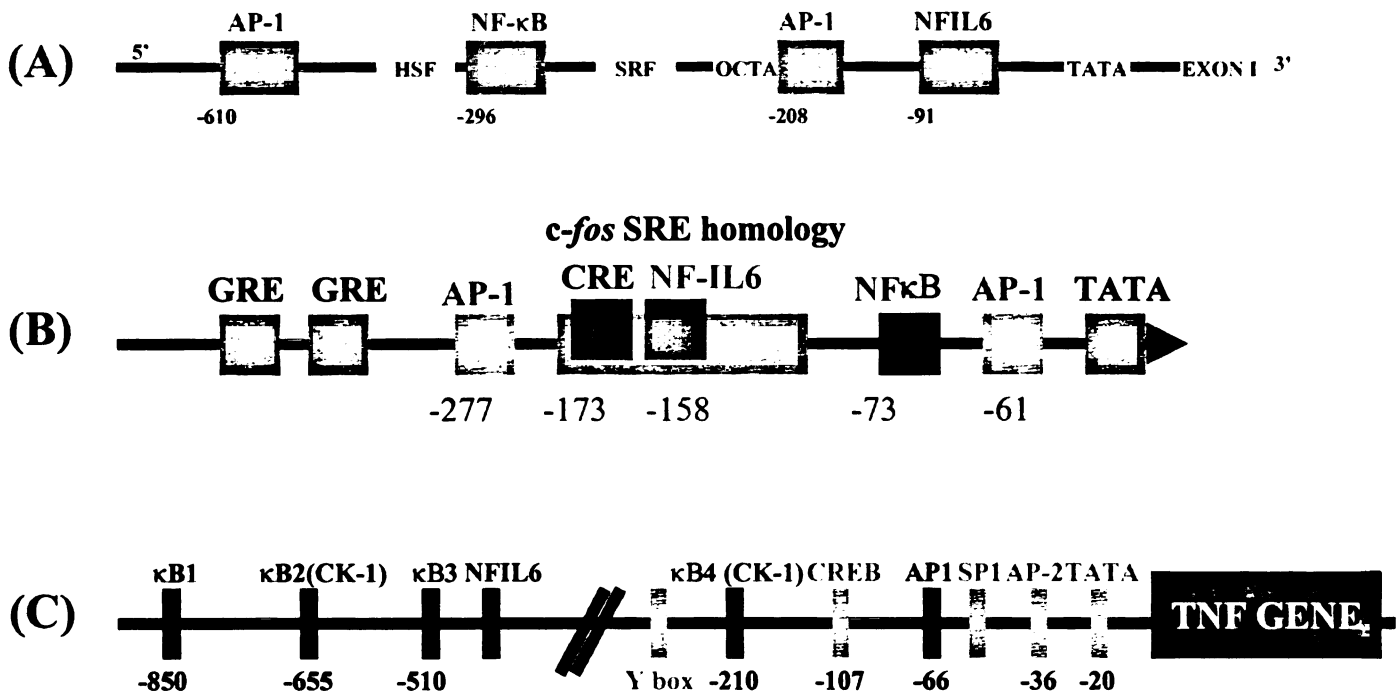


Fig.3.13. (A) Regulatory elements of IL-1β promoter; (B) Regulatory elements of IL-6 promoter; (C) Regulatory elements of TNF-α promoter.

sites are found on the IL-1β, IL-6 and TNF-α promoter (Fig. 3.13). AP-1 is primarily regulated by induction of *de novo* protein synthesis and post-translational modifications of its c-Jun and c-Fos proteins by phosphorylation and dephosphorylation (Pennypacker, 1998). The c-Jun proteins are mainly phosphorylated by a class of mitogen activated protein kinase (MAPK), known as the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPK) (Karin *et al.*, 1997; Liebermann *et al.*, 1998; Wisdom, 1999). Transactivations of *c-fos* gene are mediated via phosphorylation by extracellular-signal-regulated kinase (ERK) as well as JNK of MAPK (Karin, 1995; Karin *et al.*, 1997). Phosphorylation of c-Jun and c-Fos protein has been shown to increase the transactivation of AP-1 (Bohmann *et al.*, 1987; Angel *et al.*, 1988). Here, it has been shown that VT alone was able to induce AP-1 binding activity and the induction involved a major activation of the c-Jun and c-Fos components. In mammalian cells, c-Jun is constitutively present in the cells in an inactive form in cytoplasm and the major increases of this protein in nucleus might reflect the ability of VT to promote JNK and/or ERK activities. Consistent with this possibility, our laboratory has found that VT was able to enhance the MAP kinase activities in RAW 264.7 cells (Yang *et al.*, 2000).

In this study, we identified two major complexes of NF-κB. A VT-mediated increased in the lower complex (faster migrating band) was observed in the absence of LPS. On the contrary, the upper complexes (slower migrating band) were induced only upon stimulation with LPS, and VT was able to further enhance the binding in both synchronous and delayed models. Therefore, the lower complexes might be formed by the binding of the constitutively expressed NF-κB proteins, whereas the upper complexes are

those NF-κB proteins induced only in the presence of a co-stimulus. There are four major κB sites on the promoters of murine TNF-α (Pauli, 1994), and κB sites have also been found on the promoters of IL-1 and IL-6 genes (Hirano, 1994; Dinarello, 1996) (Fig. 3. 13). It has also been noted that the inhibitor protein of NF-κB, IκBα, can be phosphorylated by IκB kinase (IKK)1 and IKK2 (Wallach *et al.*, 1999; Rath and Aggarwal, 1999). These IKKs are activated via phosphorylation by NIK, a protein kinase homologous to MEKK (MAP kinase kinase) (Rath and Aggarwal, 1999) . Phosphorylation of the IκBα will result in activation of NF-κB, which might be involved in inflammatory response and in inhibiting apoptosis caused by TNF-α.

Activation of leukocytes by different signals always leads to the release of different subunits of NF-κB from the cytoplasm to nucleus. In this study, we found that VT has effects on p50 and c-Rel subunits by the EMSA supershift assay. p50 might be able to form its own homodimers or heterodimers with other subunits. The p50 homodimers have been suggested to be constitutively expressed in monocytes without stimulation (Ziegler-Heitbrock *et al.*, 1993; Baeuerle and Henkel, 1994). However, heterodimers of p-50/c-Rel, p50/p65, and p65/c-Rel are thought to be involved in the trans-activating activity when stimulated. Though p50 homodimers are inactive transcriptionally, they can serve as 'helper' subunits for other NF-κB proteins, such as p65 (RelA) and c-Rel by allowing them to bind at the κB site (Baeuerle and Henkel, 1994). c-Rel has been identified as another important component of NF-κB in macrophages during activation. c-Rel is involved in many biological responses of lymphoid cells such as B cell proliferation (Tumang *et al.*, 1998), activation and T cell proliferation (Liou *et al.*, 1999). c-Rel is also involved in the

cytokine and cytokine receptor expression (Liou *et al.*, 1999), and the expression of inflammatory genes in glomerulonephritis (Stylianou *et al.*, 1999). Therefore, c-Rel might also play an important role in the cytokine superinduction by VT in RAW 264.7 cells. Interestingly, we did not detect p65 (RelA) in our assay, p65 is an important regulator of inducible genes involved in inflammation, infection and stress (Baeuerle and Henkel, 1994). It is also an important transcription activator in CD28 response element in the IL-2 promoter (Lai *et al.*, 1995), and a key factor for cell proliferation and apoptosis since lack of Rel A causes embryonic lethality and liver degeneration in mice (Beg *et al.*, 1995).

NF-IL6 is also involved in the gene regulation of macrophages (Clarke and Gordon, 1998). Here, increased NF-IL6 at 2 hr in the synchronous model relates to the increased IL-6 mRNAs and proteins observed in the previous paper (Wong *et al.*, 1998). NF-IL6 also plays a critical role in both IL-6 and TNF-α gene expression (Poli, 1998). In this study, both C/EBPα and β subunits had been identified. C/EBPα is mostly found in immature granulocytes and deficiency of these genes might cause perinatal death (Poli, 1998; Lekstrom-Himes and Xanthopoulos, 1998). C/EBPβ has been shown as an important transcription factor in the myeloid cells during maturation and activation (Yamanaka *et al.* , 1998; Poli, 1998). When human monocytic cells U937 are stimulated with PMA, the NF-IL6 protein is supershifted with the C/EBPβ antisera (Combates *et al.*, 1997). C/EBPβ contributes to regulation of TNF-α gene expression in the human U937 cells (Pope *et al.*, 1994). C/EBPβ has also been found to be activated via phosporylation by MAP kinase at its threonine residues (Poli, 1998). Here, C/EBPβ was also found to be the major component involved in the cytokine superinduction by VT. Therefore, NF-IL6 may be

responsible, in part, for the superinduction of the cytokine protein and mRNA levels.

Previously, Zhou *et al.* (1997; 1998) demonstrated that enhanced IL-6 and TNF-α mRNAs and proteins are found in mice gavaged with VT. Recently, increased AP-1, NF-κB and NF-IL6 binding activities have also been observed in mice gavaged with LPS or VT alone (Zhou *et al.*, 1999). The delayed synchronous model in our study is designed to mimic the *in vivo* experiments performed previously in our lab. We found that the *in vivo* results were quite comparable to the results of our studies in the delayed model, in which increased AP-1, NF-κB and NF-IL6 are found in macrophage RAW 264.7 cells with VT. Consistent with our findings, both NF-κB and AP-1 binding activities are also enhanced by VT in the EL-4 cells up to 48 hr (Ouyang *et al.*, 1996; Li *et al.*, 2000). The reason for increases in later time periods in EL-4 cells might be due to the inherent differences in the T cell and macrophage cell lines.

It should be noted that the regulation of cytokine gene expression can also occur at the post-transcriptional level (Tracey, 1994). The repeating octameric units TTATTTTAT in the 3'-untranslated region (UTR) of the mRNA may confer some instability and superinducibility to various genes (Dofferhoff *et al.*, 1991; Pauli, 1994). The AU-rich elements have also been suggested as a factor that exerts instability on transiently expressed genes and shortens the half-life of the mRNA (Shaw and Kamen, 1986; Tracey, 1994; Ross, 1996). It is expected that stabilization of the cytokine mRNAs might play a key role in the upregulation of cytokine proteins. Recently, JNKs as well as other MAP kinases have been shown to be involved in the stabilization of cytokine and other targeted gene mRNAs (Chen *et al.*, 1998; Miyazawa *et al.*, 1998; Ming *et al.*, 1998; Winzen *et al.*, 1999;

Wang *et al.*, 1999). Therefore, stabilization of the cytokine mRNAs by VT might be an alternative mechanism responsible for the superinduction of cytokines and therefore should be evaluated.

The results of our studies have shown that VT differentially enhances AP-1, NF-κB and NF-IL6 binding activity. Changes in these activities may contributed to the cytokine superinduction by VT in macrophage cells. Further studies on VT-mediated promoter activity will be necessary to confirm the relationship between activation of the *trans*-acting elements, transcription factors, and the cytokine superinduction.

CHAPTER IV

EFFECTS OF VOMITOXIN ON TRANSCRIPTIONAL ACTIVITY OF TUMOR NECROSIS FACTOR ALPHA (TNF-α) PROMOTER IN MURINE MACROPHAGE RAW 264.7 CELLS

ABSTRACT

The trichothecene vomitoxin (VT) has been shown to superinduce tumor necrosis factor (TNF-α) protein production and mRNA expression in macrophage RAW 264.7 cell lines. The purpose of this study was to investigate the role of altered transcriptional regulation in VT-induced superinduction of TNF-α in the macrophage. A construct containing the TNF-α promoter linked to a chloramphenicol-acetyl-transferase (CAT) reporter gene was used for this purpose. Using ELISA, it was shown that CAT protein production were not significantly enhanced by VT at both 2 and 6 hr when LPS was added simultaneously (synchronous model). When cells were treated with 100 ng/ml and 1000 ng/ml LPS for 2 hr prior to the addition of 100 ng/ml of VT (delayed synchronous model), increased CAT protein was observed at 2 and 4 hr but not at 8 and 12 hr. Expression of CAT mRNA was also determined using a competitive reverse transcription-polymerase chain reaction (RT-PCR). From 0.4 - 3-fold increases were found in cells treated with VT at 0, 10, and 100 ng/ml of LPS in the synchronous model. From 0.2 - 2 fold increases were found in cells co-treated with 0 to 100 ng/ml of LPS and VT in the delayed model. No increase in CAT mRNA was found in cells co-treated with 1000 ng/ml of LPS and VT in either model. The low level of increased CAT mRNA expression under these varied treatment conditions suggest that transcription control might contribute to the superinduction of TNF-α observed previously, albeit to a small extent.

INTRODUCTION

The trichothecene vomitoxin (VT, deoxynivalenol) is a secondary metabolite

produced by species of *Fusarium* found in wheat and corn supplies throughout the world (Ueno, 1985; Vesonder and Hesseltine, 1980; Rotter *et al.*, 1996). Trichothecenes have been shown to cause an acute shock-like toxicity or chronic effects including feed refusal in experimental animals (Cundliffe *et al.*, 1974; Vesonder and Hesseltine, 1980; Bamburg, 1983). VT and other trichothecenes are potent protein synthesis inhibitors. Low and acute doses of VT have been known to cause anorexia and emetic effects in livestock (Rotter *et al.*, 1996). Dong and Pestka (1993) showed that dietary exposure of mice to VT elevates serum IgA, circulating IgA complexes and mesangial IgA deposition in the kidney. These features are highly analogous to the most common human glomerunephritis, IgA nephropathy (D'Amico, 1987).

VT is able to superinduce interleukin (IL)-1β, 2,4,5,6, and TNF-α expressiion both *in vivo* and *in vitro* (Azcona-Olivera *et al.*, 1995a; Ouyang *et al.*, 1996; Zhou *et al.*, 1997; Wong *et al.*, 1998). Previous studies have shown that other protein synthesis inhibitors, CHX and anisomycin, are able to superinduce IL-2, IL-6 IFN-γ and TNF-α mRNA both *in vivo* and *in vitro* (Efrat and Kaempfer, 1984; Zubiaga *et al.*, 1991; Cockfield *et al.*, 1993; Osipovich *et al.*, 1993; Akahane *et al.*, 1994; Biragyn and Nedospasov, 1995; and Faggioli *et al.*, 1997). Interestingly, Ouyang *et al.*(1996) have demonstrated that the activity of transcription factor NF-κB was enhanced in IL-2 gene expression in EL-4 cells treated with VT. In the previous chapter, I also demonstrated that VT increases transcription factor AP-1, NF-κB and NF-IL6 binding activities in RAW 264.7 macrophage cells. Based on these findings, it is reasonable to suggest that VT might superinduce cytokines via enhancing their gene transcription. However, the effects of VT on the activity of the *cis*-acting elements

of these cytokines in macrophages have not yet been determined.

TNF-α is a pro-inflammatory cytokine mainly produced by mononuclear cells (Vilcek and Lee, 1991; Dayer and Burger, 1994). It is among the early activated cytokines in inflammation together with interleukin(IL)-1β, interferon-gamma (IFN-γ), and IL-6 (Beutler, 1990). Various inducers such as LPS, phorbol esters, IFN-γ , IL-2, GM-CSF, ionophore, X-rays, vitamin D3 and even Sendai virus, can lead to the activation of TNF-α gene transcription. According to Beutler *et al.* (1986), TNF-α biosynthesis is controlled at the transcriptional, post-transcriptional and translational levels. In this study, we investigated the *cis* effects of VT on TNF transcription by transiently transfecting a TNF-CAT reporter construct into macrophage RAW 264.7 cells and measuring CAT gene expression.

MATERIALS AND METHODS

RAW 264.7 cell culture. RAW 264.7 cells (TIB 77, American Type Culture Collection, Rockveille, MD) (2.5 x 10^5 cells/ml) were plated in 100-mm tissue culture dishes (Corning, NY) in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 1% (v/v) NCTC 135 (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in a 5-8% CO_2 incubator at 37°C.

Cell transfection. A TNF-CAT reporter construct vector (kindly supplied by Dr. Bruce Beutler, Howard Hugh Institutes. TX) was transformed into *E.coli* XL-1 Blue MRF'

(Stratagene Co., La Jolla, CA) cells and amplified. The amplified vectors were purified using the Qiagen plasmid purification Maxi kit (Qiagen, Valencia, CA). Cells were transiently transfected by three different methods: calcium phosphate ($CaPO_4$), DEAE, and the liposome method. In the $CaPO_4$ method, cells in exponential phase were split the day before transfection according to the method of Kingston (1996). Then cells (5×10^5) were fed with 9 ml fresh medium 2 hr prior transfection. DNA (1 μg) was precipitated with ethanol, suspended in distilled water and diluted in 2.5 M calcium chloride ($CaCl_2$) solution (Kingston, 1996). After the addition, 500 μl of 2X HEPES-buffered saline (HeBS) was added to the DNA/$CaCl_2$ solution. The mixture was then distributed evenly to the cells in the tissue culture dishes for further incubation. In the DEAE method, cells (5×10^5) were plated in a 35-mm dishes prior to the transfection (Kingston, 1996). Then cells were incubated until they were confluent. Fresh medium containing the DEAE dextran (Sigma) and 1 μg plasmid were added and incubated for 1 hr at 37°C in a humidified CO_2 incubator. Afterwards, cells were washed twice with medium and fresh medium was added for further incubation in a CO_2 incubator. In the liposome method (Kingston, 1996), RAW 264.7 cells (5×10^5 cells) were grown in DMEM supplemented with 10% (v/v) FBS. Cells were then split when they reached confluence by rinsing with Dulbecco's phosphate buffer saline (DPBS; Sigma). Cells were transfected with 1 μg TNF reporter construct and 5 μl lipofectamine (Gibco BRL) in 2 ml DMEM medium for 3 hr. After the transfection, cells were incubated for an additional 12 to18 hr in culture medium. Cells were then washed with DPBS and treated with VT (0 and 100 ng/ml) and lipopolysaccharide (LPS, *Salmonella typhimurium;* Sigma) (0, 10, 100 and 1000 ng/ml) in a synchronous or a delayed

synchronous fashion for different time periods. Plasmid containing the cytomegalovirus promoter (pCMV) (kindly supplied by Dr. Tim Zacharewski, Michigan State University) with β-galactosidase reporter gene was used as a control plasmid in all the experiments. Cells which turned blue in the presence of substrate X-gal were counted by light microscopy under 40 X magnification.

CAT ELISA. CAT ELISA was performed according to the manufacturer's protocol (Boeringer Mannheim, Indianapolis, IN). Culture medium was carefully collected and cells were washed 3 times with 1 ml room temperature DPBS by pipetting. Cells were resuspended in 1 ml lysis buffer per 1 x 10^6 cells and incubated for 30 min on ice. Then cells were transferred to a microfuge tube and centrifuged for 10 min at 15,000 rpm in a refrigerated centrifuge. Supernatant was removed and an aliquot of the supernatant was used for protein concentration determination according to Bradford (1976). Two hundred microliters (200 μl) of cell extracts from each sample were used for the ELISA to determine CAT protein production. Briefly, cell extracts containing CAT were added to the wells of ELISA microtiter strips coated with anti-CAT antibodies. Then anti-CAT conjugated with digoxygenin (DIG) was added and bound to CAT. An antibody to DIG conjugated with peroxidase was added and bound to DIG. In the final step, the peroxidase substrate 2, 2'-azinobis (3-ethylbenzthiazoline-sulfonic acid) (ABTS) was added. The absorbance of the sample was measured at 405 nm and were directly correlated to the level of CAT present in the medium.

TNF-α ELISA assay. Harvested medium as mentioned earlier was used for the detection of the extracellular TNF-α. TNF-α concentrations were measured by ELISA

as described by Dong *et al* (1994). The TNF-α assay was performed by a modified ELISA in which Immunolon IV Removawell microtiter strips (Dynatech Laboratories., Chantilly, VA) were coated with 50 µl/well of monoclonal rat anti-TNF-α antibody (Pharmingen) diluted in phosphate buffered saline (PBS)and incubated at 4°C by overnight. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (Sigma) (PBS-T). To reduce non-specific protein binding, 300 µl PBS containing 1 % (w/v) bovine serum albumin (BSA) was added to each well, incubated for 30 min at 37°C and then washed four times with PBS-T. Reference TNF-α or samples were diluted in DMEM with 10 % (v/v) FBS, and 50 µl was added to appropriate wells. Plates were incubated for 60 min at 37°C and then washed four more times with PBS-T. Rabbit anti-TNF-α antibody (Pharmingen), diluted in PBS containing 1 % (w/v) BSA, was added to each well and the plate incubated at room temperature for 60 min. The plates were then washed six times with PBS-T, and 50 µl of goat anti rabbit IgG antibody conjugated with horse-radish peroxidase (Cappel Laboratories, Durham, NC) diluted 1 : 5000 in PBS with 1% (w/v) BSA was added to each well and incubated for room temperature for 60 min. ELISA plates were washed eight times with PBS-T. Substrate (100 µl) consisting of 3', 3', 5', 5'- tetramethyl benzidine (0.1 mg/ml; Fluka Chemical Corp., Ronkonkoma, NY) in 0.1 M citric-phosphate buffer (pH 5.5) and 0.003 % (w/v) hydrogen peroxide (H_2O_2) was added to each well and incubated 20 minutes at room temperature for color development. The reaction was terminated with 100 µl of 6 N sulfuric acid (H_2SO_4). Absorbance was measured at 450 nm with Vmax kinetic microplate Reader (Molecular devices, Menlo Park, CA) and interleukins production quantified using the Softmax software (Molecular Devices).

RNA Extraction and Reverse Transcription (RT). Total RNA was extracted from the transfected RAW cells as previously described (Wong *et al.*, 1998) using the RNA Stat-60 (Tel-test "B" inc., Friendswood, TX). First strand cDNA synthesis was carried out by the procedures of Kawasaki (1991) with some modifications. For RT, RNA (2 μg) in a maximum of 18.8 μl of DEPC-H_2O was mixed with 1.1 μl of 45 mM oligo d $(T)_{15}$ (Promega Co., Madison, WI) and incubated for 10 min at 70 °C. Immediately following the incubation, 10.1 μl of RT master mix [comprised of 6 μl of 5X RT buffer (Gibco BRL), 1.5 μl 10 mM of dNTP mix (Gibco BRL), 0.6 μl of RNase inhibitor (40 U/μl; Boehringer Mannheim) and 2 μl of M-MLV reverse transcriptase (200 U/μl; Gibco BRL), were added, mixed and then incubated at 42 °C for 1 hr. The reaction was terminated by incubation at 70 °C for 10 min and cooling on ice. The RT products were stored at -80 °C.

Construction of competitor DNA. Synthesis of the internal standard (competitor or CAT mimic) for the semi-quantitative CAT-specific RT-PCR was performed by using the overlap-extension gene splicing method (Horton *et al.*, 1989). Briefly, intermediate PCR template, fragment **A**, was amplified by using primers **a** and **b**; and fragment **B**, using primers **c** and **d** (Fig.4.1). Both PCR products of fragment **A** and **B** were combined in equal concentrations in a new PCR. The primers **b** and **c** had complementary 5' ends. Two fragments were mixed along with excess **a** and **d**, denatured, re-annealed, and primer extended by DNA-polymerase to generate the competitor DNA. The PCR product was analyzed on a 2% agarose gel, excised, and purified using the QIAEX II purification kit (Qiagen). The concentration of the cDNA was determined by spectrophotometry. The competitor was 60 bp less than the native CAT gene (562 bp). Native CAT gene can be

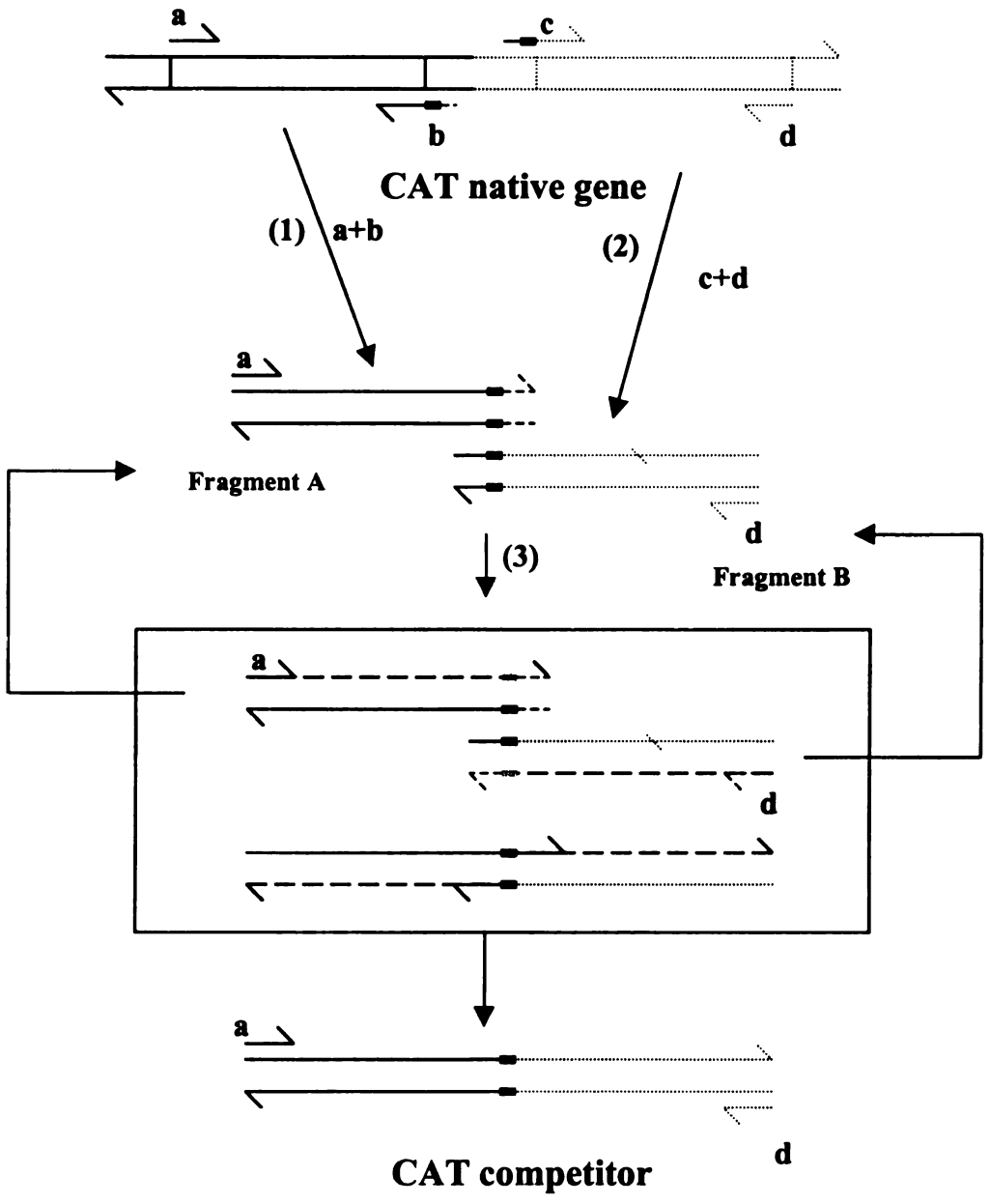


Fig. 4.1. Construction of the CAT competitor by using internal primers with the splicing by overlap extension (SOE) method modified from Horton *et al.* (1989).

amplified by the primers **a** and **d** after the RT step, which has a fragment size around 562 bp.

Oligonucleotide Synthesis. Oligonucleotides used for PCR primers were synthesized at the Macromolecular Structure Facility (Michigan State University). Sequences for the external primers were: **a**: 5'-CCC AAT GGC ATC GTA AAG AAC-3', **d**: 5'-GAC ATG GAA GCC ATC ACA GA-3'; internal primers: **b**: 5'-TGA CGC CAT CGC TCT CGG TGT AAC AAG GGT GAA CAC-3', **c**: 5'- AGA GCG ATG GCG TCA AAG ATG TGG CGT GTT ACG GT-3'.

Semi-competitive PCR. The semi-competitive PCR reactions were performed by adding a fixed amount of the sample cDNA (4 ng) with titrated known amounts of competitor (CAT mimic) (3.5 pg to 250 pg) to a PCR master mix comprised of 6 µl of 25 mM $MgCl_2$ (Perkin Elmer Gene Amp PCR System, Norwalk, CT), 5 µl of 10X PCR buffer (Perkin Elmer), 4 µl of 10 mM dNTP mix (Gibco BRL), 400 ng of primer **a**, 400 ng of primer **d**, 0.5 µl of Taq polymerase (5 U/µl; Gibco BRL) and variable amount of DEPC-H_2O to make up the final volume of 50 µl. Following mixing, PCR amplification was performed in a 9600 Perkin Elmer Cycler (Perkin Elmer Corp., Norwalk, CT) as follows: one cycle of denaturation at 95 °C for 5 min; 21 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min. The final cycle was concluded with a 10 min incubation at 72 °C. All PCR products were separated on a 2% agarose gel and visualized by staining with ethidium bromide and quantified using the FX Phospho-Imager (Bio-Rad Laboratories Inc., Melville, NY). If the amount of the competitor DNA and native cDNA were equal, then that amount represented the

concentration of the CAT mRNA present in the sample for detection. A standard curve was plotted to quantify the CAT mRNA in the transfected cells.

Statistical analysis. A one way analysis of variance (ANOVA) using Student-Newman-Keuls tests and Dunnett's test or Kruskal Wallis ANOVA on ranks was applied to parametric data and non-parametric data with application of Sigma-Stat Analysis System (Jandel Scientific, San Rafael, CA). A p vale of less than 0.05 was considered statistically significant.

RESULTS

Transfection efficiency Transient transfection with the $CaPO_4$, DEAE and liposome methods were performed by using the control plasmid pCMV containing the β-gal gene. Optimal conditions were established by trying different concentrations of DNA, cell numbers, and time periods. The efficiency was determined by incubating cells with beta-galactosidase substrate. With $CaPO_4$, the transient transfection efficiency was low (less than 2 %) (Fig. 4.2). The transfection efficiency by the DEAE method was somewhat greater than that for the $CaPO_4$ method (5-9%) (Fig. 4.3). The highest transfection efficiency (around 10-15%) was observed with cells transiently transfected using the liposome method (Fig. 4.4). Transient transfection with liposomes was a faster method. This method was used for further experiments described in this study.

Effects of VT on CAT and TNF-α protein expression. In order to determine the effects of VT on the promoter activity of the TNF-α, RAW 264.7 cells were transiently transfected with plasmids containing the TNF promoter linked to a CAT reporter gene. Transfected cells were treated with LPS and VT in a synchronous or a delayed synchronous

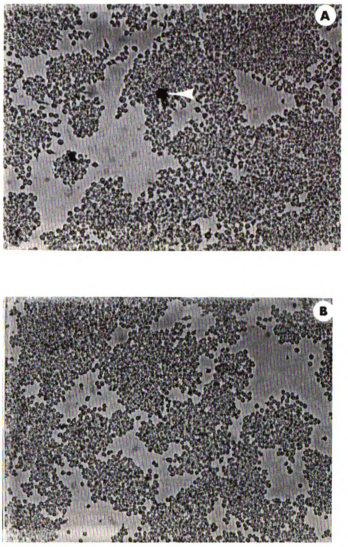


Fig. 4.2. Cells transfected with $CaPO_4$ for 16 hr and then incubated for 12 hr before staining for the β-galactosidase activity (**arrows**) of pCMV plasmid. (A) Cells treated with 1 μg pCMV plasmid; (B) Control cells without pCMV.

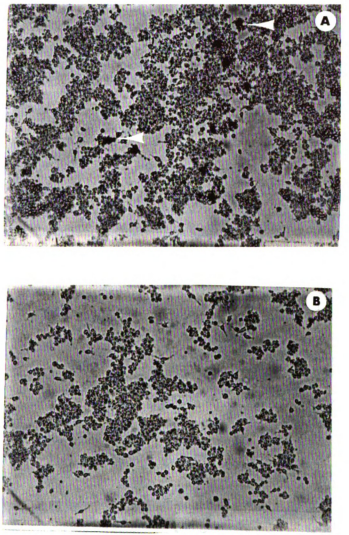


Fig. 4.3. Cells transfected with DEAE for 3 hr and then incubated for another 24 hr before staining for β-galactosidase. (A) Cells transfected with pCMV; (B) Cells without pCMV.

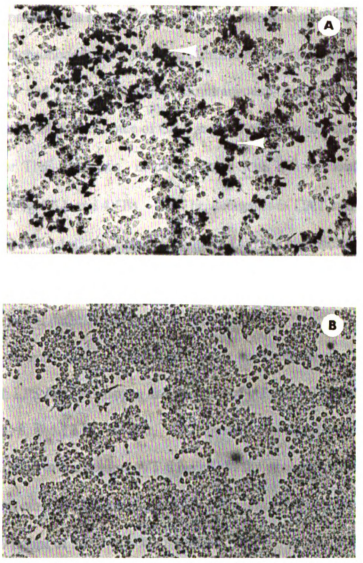


Fig. 4.4. Cells transfected using the liposome method for 3 hr and then incubated for another 12 hr before staining for β-galactosidase. (A) Cells transfected with pCMV; (B) Cells without pCMV.

fashions. CAT protein was measured to assess the response on TNF-α transcription.

In the synchronous model, cells were transfected and incubated with LPS (0, 10, 100 and 1000 ng/ml) and VT (0, 100 ng/ml) simultaneously for 2 and 6 hr. Cells were then lysed and detected for the presence of CAT protein. At 2 hr, effects were not observed in CAT protein or TNF-α protein in cells treated with and without VT at all the LPS concentrations (Fig.4.5A and 4.5B). At 6 hr, decreases in CAT and TNF-α protein were observed in cells treated with VT and 10 ng/ml of LPS but significant differences were not observed at other LPS concentrations (Fig.4.6A, B). Intracellular TNF-α protein was also analyzed but was not detectable (Data not shown).

In the delayed synchronous model, cells were transiently transfected and pre-stimulated with LPS for 2 hr prior to the addition of VT (0, 100 ng/ml) and then incubated for another 2, 4, 8, and 12 hr. At 2 hr, CAT protein was significantly increased with VT treatment compared to the control (VT 0 ng/ml) in the presence of 100 ng/ml of LPS but unaffected under other conditions (Fig. 4.7A). A significant increase of TNF-α protein was observed at 10 ng/ml of LPS with VT treatment (Fig. 4.7B). At 4 hr, CAT protein was significantly induced in cells treated with VT in the presence of 1000 ng/ml LPS (Fig.4. 8A). A significant increase of TNF-α was also found in VT-treated cells at 10 ng/ml of LPS (Fig. 4.8B). At later time points, 8 and 12 hr, no significant increase of CAT protein was found in cells treated with VT at all LPS concentrations (Fig. 4.9A, 10A). For the 8 hr time point, TNF protein expression was not significantly enhanced at cells treated with VT at all LPS concentrations (Fig. 4.9B). Significant increases of TNF-α were found in VT-treated cells incubated with 0, 10, 100 and 1000 ng/ml of LPS at 12 hr (Fig. 4.10B).

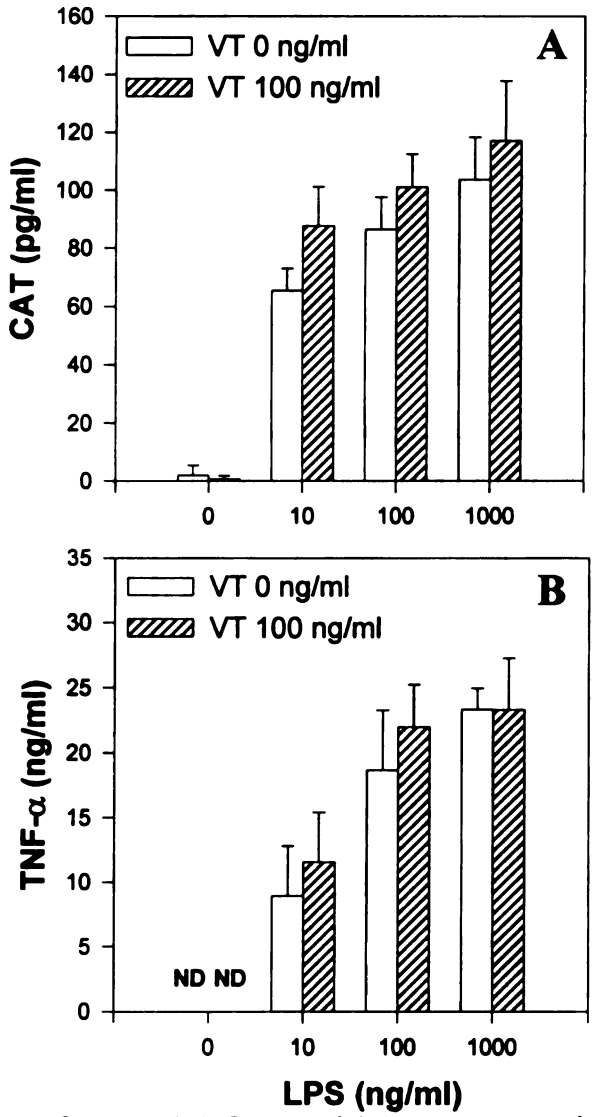


Fig. 4.5. Effect of VT on (A) CAT and (B) TNF-α protein expression in the presence of LPS for 2 hr in synchronous model. Cells were transfected with the pTNF-CAT plasmid construct and then treated with VT (0, 100 ng/ml) and LPS (0, 10, 100 and 1000 ng/ml) simultaneously. Then cells were lysed and protein extract were analyzed by CAT ELISA. For the detection of TNF-α, supernatant from each samples were collected and analyzed by sandwich ELISA.

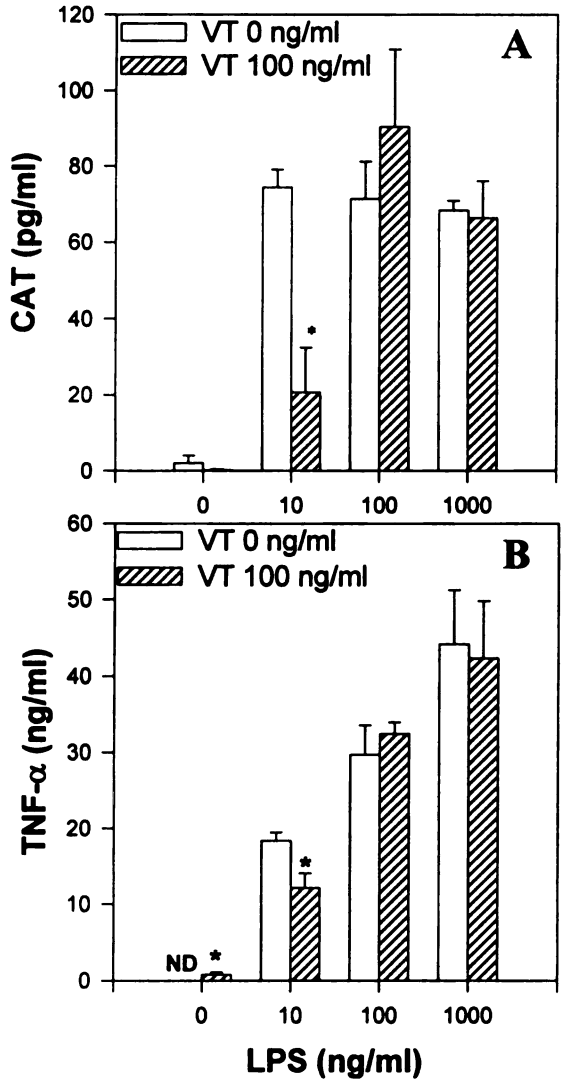


Fig. 4.6. Effect of VT on (A) CAT and (B) TNF-α protein expression in the presence of LPS for 6 hr in the synchronous model. Experiments were performed as described in the legend of Fig.4.5. * means significant different ($P < 0.05$) between the VT-treated group and control group at all LPS concentrations.

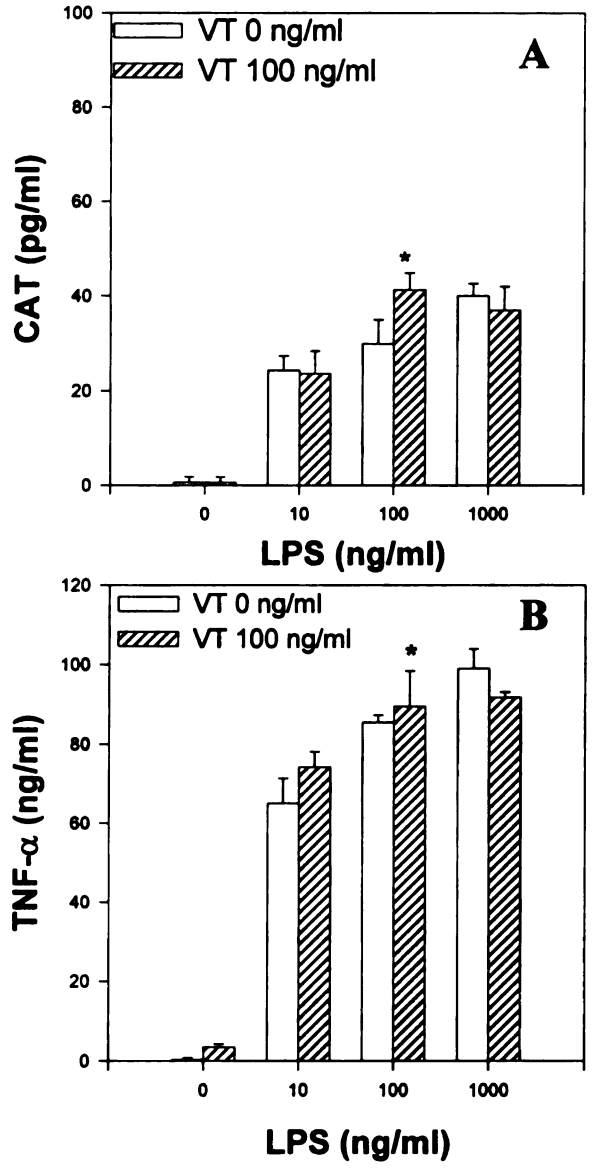


Fig. 4.7. Effect of VT on (A) CAT and (B) TNF-α protein expression in the presence of LPS for 2 hr in the delayed synchronous model. Cells were transfected with pTNF -CAT plasmid construct, prestimulated with LPS(0, 10, 100 and 1000 ng/ml)and then VT (0, 100 ng/ml) were added and incubated for another 2 hr. Then cells were lysed and protein extract were analyzed by CAT ELISA. For the detection of TNF-α, supernatant from each samples were collected and analyzed by sandwich ELISA. * means significant different ($P < 0.05$) between the VT-treated group and control (VT 0) group at all LPS concentrations.

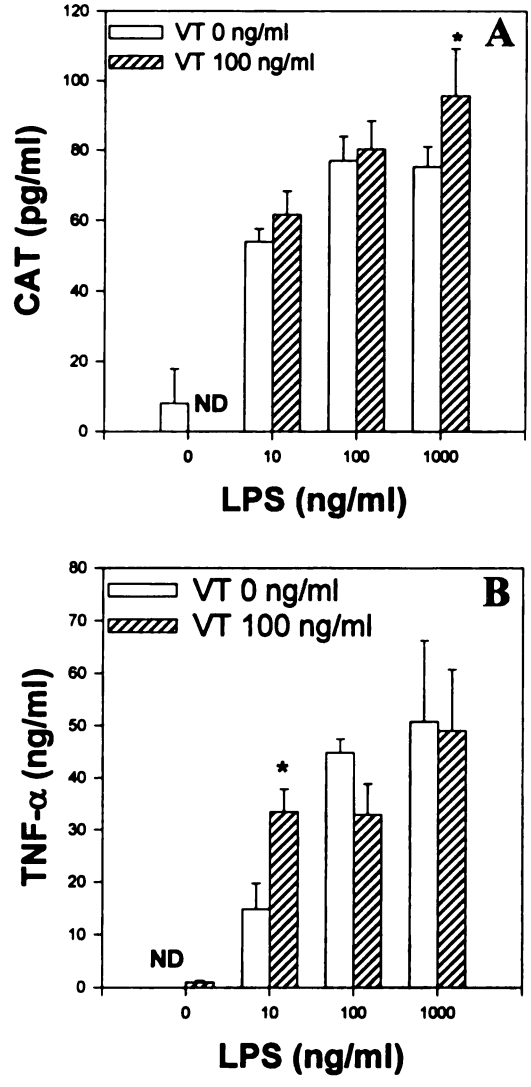


Fig. 4.8. Effect of VT on CAT and (B) TNF-α protein expression in the presence of LPS for 4 hr in delayed synchronous model. Experiments were performed as described in the legend of Fig. 4.7.

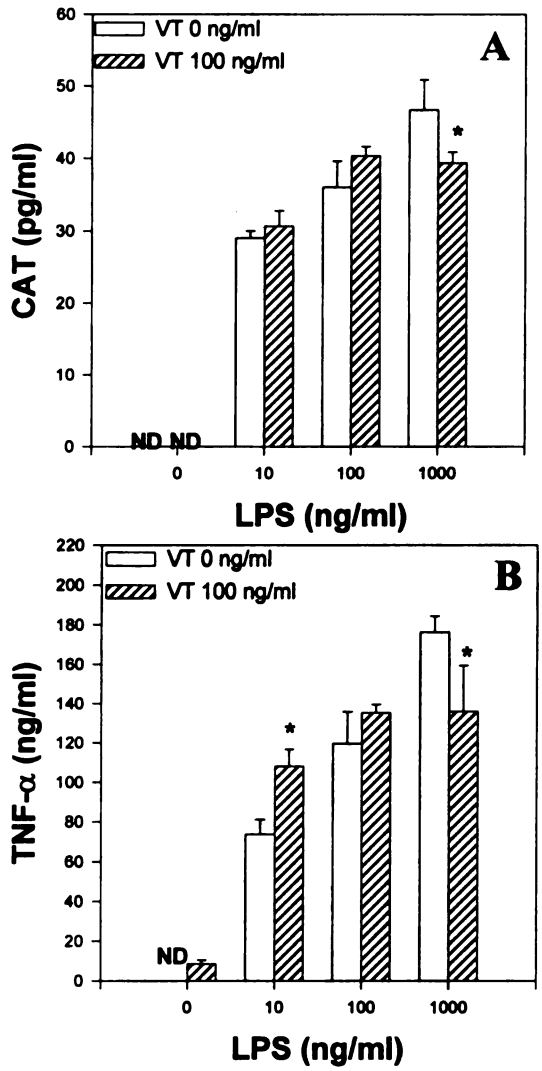


Fig. 4.9. Effect of VT on (A) CAT and (B) TNF-α protein expression in the presence of LPS for 8 hr in delayed synchronous model. Experiments were performed as described in the legend of Fig. 4.7.

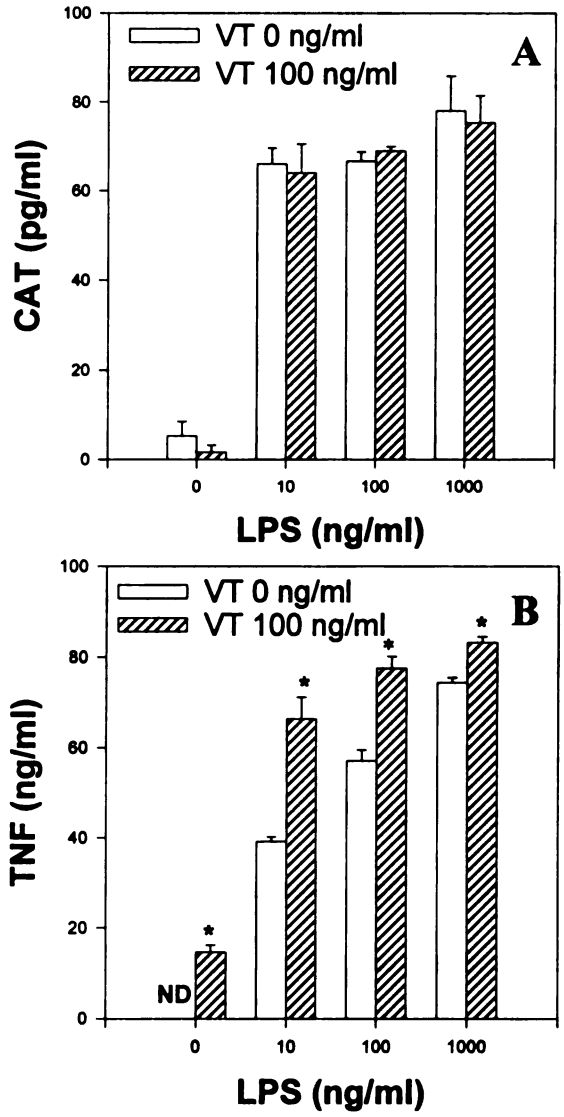


Fig. 4.10. Effect of VT on (A) CAT and (B) TNF-α protein expression in the presence of LPS for 12 hr in delayed synchronous model. Experiments were performed as described in the legend of Fig. 4.7.

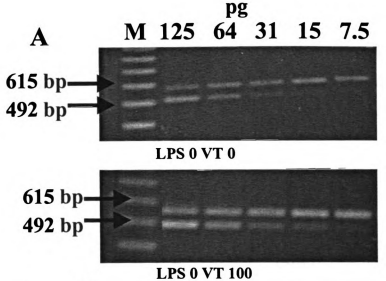
Effects of VT on CAT mRNA expression. The synthesis of the CAT protein might be inhibited by VT, a protein synthesis inhibitor, thus complicating the resultant analysis. Therefore, we further assessed the CAT mRNA by competitive RT-PCR. In the synchronous model, transfected cells were incubated with different concentrations of LPS and VT(0, 100 ng/ml) for 2 hr and then total RNAs were isolated. In this study, two separate experiments were performed at different times (Fig. 4.11A, B). In the first experiment, CAT cDNA was slightly increased in cells treated with VT compared to the control at 0, 10, and 100 ng/ml of LPS (Fig. 4.11C). In the second experiment, the amount of CAT cDNA increased to a larger extent by VT compared with the control (VT 0 ng/ml) (Fig. 4.11C). Approximately 1.3-, 1.8- and 3.2-fold increases were observed at 0, 10 and 100 ng/ml LPS, respectively in the VT-treated cells. No difference was found in the presence of 1000 ng/ml of LPS.

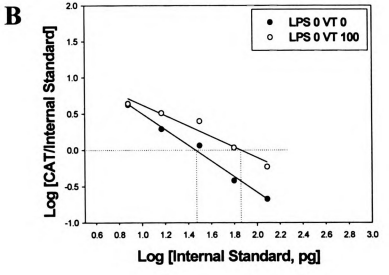
In the delayed synchronous model, cells were pre-stimulated with LPS for 2 hr, and then VT was added and incubated for another 2 hr. In the first experiment, a 1.4- fold of increase was observed with VT-treated cells in the absence of LPS (Fig. 4.12) . When cells were treated with LPS, the increase was not as high as in the 0 ng/ml of LPS. A decrease was observed when cells treated with 1000 ng/ml of LPS. In the second experiment, 2-, 0.2-, and 0.4- fold increases were observed with cells treated with LPS 0, 10, and 100 ng/ml, respectively. VT had no effects on cells treated with 1000 ng/ml of LPS.

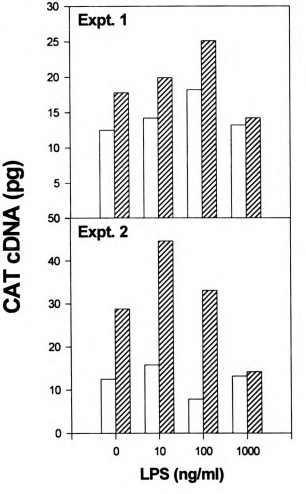
DISCUSSION

Transfection with a plasmid containing a desired promoter has been frequently used

Fig.4.11. Effect of VT on TNF-α transcativation in the synchronous model. Cells were transiently transfected with a pTNF-CAT construct, and LPS and VT were added and incubated for 2 hr. RNA was extracted from each samples and semi-quantitative RT-PCR was performed as described in the Materials and Methods. (A) Ethidium bromide staining of samples; (B) standard curve for representative samples; (C) the CAT cDNA concentration calculated from the standard curve in two separate experiments.


A
pg
M 125 64 31 15 7.5
615 bp
492 bp
LPS 0 VT 0
615 bp
492 bp
LPS 0 VT 100


B
LPS 0 VT 0
LPS 0 VT 100
Log [CAT/Internal Standard]
Log [Internal Standard, pg]


C
Expt. 1
Expt. 2
CAT cDNA (pg)
LPS (ng/ml)

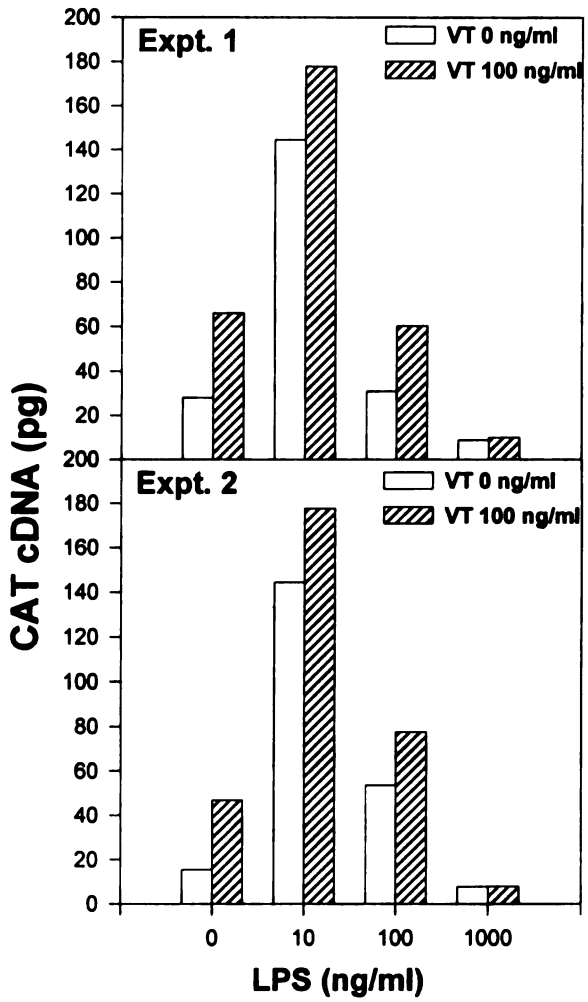


Fig. 4.12. Effect of VT on TNF-α transactivation in the delayed synchronous model. Cells were transiently transfected with pTNF-CAT construct, and pre-stimulated with LPS for 2 hr prior the addition of VT and incubated for another 2 hr. RNA was isolated and semi-quantitative RT-PCR was performed as described in the Materials and Methods.

as a tool for studying transcription in mammalian cells. VT has been shown to enhance binding activities of transcription factors in macrophage RAW 264.7 cells (Chapter 3). The data presented here revealed that transcription of CAT DNA under control of the TNF-α promoter was enhanced, albeit to a small extent, by VT in transiently transfected RAW 264.7 cells. However, protein expression was not affected.

Previously, Economou *et al.* (1989) studied the promoter regions of the TNF-α gene with a recombinant construct containing the human TNF-α promoter linked to a luciferase (Luc) reporter gene using a transient transfection system. Beutler and his colleagues (Han *et al.*, 1990; Bazzoni *et al.*, 1994) also studied the effects of LPS or UV by using plasmid constructs containing the promoters of the TNF-α gene and CAT reporter gene. Recently, Roger *et al.* (1998b) used transient transfection with a construct controlled strictly by its relevant promoter to assess the role of transcription in the superinduction of IL-8. Here, we used CAT as a reporter to determine the response of the TNF transcription because it is stable and non-secreted. The increase of CAT protein after the exposure of the cells to LPS verified the efficacy of the transfection. However, the results of the CAT protein analysis showed that VT could only slightly increase the CAT protein production compared with the control. Since VT is a protein synthesis inhibitor (Rotter *et al.*, 1996), the synthesis of CAT proteins might have been inhibited by VT to a certain extent. Thus CAT proteins might not reflect the actual transcription effects. By using a competitive RT-PCR technique, it should be possible to verify if CAT transcript was increased with VT treatment. Previously, Wong *et al.* (1998) showed that 100 ng/ml of VT was able to enhance TNF-α protein production by 1.5 and 3 fold and TNF mRNA by 3 fold in the

absence of LPS. In the presence of 1000 ng/ml of LPS, 1.5 and 1.8 fold increases of TNF-α protein was observed in VT-treated cells. However, only 0.4 and 1.3 fold of CAT transcripts were enhanced with VT (100 ng/ml) alone in the synchronous model of this study. A negligible increment was found in cells treated with 100 ng/ml of VT and 1000 ng/ml of LPS in the synchronous model (Fig. 10). Then, transcription may play a minor role in the superinduction of the TNF-α protein and mRNA levels.

The increases in binding activities of transcription factors NF-κB and NF-IL6 shown in previous studies may have a role for transcription in cytokine superinduction by VT (Wong *et al.*, unpublished data). The results of this transfection assay showed that increased binding activities of transcription factors might not necessarily increase transcription of the associated genes. Transcriptional control is the result of complex cross-talk between transcriptional activators and basic transcription machinery (Zawel and Reinberg, 1993). Notably, most of the transcription factors are regulated at both transcriptional and post-transcriptional levels (Baeuerle, 1991). Thus, the availability of the transcription factors is a key factor for further transcription of the responsive genes.

Cooperation between each transcription activator in binding is important in activating transcription of many genes and provides chances for diversity in transcription regulation during macrophage activation (LeClair *et al.*, 1992; Ohmori and Hamilton, 1994). Previously, Sanceau *et al.* (1995) showed that triggering of the human IL-6 gene by IFN-γ and TNF-α in monocytic cells required cooperation between transcription factors IRF-1 (interferon regulatory factor-1), NF-κB and SP-1. Also, the presence of transcription factors as negative regulators might interfere with the binding of the positive regulators to

the *cis*-acting elements on the promoters of many genes. *c-Rel*, a subunit of NF-κB, has been shown to act as an activator of TNF-α and inducible nitric oxide synthase (iNOs) genes, but also act as a repressor of GM-CSF, G-CSF and IL-6 genes (Grigoriadis *et al.*, 1996). Negative regulation might impact the transcription of cytokines in the presence of VT.

Alternative effects, such as increased stability of the mRNA, might be involved in the regulation of the cytokine superinduction by VT in macrophage cells. IL-8 mRNA superinduction had been shown in the lung epithelial cells with CHX to be due to the effects of increased mRNA half-life (Roger *et al.*, 1998b). Granulocyte-macrophage colony stimulating factor (GM-CSF) production in both murine EL-4 cells and human blood monocytes was also regulated by modulation of cytoplasmic mRNA half-life (Bickel *et al.*, 1990; Ernst *et al.*, 1989). IL-2 mRNA is stabilized by VT in the EL-4 cells (Li *et al.*, 1997). Therefore, it is reasonable to suggest that stability of mRNA may play a key role in the VT-induced superinduction. The presence of AU rich sequences in the 3' untranslated region (UTR) of mRNA's (Caput *et al.*, 1986) in many genes of transiently-expressed cytokines may comprise a translational regulatory element involved in the control of TNF-α gene expression. This sequence might confer instability and also confer superinducibility with alteration of the access to translation (Shaw and Kamen, 1986; Dofferhoff *et al.*, 1991; Pauli, 1994).

In conclusion, the results of this study showed that transcriptional regulation may play a small role in VT-induced cytokine superinduction. Further experiments on the role of post-transcriptional regulation of VT on TNF-α gene expression will be needed for a better understanding of the mechanisms involved in TNF-α superinduction.

CHAPTER V

STABILIZING EFFECTS OF VOMITOXIN ON CYTOKINE mRNA HALF LIFE IN MURINE RAW 264.7 MACROPHAGE CELLS

ABSTRACT

Vomitoxin (VT), a trichothecene, superinduces cytokine gene expression in the RAW 264.7 macrophage cell line. To better understand the underlying molecular mechanisms for this observation, post-transcriptional effects of VT on both TNF-α and IL-6 gene expression were studied in lipopolysaccharide (LPS)-stimulated macrophage RAW cells. ELISA revealed that VT could enhance both TNF-α and IL-6 protein secretion in the presence of LPS. With the addition of the transcriptional inhibitor, 5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole (DRB), secretion of both cytokine proteins was inhibited. Using Northern analysis, the mRNA stabilities of TNF-α and IL-6 were studied in DRB-treated RAW 264.7 cells exposed to VT and LPS in both asynchronous and delayed synchronous fashions. In the asynchronous model, cells were pre-treated with LPS for 2 hr, medium removed and medium containing DRB and VT then added. In the delayed synchronous model, cells were pretreated with LPS for 2 hr and then DRB and VT added. TNF-α and IL-6 mRNA were rapidly stabilized by VT (100 and 250 ng/ml) in both asynchronous and delayed synchronous models. In the asynchronous model, TNF-α mRNA half-life (t1/2) was 25 min and stabilized by both 100 and 250 ng/ml of VT to half-lives greater than 3 hr; VT also extended half-life of IL-6 mRNA from 60 min to greater than 3 hr with 100 and 250 ng/ml of VT. In the delayed synchronous model, the t1/2 for TNF-α mRNA of 1.3 hr was extended to half-lives greater than 3 hr with 100 ng/ml and 250 ng/ml of VT. The half-life for IL-6 mRNA level in this model was also prolonged by VT, from 90 min to > 3 hr at concentration of 100 and 250 ng/ml. The results suggest that the

post-transcriptional control via enhancement of mRNA stability is likely to contribute to cytokine superinduction in macrophages by VT and other trichothecenes.

INTRODUCTION

The trichothecene mycotoxin VT is a secondary metabolite produced by *Fusarium graminearum* and *F. culmarum* during growth on agricultural staples such as wheat and corn (Vesonder *et al.*, 1973; Vesonder and Hesseltine, 1980). Trichothecenes can either be immunosuppressive or immunostimulatory depending on the dose and timing of exposure (Pestka and Bondy, 1994). Previous studies have shown that *in vivo* trichothecene exposure inhibits DNA and protein synthesis in bone marrow, lymph nodes, spleen and thymus (Rosenstein and Larfarge-Frayssinet, 1983; Azcona-Olivera *et al.*, 1995a; Rotter *et al.*, 1996). *In vitro* exposure to VT also depresses mitogen-induced proliferation of the murine lympohocytes (Robbana-Barnat *et al.*, 1988; Tryphonas *et al.*, 1986). In contrast, both single and multiple gavage of mice with trichothecene VT have been shown to increase resistance to the mastitis pathogen *Mycobacterium* and to *Staphylococcus* (Atroshi *et al.*, 1994). Dong and Pestka (1993)demonstrated that the dietary exposure of mice to VT elevated serum IgA, circulating IgA immune complexes and mesangial IgA deposition in the kidney. These latter features are highly analogous to the human IgA nephropathy (D'Amico, 1987).

VT may enhance immune function by upregulating cytokine production. Increased mRNAs of interleukin-2, -4, -5 and/or IL-6 and their secreted proteins have been observed upon the exposure to VT or cycloheximide (CHX) in T cells (Warner *et al.*, 1994; Ouyang

et al., 1996). Superinduction of inflammatory cytokines such as IL-1 has been observed in peritoneal macrophages treated with VT (Miller and Atkinson, 1986). Increased IL-6 and TNF-α mRNA and proteins have also been found in RAW 264.7 macrophage cells treated with VT (Wong *et al.*, 1998). Transient increases of mRNAs for cytokines IL-1β, IL-6, and TNF-α associated with macrophages have been found in mice exposed to VT orally within 2 hr (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997). Notably, because IL-6 drives IgA production when added to the Peyer's patch B cell cultures (Yan *et al.*, 1997), its upregulation by VT may provide help for B cells to over-produce IgA driving VT-induced IgA nephropathy.

Although the exact mechanisms of the superinduction of macrophage cytokine gene expression by VT are not fully understood, transcriptional regulation may be contributory (Lu-Kuo *et al.*, 1996; Roger *et al.*, 1998a). In support of this contention, increased binding activities of transcription factors NF-κB and NF-IL6 by VT have been demonstrated in macrophage RAW 264.7 cells (Wong *et al.*, chapter III). However, the results of transfection assays with a TNF promoter -CAT construct indicated that the transcription of the reporter was not increased dramatically. Interestingly, post-transcriptional control has also been previously implicated as an important in upregulation in the cytokine induction through protein synthesis inhibitors. For example, in the presence of a transcription inhibitor, actinomycin D (Act-D), CHX increases the half-life of TNF-α mRNA in macrophages (Celada *et al.*, 1989; Taffet *et al.*, 1989; Zuckerman *et al.*, 1991). Upon the removal of this protein synthesis inhibitor, cytokine mRNA degradation seems to resume quickly (Fort *et al.*, 1987). Roger *et al.* (1998a) has also shown that the increased mRNA

half-life contributes to IL-6 superinduction by CHX in lung epithelial cells. It was hypothesized that stabilization of cytokine mRNAs might play a key role in the proinflammatory cytokine superinduction by VT in macrophage cells. The objective of this study was to assess the effects of VT on cytokine mRNA half-life in the presence of a transcription inhibitor, 5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole (DRB). The results showed that VT can stabilize both TNF-α and IL-6 mRNA in macrophage RAW 264.7 cells.

MATERIALS AND METHODS

RAW 264.7 cell culture. RAW 264.7 cells (TIB 77, American Type Culture Collection, Rockveille, MD) (2.5 x 10^5 cells/ml) were plated on 100-mm diameter tissue culture dishes (Corning, NY) in Dulbecco's modified Eagle medium (DMEM; Sigma Co., St. Louis, MO) supplemented with 10%(v/v) fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 1% (v/v) NCTC 135 (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL) , 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in a 5-8% CO_2 incubator at 37 ° C. Two exposure models were used. An *asynchronous* model was employed in which cells were pre-stimulated with 1 μg/ml of LPS (*Salmonella typhiumurium*; Sigma; stimulation index: 110.7) for 2 hr and the supernatant was removed. Then, VT (0 to 250 ng/ml) was added in the presence and absence of transcriptional inhibitor, 5,6-dichloro-1-beta-D-furanosyl benzimidine (DRB, Fluka Co., Madison, WI), for various time intervals. A *delayed* synchronous model was also used. Whereby, cells were preincubated with 1 μg/ml LPS for 2 hr and VT and DRB were added and incubated

for appropriate time intervals.

Cytokine assays. IL-6 and TNF-α concentrations were measured by ELISA as described by Dong *et al* (1994). Briefly, Immunolon IV Removawell microtiter strips (Dynatech Laboratories., Chantilly, VA) were coated with 50 µl/well of monoclonal rat anti-mouse IL-6 or TNF-α antibody (Pharmingen, San Diego, CA) diluted to 1 µg/ml in phosphate buffered saline (PBS; pH 7.2) and incubated at 4°C overnight. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (Sigma) (PBS-T). To reduce non-specific protein binding, wells were blocked with 300 µl PBS containing 1% (w/v) bovine serum albumin (BSA), incubated for 30 min at 37°C and then washed four times with PBS-T. Reference IL-6 or TNF-α and samples were diluted in DMEM with 10% (v/v) FBS, and 50 µl was added to appropriate wells. Plates were incubated for 60 min at 37 °C and then washed four more times with PBS-T. Biotinylated IL-6 or TNF-α antibody (Pharmingen), diluted to 1 µg/ml in PBS containing 1% (w/v) BSA, were added to each well and the plate was incubated at room temperature for 60 min. Plates were then washed six times with PBS-T and 50 µl streptavidin hoseradish peroxidase (Sigma), diluted 1:1000 in PBS with 1% (w/v) BSA, was added to each well and incubated at room temperature for 60 min. ELISA plates were washed eight times with PBS-T. Substrate (100 µl) consisting of 3', 3', 5', 5'- tetramethyl benzidine (0.1 mg/ml; Fluka Chemical Corp., Ronkonkoma, NY) in 0.1 M citric-phosphate buffer (pH 5.5) and 0.003% (w/v) hydrogen peroxide (H_2O_2) was added to each well and incubated for 20 minutes at room temperature. The reaction was terminated with 100 µl of 6 N sulfuric acid (H_2SO_4). Absorbance was measured at 450 nm with Vmax kinetic microplate Reader (Molecular Devices, Menlo Park, CA) and interleukin

concentration was quantified using the Softmax software (Molecular Devices).

cDNA probe preparation. Plasmids encoding IL-6, TNF-α and GAPDH were kindly supplied by Dr. Richard Schwartz (Michigan State University). The plasmids were transformed into *E.coli* DH-5α strain by the heat shock method (Sambrook *et al.*, 1989). Briefly, 5 μl of DNA were added to 100 μl DH-5α competent cells and these were kept on ice for 30 min. Then the mixture was transferred to 42 °C for 2 min and 0.9 ml of the SOC medium were added. Bacterial cells were spread on agar plates for inoculation. The transformants were inoculated in flasks containing 250-ml LB(Laurie Bertani) medium and plasmids encoding the cDNAs were purified by using the Qiagen Maxi kit (Qiagen, Valencia, CA). After purification, plasmids were digested with restriction enzymes, *Pst I* and *Bam HI* (for 1.3 kb of TNF-α probe); *Pst I* (for 0.65 kb of IL-6 probe); and *Pst I* (for 1 kb of GAPDH probe). The digested products were run on a 1% agarose gel and purified by using the Genelute spin column (Sigma). Collected cDNAs were used as probes in the further experiments. The housekeeping gene GAPDH cDNA was used as a positive control.

Northern blotting and mRNA stability analysis. Total cellular RNA was extracted from RAW 264.7 cells by the modification of the single-step method (Chomczynski and Sacchi, 1987) using RNA-Stat-60 isolation reagent (Tel-test "B" inc., Friendswood, TX). Total RNA (10 μg) was separated by electrophoresis on a 1.5% (wt/vol) agarose, 0.6 M formaldehyde (Sigma), 0.02 M MOPS (Sigma) gel at 80 Volt for 3 hr. The gel was stained with ethidium bromide to verify for the integrity and equal RNA loading, and the RNA was transferred to a nitrocellulose membrane (Nytran, Schleicher & Schuell Inc., Keene, NH) with 20X SSC according to Sambrook *et al.* (1989). ^{32}P-labeled cDNA

probes were prepared by nick translation using the random priming kit (Boehringer Manheim, Indianapolis, IN). After auto-cross-linking for few minutes, the membrane was pre-hybridized at 42 °C in Denhardt's hybridization buffer (Kingston, 1997) for 2 hr. Blots were hybridized at the same temperature overnight in a hybridization incubator (Robbins Scientific, Sunnyvale, CA). The hybridized membrane then was washed with SSPE buffer and exposed to Amersham hyperfilm MP(Amersham, Arlington Heights, IL) with an intensifying screen at -80 °C for variable time periods depending on the signal intensity. Blots were scanned with the Image Quantity (Molecular Dynamics) and the intensities of the signals were analyzed. Relative mRNA levels were determined by the normalization of the signal intensity of TNF-α and IL-6 mRNA against GAPDH mRNA intensity.

Statistical analysis. A one way analysis of variance (ANOVA) using Student-Newman-Keuls tests and Dunnett's test or Kruskal Wallis ANOVA on ranks was applied to parametric data and non-parametric data with application of Sigma-Stat Analysis System (Jandel Scientific, San Rafael, CA). A p value of less than 0.05 was considered statistically significant.

RESULTS

Effects of DRB and VT on cytokine protein expression. An asynchronous model was used whereby RAW 264.7 cells were pre-stimulated with 1 μg/ml of LPS for 2 hr prior to the addition of the transcription inhibitor, DRB, and VT (0, 100 and 250 ng/ml) in fresh DMEM medium for 2, 4, 8 and 24 hr. Transcription inhibitors such as actinomycin D (Act-D) and immunosupressive drugs such as cyclosporin A (CsA) and dexamethasone effectively

inhibit both IL-6 and TNF-α expression in macrophages, fibroblasts, and mast cells (Williams and Coleman, 1995; Lu-Kuo *et al.*, 1996; Witowski *et al.*, 1996; Nguyen *et al.*, 1990). DRB, an inhibitor for RNA polymerase during RNA synthesis (Chodosh *et al.*, 1989), has been used frequently to inhibit transcription of cytokine mRNAs including those for interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bickel *et al.*, 1990). In preliminaries study we found that neither Act-D or CsA profoundly inhibited transcription of TNF-α or IL-6 proteins (data not shown). However, DRB effectively blocked cytokine transcription and was not as cytotoxic as Act-D. In the absence of DRB, a significant increase of TNF-α protein was observed with 100 ng/ml of VT at 2, 4, 8 and 24 hr. TNF-α protein production was completely suppressed by DRB (Fig. 5.1) at all the time points. In the absence of DRB, IL-6 protein expression of was also enhanced by addition of VT (Fig. 5.2). DRB significantly inhibited IL-6 protein production at all time points. DRB was subsequently used as the transcriptional inhibitor in studying the mRNA stability.

Effects of VT on TNF-α and IL-6 mRNA stability in asynchronous model. In the asynchronous model, cells were treated with 1 μg/ml of LPS for 2 hr and the medium was removed. Cultures were washed by pipetting and fresh 15 ml DMEM medium containing 100 μM DRB and VT (100 and 250 ng/ml) or medium alone were added, cells were incubated for different time intervals to monitor the cytokine mRNA expression by Northern analysis. In the absence of VT, TNF-α mRNA levels were decreased by the addition of DRB at all time-points tested (30, 90, 180 and 300 min) (Fig. 5. 3A,B). Degradation of mRNA in the VT-treated cells was less than in untreated cells with mRNA levels remaining

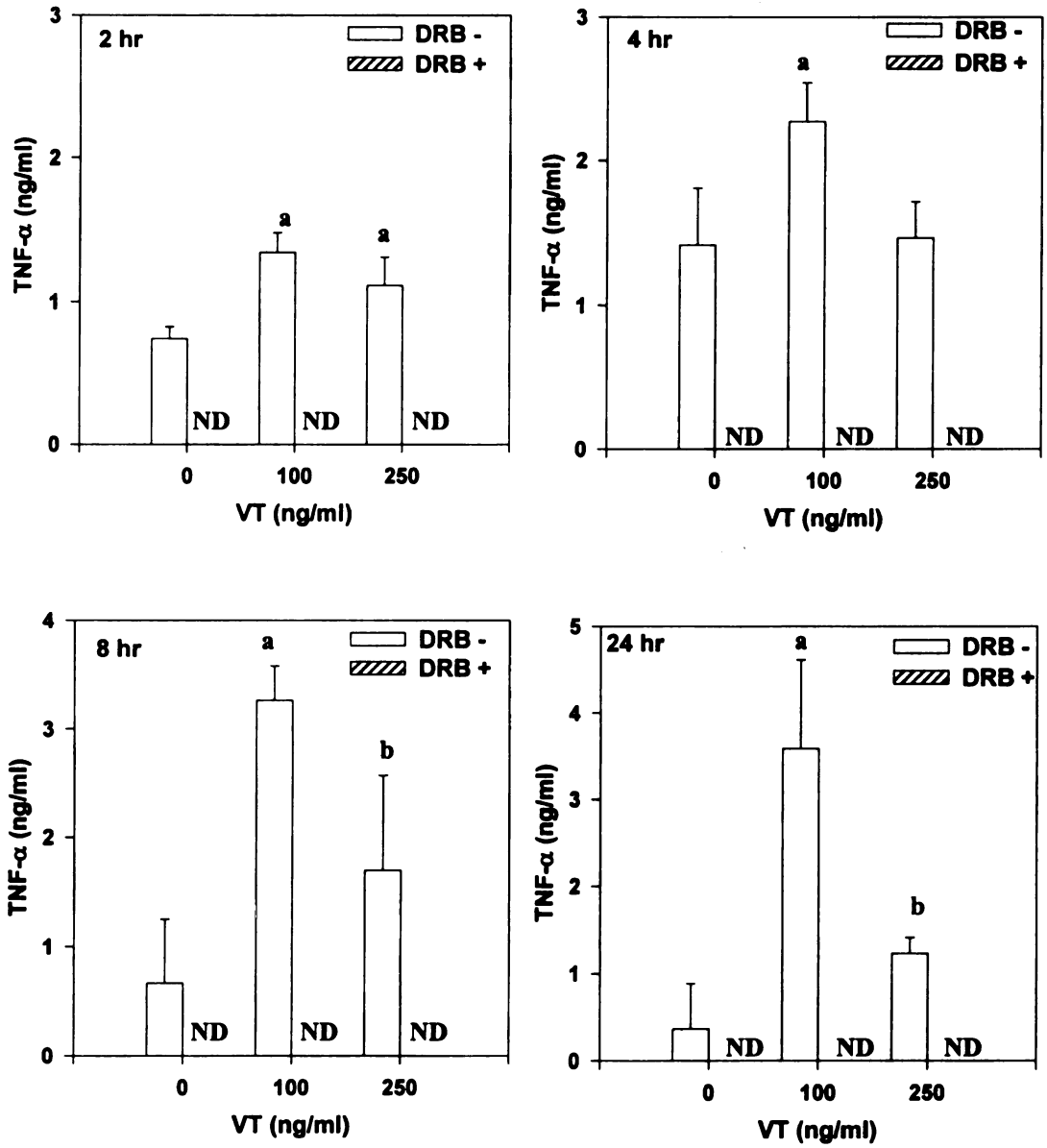


Fig.5.1. Effect of VT on TNF-α production in LPS-treated RAW 264.7 cells. Cells were pre-treated with 1 μg/ml LPS for 2 hr prior to the addition of VT (0, 100 and 250 ng/ml) in the absence or presence of 100 μM DRB for further incubations. "**a**" denotes significant difference from control (V0) in the absence of DRB "**b**" denotes significant difference between each other of different VT treated group in the absence of DRB. Data are representative of two separate experiments.

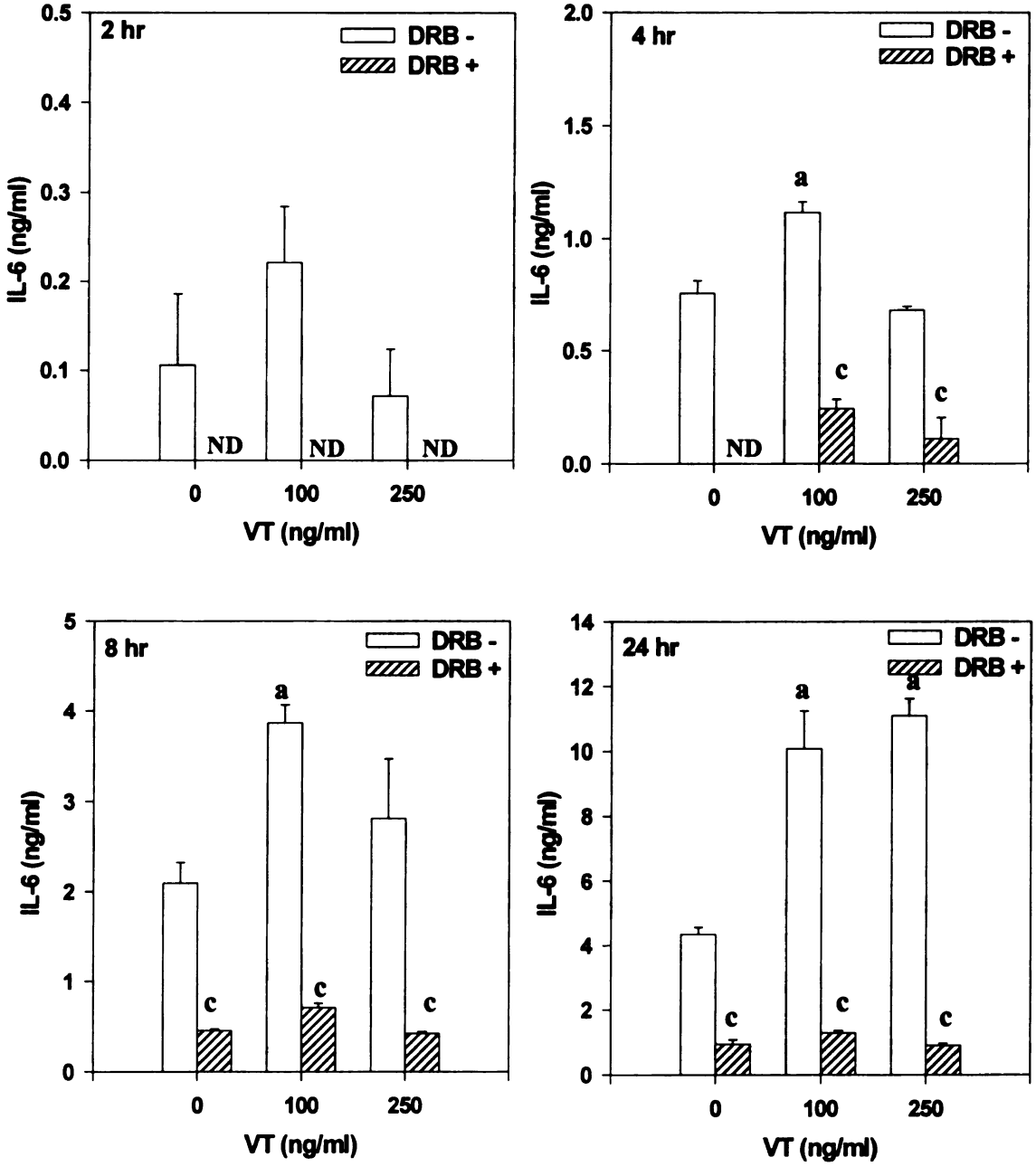


Fig.5.2. Effect of VT on IL-6 protein production in LPS-treated RAW 264.7 cells. Experiments were performed as described in the legend of Fig.5.1. Data are representative of two separate experiments. "**a**" denotes significant difference from control (V0) in the absence of DRB; "**c**" denotes significant difference between the DRB-treated group and the control group (DRB 0) in all VT concentrations.

relatively constant until 180 min. After 300 min, a slight decrease in TNF-α mRNA level was evident in VT-treated cells but the mRNA level of the treatment group was still higher than the control group (0 ng/ml of VT). At the 300 time period, the GAPDH mRNA level was not constant but depleted immediately at all the samples. Therefore, the data could not be used for the calculation of half-life as the GAPDH expression at this time point seemed to be affected by the inhibitor to a broader range. The TNF-α half-life (t1/2) for the control group (0 ng/ml VT) was calculated as 25 min in the asynchronous model. The half-life was longer than 180 min (>180 min) with the addition of the 100 and 250 ng/ml of VT.

In the asynchronous model, control IL-6 mRNA level increased over time upon the removal of LPS and in the absence of DRB (Fig. 5.3A, C). With the addition of DRB, the IL-6 mRNAs were depleted as early as 30 min in the absence of VT treatment. In contrast, the IL-6 mRNA levels were stabilized by the addition of VT at all the time periods (30 to 300 min). The GAPDH mRNA level in the asynchronous model remained constant at all VT concentrations except at 300 min. For the IL-6 half-life study, 100 and 250 ng/ml of VT was able to extend the mRNA half-life from 60 min to greater than 3 hr.

Effects of VT on TNF-α and IL-6 mRNA stability in delayed synchronous model. In the delayed synchronous model, cells were pre-treated with 1 μg/ml of LPS for 2 hr prior to the addition of VT (100 and 250 ng/ml)or vehicle alone and 100 μM DRB. Cells were incubated and TNF-α and IL-6 mRNA were monitored over 300 min. Again, DRB depressed TNF-α mRNA expression at 30, 90, 180 and 300 min (Fig. 5. 4A, B). TNF-α mRNA levels in DRB-treated cells were higher in the presence of both 100 and 250 ng/ml VT compared to the controls at 30, 90, 180 and 300 min. TNF-α half-life of the untreated

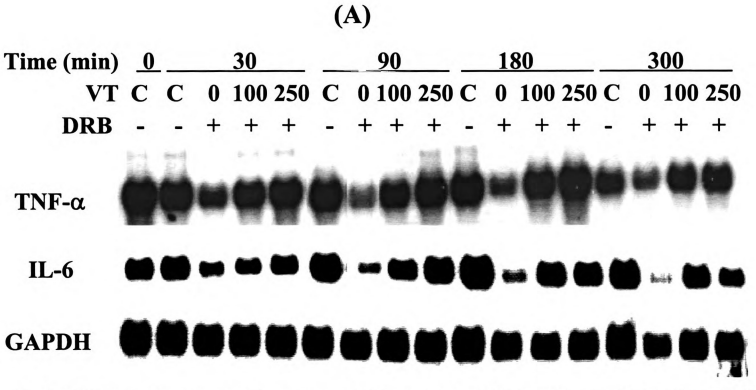


Fig. 5.3 (A)Effects of asynchronous VT-exposure on TNF-α and IL-6 mRNA expression in LPS-treated RAW 264.7 cells. Cells were pre-treated with 1 μg/ml LPS for 2 hr and the medium was then removed. Fresh medium containing VT (0,100 and 250 ng/ml) and/or DRB (100 μM) was added for further incubation of different time periods as indicated in the figure. The house keeping gene GAPDH was used as an internal control. Data are representative of two separate experiments.

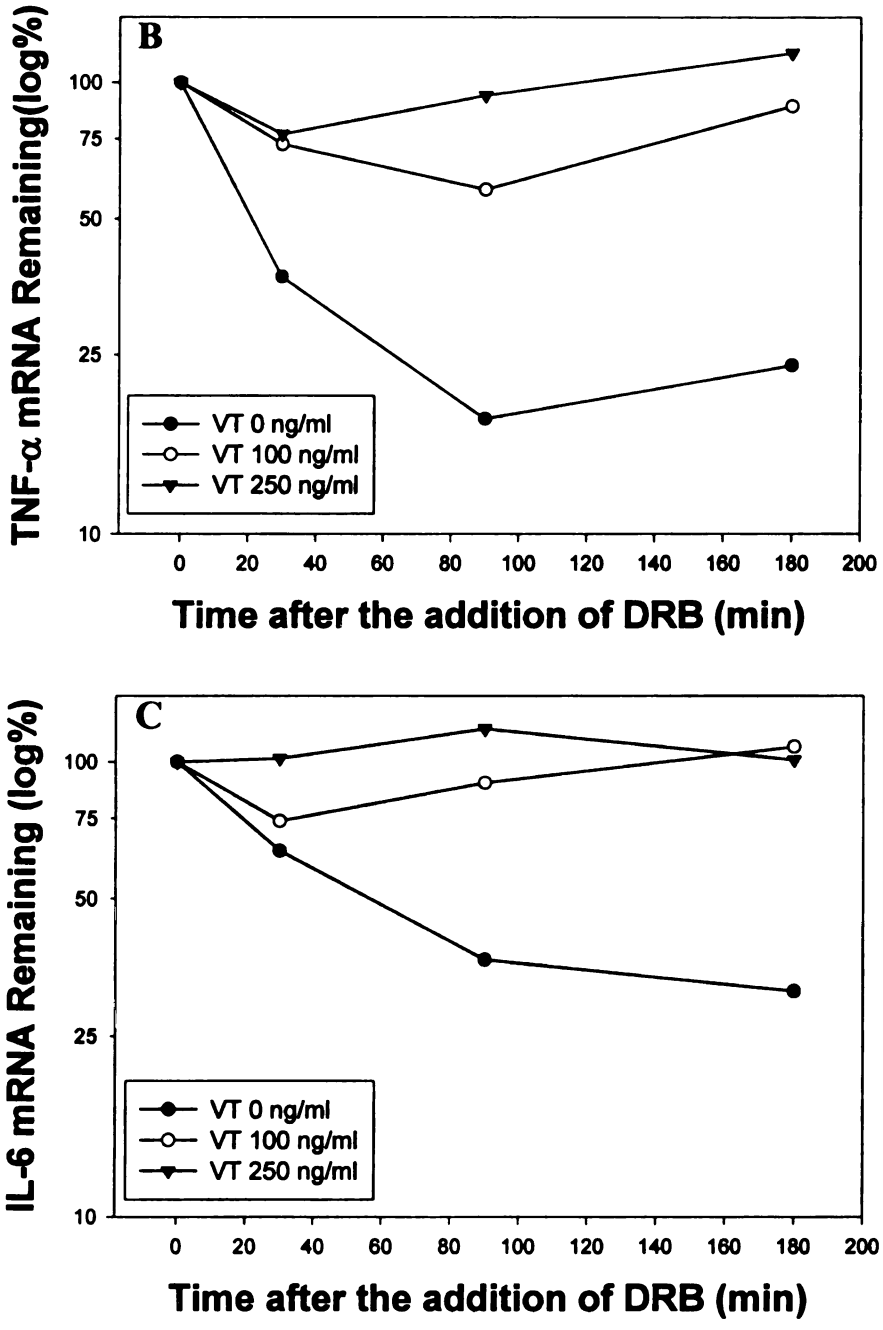


Fig.5.3 (B) mRNA stability for TNF-α in an asynchronous model; (C) mRNA stability for IL-6 in an asynchronous model.

group was 1.3 hr, and it was extended beyond 180 min by both 100 and 250 ng/ml of VT. DRB also depressed IL-6 mRNA expression(Fig. 5. 5A, B). However, IL-6 mRNA levels were higher in the VT-treated cells than in the control group at 30, 90 and 180 min time periods with DRB. IL-6 mRNA half-life was 90 min in the absence of VT in this model but was extended to longer than 3 hr in the presence of 100 and 250 ng/ml of VT.

DISCUSSION

Superinduction of cytokines has been implicated to have a major contribution in toxicological and immunological pathogenesis caused by VT, such as IgA deposition in the kidneys of mice (Dong *et al.*, 1994). Cytokine superinduction has previously been observed in macrophage RAW 264.7 cells treated with VT (Wong *et al.*, 1998). In the present study, VT was found to increase both TNF-α and IL-6 mRNA half-life in this *in vitro* model. These data suggest that increased cytokine mRNA stability might be responsible for the superinduction of TNF-α and IL-6 gene expression.

The asynchronous model was employed here to study the stabilizing effects of VT without interference from continual stimulation of LPS. The results suggested that VT alone can stabilize both TNF-α and IL-6 mRNA level in this model. These result are consistent with my previous study (Wong *et al.*, 1998), in which both TNF-α and IL-6 mRNA were markedly increased with VT in RAW 264.7 cells. To mimic *in vivo* experiments, a delayed synchronous model was also employed in this study. When LPS was retained in cell cultures during treatment of DRB and VT, stronger induction in the TNF-α mRNA expression was observed compared to the asynchronous model. Nevertheless, VT

Fig.5.4 (A) Effects of delayed synchronous VT-exposure on TNF-α mRNA expression in LPS-treated RAW 264.7 cells. Cells were pre-treated with 1 μg LPS for 2 hr prior to the addition of VT (0, 100 and 250 ng/ml) and/or DRB (100 μM). GAPDH gene was used as an internal control. (B) mRNA stability for TNF-α mRNA in a delayed synchronous model. Data are representative of two separate experiments.

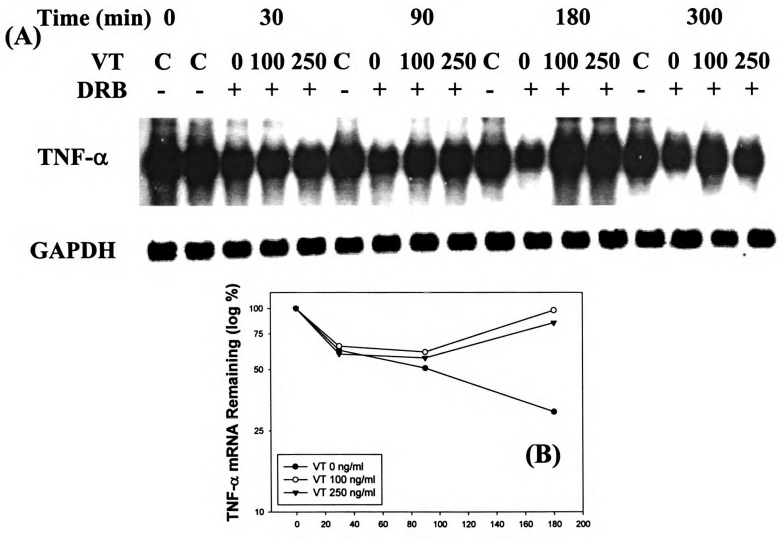
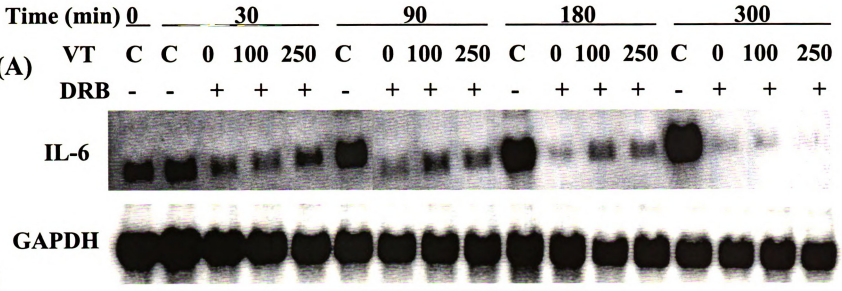

(A)
Time (min) 0 30 90 180 300
VT C C 0 100 250 C 0 100 250 C 0 100 250 C 0 100 250
DRB - - + + + - + + + - + + + - + + +
TNF-α
GAPDH
(B)
TNF-α mRNA Remaining (log %)
100
75
50
25
10
VT 0 ng/ml
VT 100 ng/ml
VT 250 ng/ml
0 20 40 60 80 100 120 140 160 180 200
Time after the addition of DRB (min)

Fig.5.5 (A) Effects of delayed synchronous VT-exposure on IL-6 mRNA expression in LPS-treated RAW 264.7 cells. (B) mRNA stability for IL-6 mRNA in a delayed synchronous model. Experiments were performed as described in the legend of Fig.5.4. Data are representative of three separate experiments.

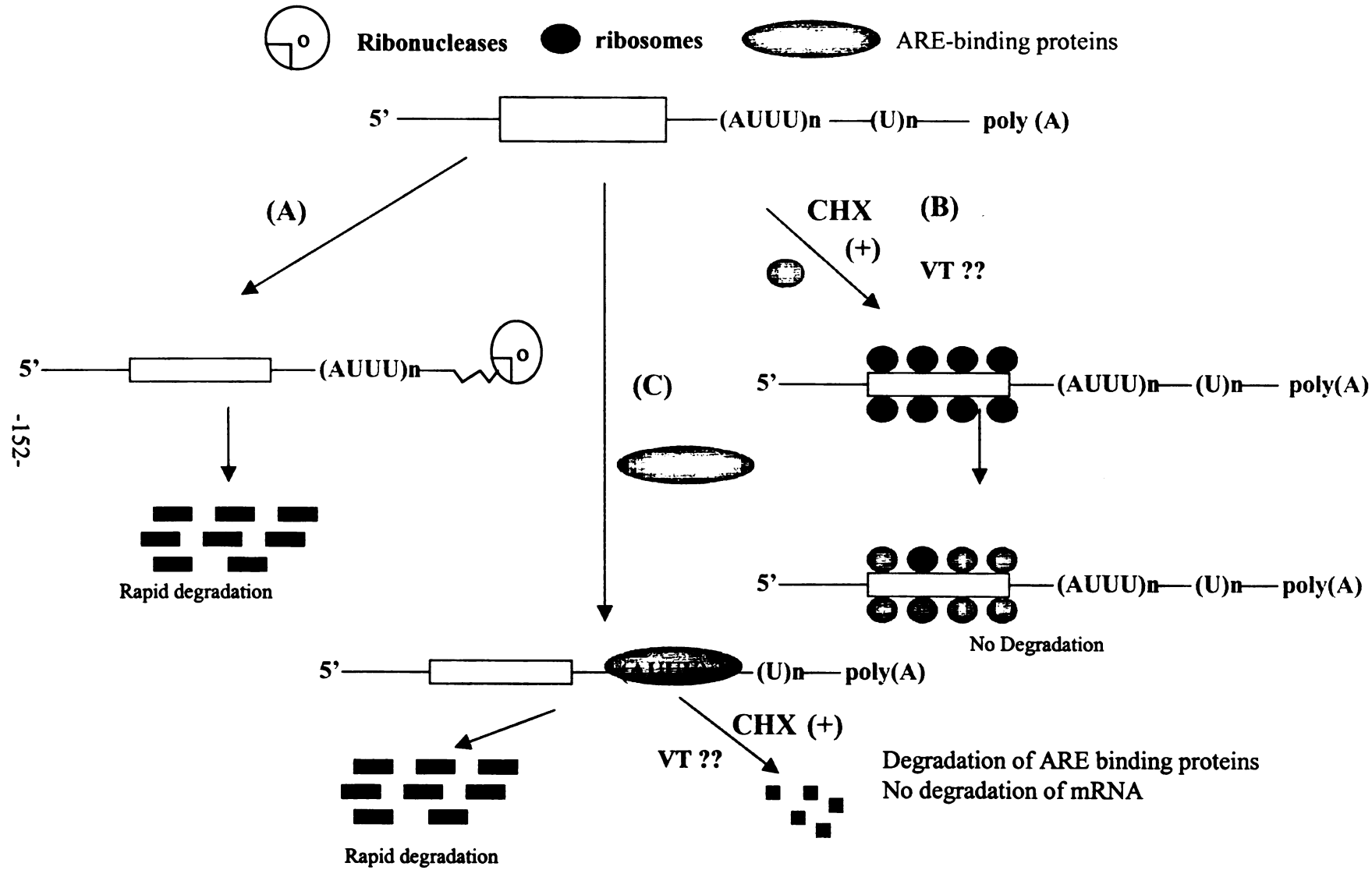

Time (min) 0
30
90
180
300
(A)
VT C C 0 100 250 C 0 100 250 C 0 100 250 C 0 100 250
DRB - - + + + - + + + - + + + - + + +
IL-6
GAPDH
B
IL-6 mRNA Remaining (log%)
100
75
50
25
10
VT 0 ng/ml
VT 100 ng/ml
VT 250 ng/ml
0 20 40 60 80 100 120 140 160 180 200
Time after the addition of DRB (min)

again prolonged the half-life of TNF-α and IL-6 mRNA.

CHX, another protein synthesis inhibitor, has been found to superinduce cytokine mRNA expression by modulating the mRNA degradation system (Zuckerman *et al.*, 1991; Roger *et al.*, 1998a, b). Genes encoding inflammatory cytokines such as TNF-α and IL-6, contain several copies of an AU-rich sequence elements (ARE) in their 3'-untranslated region (UTR) (Caput *et al.*, 1986; Chen and Shyu, 1995). The AU rich motif has been suggested to play an important role in destabilizing cytokine mRNA (Fig. 5.6A) (Shaw and Kamen, 1986; Pauli, 1994; Day and Tuite, 1998) and this is the *cis* determinants of cytoplasmic mRNA stability. Insertion of this sequence into the UTRs of genes with stable mRNAs causes instability to the mRNAs (Shaw and Kamen, 1986). In contrast, deletion of this sequence from the UTRs of short-lived genes such as GM-CSF enhances their stability. Thus, in a similar mechanism, VT may prolong the half-lives of these cytokines by protecting the ARE motifs from degradation by endonucleases or exonucleases (Ross, 1996; Ross, 1997). It has been shown that CHX stabilizes mRNA by causing ribosomes to "freeze" or "cluster" on the mRNA, and ribosomes shield the mRNA from cleavage by ribonucleases in the cytoplasm (Fig.5.6B) (Vazquez, 1979; Ross, 1995). It is suspected that VT, which is also a translational inhibitor, may stabilize mRNA via a similar mechanism.

VT could also stabilize cytokine mRNA via a "*trans*" effect. In the *trans* pathway, a labile protein which is required for degradation of mRNA is inactivated or its synthesis inhibited by the translational inhibitor (Fig. 5. 6C). Examples of proteins that recognize the ARE sequence include AU-A, AU-B, AU-C, and hnRNP (heterogeneous nuclear ribo-nucleoprotein)or also called as AUF1 (Bohjanen *et al.*, 1991; Chen and Shyu, 1995; Ross,

Fig.5.6. (A) The AU-rich elements on the 3'-untranslated region of cytokine gene. The genes with this elements are targets of the ribonucleases; (B) With the addition of the translational inhibitors such as cycloheximide (CHX), ribosomes are freezed on the gene; (C) Many proteins recognize the AU-rich elements, they are called ARE-binding proteins.


Ribonucleases
ribosomes
ARE-binding proteins
5'
(AUUU)n
(U)n
poly (A)
(A)
CHX
(+)
(B)
VT ??
5'
(AUUU)n
(C)
5'
(AUUU)n
(U)n
poly(A)
Rapid degradation
5'
(AUUU)n
(U)n
poly(A)
No Degradation
5'
(U)n
poly(A)
CHX (+)
VT ??
Degradation of ARE binding proteins
No degradation of mRNA
Rapid degradation

1995). AU-A is mostly abundant in nuclear extracts and shuttles between nucleus and cytoplasm in the absence of RNA polymerase II transcription (Katz *et al.*, 1994). AU-A was found to induce stabilization of c-*myc* mRNA *in vitro* by binding to the AU-rich sequence (Ross, 1996). In contrast, the protein AUF1 enhances mRNA degradation by binding to the ARE site in an *in vitro* assay (DeMaria and Brewer, 1996; Kiledjian *et al.*, 1997). AU-B, another destabilization protein, is a cytoplasmic factor that binds ARE in the 3' UTR of cytokine mRNAs and causes degradation of their mRNAs (Bohjanen *et al.*, 1991). The induction of this protein requires protein synthesis. With translational inhibitors, such as cycloheximide, the stabilization of the mRNAs can be achieved as it inactivates the translation of these critical degradation factors such as AU-B and AUF1. It is suspected that VT might act similarly in stabilizing the cytokine mRNA levels.

Interestingly, both transcription and translation rates decline during mitosis (Ross, 1997). During this period, labile mRNAs are stabilized before entering into the G1 phase. These labile mRNAs encode labile proteins which are essential for cell survival. From these observations, it seems that mRNA stabilization is a process important for cell viability or survival during mitosis. Therefore, mRNA stabilization in the cells treated with VT may be also vital for the cell survival and preventing them from apoptosis.

Meanwhile, it has been shown that with the addition of transcriptional inhibitors, Act-D or DRB, the AU-rich binding proteins (AUBPs) may be transported from the nucleus to the cytoplasm and lead to and increase in the cytopalsmic level of these proteins (Fig. 5.6D) (Chen *et al.*, 1995). This may result in a disturbance of other AUBPs (such as proteins for degradation) and change the equilibrium between these proteins and AREs.

Ultimately, this will lead to the disruption of the ARE-protein complexes needed for ARE-induced mRNA degradation (Chen *et al.*, 1995; Sirenko *et al.*, 1997). Thus, inhibition of transcription is also able to stabilize mRNA. Therefore, the choice of transcription inhibitors is critical in mRNA stability study. In this study, DRB seems to have more inhibition effect rather than the stabilization effect.

Earlier in this thesis, it was shown that transcriptional regulation may contribute a small positive effect on the cytokine superinduction in macrophage cells (Wong *et al.*, Chapter IV). In this study, stabilization of cytokine mRNA was found to possibly play a more major role in the cytokine superinduction by VT in RAW macrophage cells. These findings are consistent with the observations in EL-4 cells that VT not only induces NF-κB and AP-1 binding activity but can stabilize the IL-2 mRNA (Ouyang *et al.*, 1996; Li *et al.*, 1997). The stabilization of these cytokine mRNAs might be related to the enhancement of the MAP kinase activities found during *in vivo* and *in vitro* studies recently (Zhou *et al.*, personal communication; Yang *et al,* 2000). JNKs as well as other MAP kinases have been shown to be involved in the stabilization of cytokine and other targeted gene mRNAs (Chen *et al.,* 1998; Miyazawa *et al.*, 1998; Ming *et al.*, 1998; Winzen *et al.*, 1999; Wang *et al.*, 1999). It has been implied that ARE binding activity is regulated by phosphorylation of these AUBPs by MAP kinases and protein tyrosine kinases (Sirenko *et al.*, 1997). The exact mechanisms by which these kinases contribute to the stabilization of cytokine mRNA levels are still unclear. Further research on these mechanisms will be necessary.

In conclusion, VT was found to stabilize mRNA levels of pro-inflammatory cytokines in RAW 264.7 cells. Hence, stabilization may play a major role in the

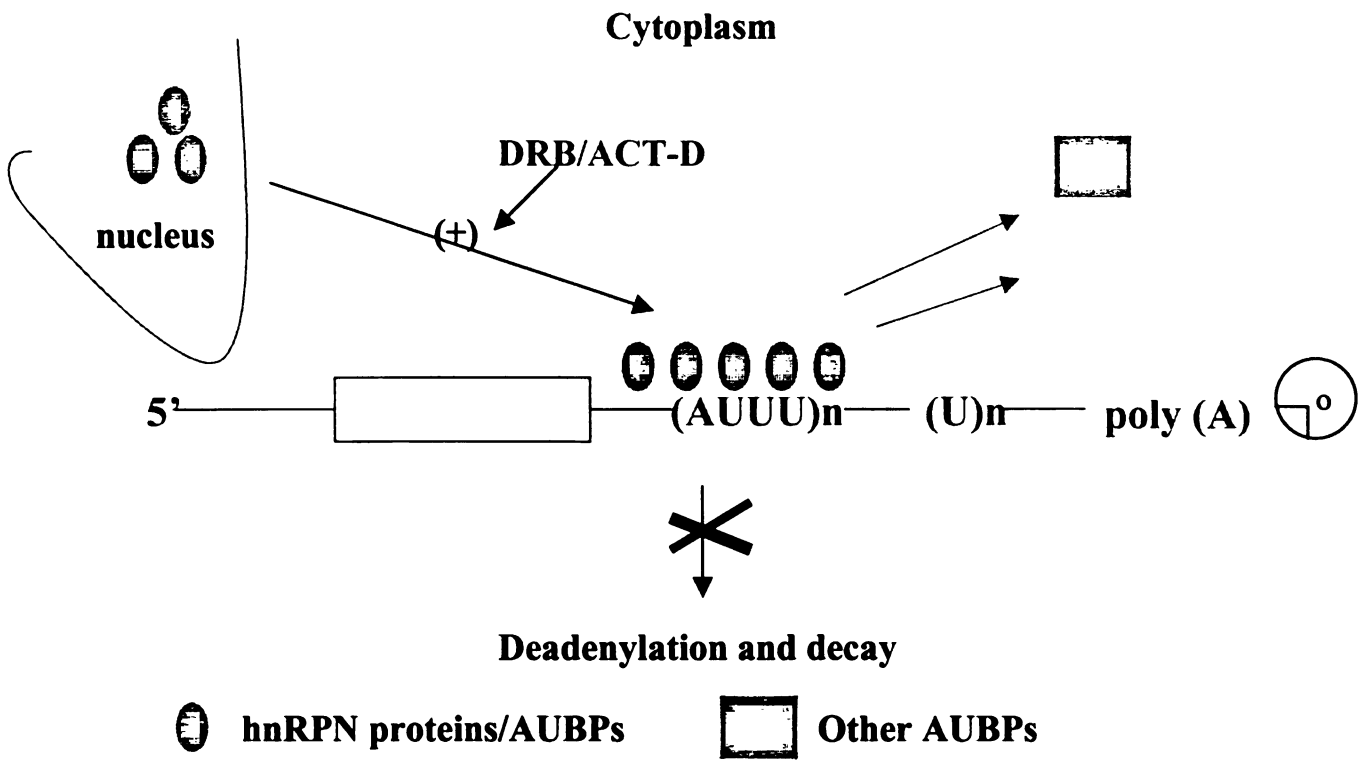


Fig.5.6 D. DRB and other transcriptional inhibitors transfer hnRPN proteins or AU-binding proteins (AUBPs) to the AU regions.

superinduction of these cytokines and subsequent pathologic sequelae.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This study suggests that trichothecenes can superinduce IL-1β, IL-6 and TNF-α gene expression in murine macrophage RAW 264.7 cells. VT could differentially superinduce cytokines by either increasing numbers of cytokine secreting cells or increasing cytokine output per cell. Further investigations into the underlying mechanism of cytokine superinduction showed that the binding activities of transcription factors AP-1, NF-κB and NF-IL6 (C/EBP) were differentially enhanced; and VT specifically targeted the phosporylated c-Jun, JunB, c-Fos, and Fra-2 subunits of the AP-1 components; p50 and c-Rel of NF-κB; and C/EBPα and C/EBPβ of NF-IL6. However, further studies into the *cis*-acting elements of the promoter of the TNF-α showed that CAT mRNA expression of the TNF-α-CAT reporter construct was increased only to a small extent by VT in transient transfection assays. Since transcriptional activation might not be the primary mechanism for VT-induced cytokine superinduction in macrophage cells, post-transcriptional regulation was also assessed. In these studies, VT was shown to markedly increase mRNA half-life of both TNF-α and IL-6 in both asynchronous and delayed synchronous models. VT markedly extended the half-life of the TNF-α mRNAs by more than 2-fold in both experimental models. Therefore, post-transcriptional control might be critical to the the VT-mediated TNF-α superinduction in macrophages. In the case of IL-6, its half-life were also extended by at least 10-fold in the asynchronous model; and 2- and 4-fold in the delayed synchronous model. Though IL-6 promoter activity has not yet been investigated, I believe that the stabilization effects of VT might also play an important role in the IL-6 superinduction in macrophages.

Since these cytokines play pivotal roles in regulating the VT-induced IgA

hyperelevation and its related toxicity, further assessment of the mechanisms are needed to be addressed. First, the effects of VT on the promoter activity of IL-6 could be assessed by using a plasmid containing the IL-6 promoter linked to a reporter gene transfected in the macrophage RAW 264.7 cells. This set of experiments will further determine the role of transcription in the IL-6 superinduction. Secondly, MAP kinases have been suggested to play a role in the stabilization of mRNA half-life in many cell models, but there is not yet direct evidence how these VT-induced MAP kinases stabilize these cytokines. Therefore, experiments could address how VT exerts its effects on MAP kinases and their related downstream proteins and how these proteins modulate the mRNA half-life of those cytokines. A direct evidence of the relationship of the MAP kinase and mRNA stability should be established.. Thirdly, mRNA stability study of these cytokines by VT should be extended to the *in vivo* model. Over the long term, it will be necessary to understand the physiological significance of all these observations in animals and humans.

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