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Studies on the effects of propanil on macrophage function and signaling

Laura Lynn Frost
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**STUDIES ON THE EFFECTS OF PROPANIL ON MACROPHAGE FUNCTION
AND SIGNALING**

Laura Lynn Frost

DISSERTATION

**Submitted to the School of Medicine
at
West Virginia University
In partial fulfillment of the requirements
for the degree of**

Doctor of Philosophy

in

Microbiology, Immunology and Cell Biology

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Rosana Schafer, Ph.D.
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Stephen Graber, Ph.D.
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NF- κ B, NADPH oxidase

GENERAL ABSTRACT

Studies On The Effects Of Propanil On Macrophage Function And Signaling

Laura Lynn Frost

Propanil has been shown to induce numerous manifestations of impaired immune responses. This dissertation focuses on the effects of propanil on TNF- α production and NF- κ B activation in murine and human macrophages in response to LPS stimulation. Initial studies demonstrated that propanil treatment of the murine peritoneal macrophage cell line, IC-21 resulted in decreased NF- κ B activation and transcriptional activity as determined by Western blot and NF- κ B-dependent reporter gene transcription, respectively. Nuclear localization of NF- κ B was also shown to be decreased by immunofluorescence. Additional studies to confirm these effects in the human cell line, THP-1 also showed decreased nuclear levels of NF- κ B in propanil-treated cells. Additionally, NF- κ B levels were determined from peritoneal exudate cells (PEC) harvested from mice treated with 200mg/kg propanil *in vivo*, and shown to be decreased compared to the corn oil (vehicle) control. However, analysis of the inhibitor of NF- κ B, I κ B α , demonstrated no differences in its degradation between propanil-treated and ethanol (vehicle) control cells in THP-1 cells. Therefore, propanil alters nuclear localization of NF- κ B independently of degradation of I κ B α .

Effects of propanil on macrophage functions were also evaluated. Propanil reduced phagocytic abilities of macrophages, as determined by phagocytosis of 2 μ m fluorescent spheres. Additionally, propanil abrogated the phorbol ester-induced NADPH oxidase activity of PEC and THP-1 cells. Listericidal ability of PEC and THP-1 cells were also evaluated following propanil treatment. Results indicated that propanil treatment significantly reduced the ability of activated macrophages to kill intracellular *Listeria monocytogenes*. Because bacterial killing is dependent on superoxide production, the decreased listericidal capabilities of propanil-treated macrophages was expected.

The data presented herein extend our knowledge of propanil's immunotoxic effects, and begin to mechanistically dissect the effects of propanil on specific signal transduction pathways. In light of the reduction of TNF- α production, NF- κ B and respiratory burst activity of macrophages, however, this study also demonstrates a possible beneficial role for propanil as a potent anti-inflammatory compound.

DEDICATION

It is with the greatest gratitude, appreciation and love that I dedicate this thesis to my parents. They instilled in me a love of learning throughout my childhood, and encouraged and supported me in every way possible. I can never thank them enough for all they have done to ensure my success.

I'm certain that I would not have succeeded in this endeavor had it not been for the love and support of my husband, Brett. I am eternally indebted to him - his love, encouragement, support and countless sacrifices have enabled the accomplishment of this body of work. I sincerely and gratefully acknowledge his commitment to me, my education, and the completion of this thesis. I dedicate this thesis to my husband, with immense appreciation, respect and love.

My greatest accomplishment to date is my son, Nicholas. I dedicate this thesis to him. It is my intention to instill in Nicholas the love of learning and quest for knowledge as my parents and grandparents did for me. I hope that Nicholas finds that with the love and support of his family, no obstacle is insurmountable.

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LITERATURE REVIEW

I. Propanil

General usage and environmental concerns

Propanil, 3,4 dichloropropionanilide, is a post-emergent herbicide used extensively for broad-spectrum weed control in the production of rice and wheat (Dahchour, A. *et al.* (1986)). Multiple applications of propanil during a growing season are used to control weeds. Crops such as rice and wheat are capable of enzymatically detoxifying propanil, and are resistant to the herbicidal effects (Mateunaka, S. (1968); Still, C. C. *et al.* (1967); Still, C. C. (1968)). The US EPA estimates that approximately 7-9 million pounds of propanil are applied to US soils annually (United States Environmental Protection Agency, 1987).

The major environmental concern regarding propanil is ground water contamination and subsequent runoff. Various aquatic animal species have been shown to be sensitive to propanil toxicity. Studies of propanil toxicity to catfish have demonstrated that concentrations as low as 10 ppm can be fatal (McCorkle, F. M. *et al.* (1977)). Similarly, mosquitofish were also susceptible to low ppm concentrations of propanil (Fabacher, D. L. *et al.* (1971)). Propanil caused decreased mobility of freshwater snails, and also affected development and viability of eggs (Kosanke, G. J. *et al.* (1988)).

Herbicidal effects and metabolism of propanil

Selective herbicidal effects of propanil are mediated by the presence of acylamidase, an enzyme which detoxifies the herbicide. Target crop plants have high levels of acylamidase activity, while susceptible weed species lack this enzyme and are killed by the herbicide (Still and Kuzirian

(1967)). Acylamidase-mediated hydrolysis of propanil results in formation of the primary metabolite, 3,4-dichloroaniline (DCA), which, in plants, remains associated mainly with lignin.

Propanil is also subject to photodecomposition and metabolism by various soil and aquatic microorganisms. Bacterial species capable of degrading propanil include *Pseudomonas spp.* and *Streptococcus* (recently reclassified as *Enterococcus*) *spp* (Dachour *et al.*, (1986); Zeyer and Kearney, (1984). Additionally, fungal species within the genera *Fusarium*, *Penicillium*, and *Pulullaria* are capable of biodegrading acylanilides similar to propanil (Bartha and Pramer, (1970); Chisaka and Kearney, (1970); Lanzilotta and Pramer, (1970)).

Immunotoxic effects of propanil

Hallmark manifestations of acute propanil toxicity in mammals include central nervous system (CNS) depression, loss of righting reflex and cyanosis (Singleton, S. D. *et al.* (1973)). Singleton and Murphy (1973) also demonstrated the development of methemoglobinemia in mice following propanil exposure. Another result of propanil exposure is liver damage. Santillo *et al.* (1995) measured several parameters indicative of liver functions in rats and found that propanil treatment resulted in elevated serum levels of the enzymatic indicators of liver function, aspartate aminotransferase and alkaline phosphatase (Santillo, M. *et al.* (1995)).

Human exposure to propanil is most likely for individuals who work in pesticide manufacturing, formulation or packaging, since propanil can be absorbed into the system through inhalation, ingestion and by dermal exposure (Cannon, S. B. (1978); Folland, D. S. (1978)). The CNS symptoms of propanil exposure include headache, drowsiness, dizziness and confusion. Acute toxicity is manifested by methemoglobinemia and cyanosis (Kimbrough, R. D. (1980)). According

to a study done in 1979, the symptoms most often reported by workers included acne, skin and eye irritation, jaundice and nausea or vomiting (Morse, D. L. *et al.* (1979)).

Two recent studies evaluated the concentrations of propanil in and near homes adjacent to rice fields in which propanil was used, by aerial spray, for weed control. The results indicated that although propanil concentrations could be detected by gas chromatography/mass spectroscopy from air samples outside (30 meters away from the residence), there was no evidence of propanil vapor associated with absorbent media samples from within the homes (Richards, S. M. *et al.* (2001)). In another study, blood samples collected from individuals living in homes near propanil-sprayed rice fields were compared to those from non-exposed families. The findings indicated that total blood cell counts, lymphocyte percentages, IL-2 production and NK cell functions were not altered in adults or children that had potentially been exposed to propanil (McClure, G. Y. *et al.* (2001)). However, when human umbilical cord blood samples were exposed to propanil *in vitro*, erythroid progenitors were found to be particularly sensitive to the toxic effects of propanil (Malerba, I. *et al.* (2002)), confirming the earlier reports of propanil's myelotoxicity by Blyler *et al.*, (1994), who showed that propanil, at doses of 50-200 mg/kg resulted in a reduction in the number of myeloid stem cells and early myeloid and erythroid progenitor cells.

In vivo administration of propanil to C57Bl/6 mice results in suppression of multiple immune system functions, as well as reduced thymus weight and increased spleen weight, summarized in Table 1. Spleen-to-body weight ratios (S:B) for five to six week-old female C57Bl/6 mice treated with propanil intraperitoneally (i.p.) were found to be significantly increased over vehicle control animals (Barnett, J. B. *et al.* (1989)). Further examination of the spleen showed increased cellularity, but no significant alterations in leukocyte populations (Barnett, J. B. and Gandy, J. (1989)). Conversely, thymus-to-body weight ratios (T:B) were significantly decreased upon i.p.

propanil exposure (Zhao, W. *et al.* (1995)). Analysis of the T cell populations demonstrated reductions in CD3⁺ CD4⁺ CD8⁺ (triple positive), CD3⁺CD4⁺CD8⁻, and CD3⁺ CD4⁻ CD8⁺ populations relative to vehicle controls (Zhao, W. *et al.* (1995)). Further investigation into the phenomenon of thymic atrophy induced by propanil, and the potential role of glucocorticoids, was conducted in adrenalectomized animals. Propanil exposure did not cause thymic atrophy in these animals, suggesting that the effects on thymocytes may be, in part, mediated by glucocorticoids (Cuff *et al.*, (1996)).

In vivo propanil exposure did not affect cytotoxic T lymphocyte (CTL) activity, however NK cell-mediated cytotoxicity was significantly reduced by propanil (Barnett, J. B. *et al.* (1992)). In addition, the mixed lymphocyte reaction (MLR) showed a dose-dependent decrease in propanil-treated animals (Barnett, J. B. and Gandy, J. (1989)), suggesting a possible affect on helper T cells. Barnett and Gandy (1989) also investigated the effects of propanil on T-dependent and T-independent antibody production and mitogenic responses. Their experiments concluded that doses of propanil from 50 – 400 mg/kg reduced the T-dependent antibody response to sheep red blood cells. The T-independent antibody response, as measured by plaque forming cells following injection of DNP-Ficoll was increased at the low dose of 50mg/kg propanil, but the higher dose, 200mg/kg caused significant reductions in the response (Barnett, J. B. and Gandy, J. (1989)). The mitogenic responses of splenocytes to concanavalin A (Con A) (T cell mitogen) or bacterial lipopolysaccharide (LPS) (B cell mitogen) were reduced only at the highest dose of 400mg/kg propanil (Barnett, J. B. and Gandy, J. (1989)).

In mice, resistance to the facultative intracellular pathogen, *Listeria monocytogenes*, after exposure to propanil was evaluated. Although IFN- γ production by splenocytes upon mitogenic stimulation was reduced by propanil, endogenous cytokine levels were similar (Watson *et al.*,

(2000)). Additionally, propanil exposure 2 or 7 days prior to *Listeria* infection did not alter magnitude or kinetics of the infection (Watson *et al.*, (2000)). Whereas serum IL-6 levels were elevated in mice given 200mg/kg propanil 2 days prior to *Listeria* infection, neither endogenous nor *ex vivo* elicited levels of TNF- α , IFN- γ or IL-1 β were significantly altered (Watson *et al.*, (2000)).

Production of cytokines from macrophages and T cells that had been exposed to propanil *in vivo* or *in vitro* have been investigated in several studies. Notably, IL-2, IL-6, IFN γ and GM-CSF production by spleen cell cultures established 2 days post *in vivo* propanil treatment (i.p.) were significantly decreased following mitogenic stimulation with ConA, IL-2 and IL-6 production remained reduced through 7 days, whereas IFN γ and GM-CSF production recovered by 4 days post exposure (Zhao, W. *et al.* (1998)). Similarly, *in vivo* propanil exposure (i.p.) caused reduced production of TNF- α and IL-6 from LPS-stimulated PEC harvested 3 and 7 days post exposure (Xie, Y. C. *et al.* (1997)). Additionally, Xie *et al.* (1997) demonstrated that *in vitro* propanil exposure also reduced production of these cytokines from PEC harvested from normal C57Bl/6 mice.

In vitro propanil treatment of the murine lymphoma T cell line, EL-4, confirmed the decrease in IL-2 production, as discussed above for the spleen (Zhao, W., *et al.* (1998)). Furthermore, mechanistic examination of the reduced protein production demonstrated decreased IL-2 mRNA levels in addition to a reduced rate of transcription and message stability (Zhao, W., *et al.* (1998)). Levels of IL-6 and TNF- α messenger RNA from PEC treated with propanil *in vitro* were also reduced upon LPS stimulation, however, message stability was not altered (Xie, Y. C., *et al.* (1997) and unpublished data). Therefore, propanil treatment differentially affected cytokine production; mRNA levels of IL-2, IL-6 and TNF- α were all reduced, however mRNA stability was only altered for IL-2. Thus, propanil seems to disrupt signal transduction pathways that culminate in cytokine transcription, as well as alter stability of some species of mRNA.

Table 1. Summary of immunotoxic effects of propanil

Function/Parameter	Effect	Reference
Spleen:body weight ratio	↑	Barnett & Gandy, 1989; Barnett <i>et al.</i> , 1992 Zhao <i>et al.</i> , 1995
Thymus: body weight ratio	↓	Barnett <i>et al.</i> , 1992; Zhao <i>et al.</i> , 1995
T-dependent antibody response ^a	↓	Barnett & Gandy 1989; Barnett <i>et al.</i> , 1992
T-independent antibody response ^b	↓ ^c , ↑ ^d	Barnett <i>et al.</i> , 1992
CTL activity	↔	Barnett <i>et al.</i> , 1992
MLR reaction	↓	Barnett & Gandy 1989
NK cell activity	↓	Barnett <i>et al.</i> , 1992
T cell mitogenic response (to ConA)	↓	Barnett & Gandy 1989
B cell mitogenic response (to LPS)	↓	Barnett & Gandy 1989
IL-2 production		
Splenocytes (treated <i>in vivo</i>)	↓	Zhao <i>et al.</i> , 1998
EL-4	↓	Zhao <i>et al.</i> , 1999
IL-6 production		
Splenocytes	↓	Zhao <i>et al.</i> , 1998
PEC	↓	Xie <i>et al.</i> , 1997
TNF-α production	↓	Xie <i>et al.</i> , 1997

↑ response increased relative to vehicle control
 ↓ response decreased relative to vehicle control
 ↔ response unchanged from vehicle control
^a measured by plaque assay using sheep RBCs as the antigen
^b measured by plaque assay using DNP-Ficoll as the antigen
^c at 200mg/kg
^d at 50mg/kg

II. **Macrophage activation and LPS signaling**

Macrophage activation

Macrophages are critical cells to both innate immunity and specific immunity. Because they function in tumor surveillance, pathogen phagocytosis and destruction, generation of acquired immunity, through their role as professional antigen presenting cells, and inflammation, by production of chemokines and cytokines, alterations in appropriate macrophage activation or cytokine production could have far-reaching consequences. It is critical therefore that macrophage activation is strictly regulated at all levels.

Macrophage development occurs in the bone marrow as pluripotent stem cells develop along the myeloid differentiation pathway in response to specific colony-stimulating factors (CSFs). Monocytes are released into the blood circulation, and ultimately undergo final differentiation into macrophages in tissues. Tissue macrophages differ according to functionality, surface marker expression, and MHC class II expression (reviewed in Adams, D. O. *et al.* (1984)). Monocytes and macrophages are not entirely functionally competent, and require additional activation to elicit the functions of phagocytosis, cytokine production, antigen presentation and production of reactive oxygen intermediates (ROI) (Summarized in Table 2). This process is a complex paradigm involving several levels of activation. For example, resident peritoneal macrophages are refractory to stimulatory signals, and are "resting cells" (Unanue, E. R. (1981)). However, elicitation by thioglycollate injection results in a population of peritoneal exudate cells (PEC) that readily respond to stimulation, similar to those found at sites of inflammation. These cells are referred to as "responsive" or "inflammatory" macrophages. Treatment of responsive macrophages with IFN γ induces further activation resulting in a stage termed "primed" macrophages. Primed macrophages

readily become cytolytic when exposed to LPS, a potent activator (Unanue, E. R. (1981); Adams, D. O. and Hamilton, T. A. (1984)). Higher concentrations of LPS can also compensate for IFN γ requirement and achieve the fully activated state from responsive cells.

Table 2. Macrophage activation

	Thioglycollate		IFN γ	LPS
	Resident	→ Responsive	→ Primed	→ Activated
Cytokine production	-	-	-	++++
Ag presentation	-	-	++++	++
Phagocytosis	-	-	-	++++
Tumoricidal	-	-	-	++++
Production of ROI	-	-	+++	+++
MHC Class II expression	-	-	++++	++

(Adapted from Adams & Hamilton, 1984)

LPS receptors and signal transduction

Activation of macrophages stimulates numerous cellular responses, but perhaps the most important of these are the signaling events that lead to TNF- α production. Activation of macrophage signal transduction by LPS has been known to involve the surface protein, CD14 (reviewed in Kielian, T. L. *et al.* (1995); Wright, S. D. *et al.* (1990)). The CD14 glycoprotein is expressed on monocytes, macrophages and neutrophils (reviewed in Kielian, T. L. and Blecha, F. (1995)). Synthesis and release of TNF- α , one of the earliest cytokines released by activated macrophages, was almost entirely blocked when LPS-stimulated macrophages were pre-treated with anti-CD14 antibodies (Wright, S. D., *et al.* (1990)). Binding of LPS to CD14 was shown to require association with the LPS binding protein (LBP), which is ubiquitously present in blood (Schumann, R. R. *et al.* (1990); Wright, S. D., *et al.* (1990)). However, CD14 is a glycosylphosphatidylinositol (GPI) linked

protein, hence lacks a traditional transmembrane sequence and signaling domain (for reviews see: Kielian, T. L. and Blecha, F. (1995); McMillan, D. C. *et al.* (1990)). Therefore a direct role for CD14 in LPS-induced signal transduction was a source of much controversy. However, CD14 co-precipitated with tyrosine kinase activity in monocyte whole cell lysates (Stefanova, I. *et al.* (1991)). Early studies speculated that possible mechanisms of LPS signal transduction by CD14 included internalization of the receptor complex (Jonas, L. *et al.* (1989); Jonas, L. *et al.* (1990)). Others hypothesized that because CD14 was GPI-anchored, it would have enough mobility in the plasma membrane to associate with other membrane components (reviewed in Kielian, T. L. and Blecha, F. (1995)).

A naturally occurring mutation in the LPS hyporesponsive, C3H/HeJ strain of mice was traced to the gene encoding a toll-like receptor (TLR), a homolog of the toll receptors involved in innate immunity in *Drosophila* (Poltorak, A. *et al.* (1998); Qureshi, S. T. *et al.* (1999); Vogel, S. N. *et al.* (1999)). These discoveries led to speculation that TLR4 might be the elusive LPS co-receptor, since TLR proteins have intracellular signaling domains. A technique called FRET (fluorescence resonance energy transfer) in which two proteins are fluorescently tagged in a manner that only allows fluorescence of one of the proteins when energized by the fluorescence emitted by the other, thus, the intensity is dependent on the proximity of the two proteins has been recently described. Using this technique, Jiang *et al.* (2000) demonstrated that LPS/LBP induced a situation, in human monocytes, which reduced the physical proximity between CD14 and TLR4, supporting the hypothesis that TLR 4 was the LPS co-receptor. Importantly, Jiang *et al.* (2000) also demonstrated that this association occurred prior to the nuclear translocation of NF- κ B, another crucial component to LPS-induced signal transduction.

Binding of LPS to CD14 and its co-receptor, TLR4, induces a complex series of signaling events in macrophages. Early events include the activation of phospholipase C γ (PLC γ) which in turn cleaves phosphatidylinositol bisphosphate (PIP₂), into the signaling intermediates, diacylglycerol (DAG) and inositol trisphosphate (IP₃). IP₃ binds to the IP₃ receptor inducing release of calcium from the endoplasmic reticulum stores. This initial calcium release alters plasma membrane associated calcium channels inducing a capacitative calcium entry (reviewed in Berridge, M. J. *et al.* (1994); Berridge, M. J. (1997)). Capacitative calcium entry is governed by store operated channels (SOC) that respond to emptied internal calcium sources by allowing calcium influx over the plasma membrane. This process of emptying the internal stores of calcium, and refilling them through the capacitative calcium entry sets up a situation of waves, or oscillations of calcium spikes in the cytosol (reviewed in Berridge, M. J. (1997)). The oscillating increases in cytosolic calcium have been implicated in several aspects of leukocyte cell biology, including activation of calcium-dependent kinases (e.g. calmodulin, CAM kinase), regulation of proliferation, apoptosis and gene transcription through activation of transcription factors, and specifically in macrophages and neutrophils, activation of the NADPH oxidase (reviewed in Berridge, M. J. (1997); Shaffer, A. L. *et al.* (2001); Granfeldt, D. *et al.* (2002)).

Bifurcation of the signal initiated by LPS occurs early in the transduction process, with the generation of DAG and IP₃ from PIP₂. Whereas IP₃ invokes calcium fluxes and many subsequent calcium-dependent events, DAG acts directly on protein kinase C (PKC). LPS activation of PKC requires CD14 (Liu, M. K. *et al.* (1994)). Several isoforms of PKC have been identified: classical PKC (cPKCs), which are calcium-dependent; new PKCs (nPKCs) and atypical PKCs (aPKCs), which are calcium-independent (Nishizuka, Y. (1988)). The cPKCs (PKC- α , β I, β II, and γ) have been implicated in NF- κ B activation (Carter, A. B. *et al.* (1998)). There is, however, some

controversy as to the role of PKCs in NF- κ B activation (Feuillard, J. *et al.* (1991); Saijo, K. *et al.* (2002)).

LPS also induces activation of the mitogen activated protein kinase pathway (MAPK) via receptor tyrosine kinase activation of ras and raf-1 (reviewed in Sweet & Hume 1996; Reimann *et al.*, 1994). Geppert *et al.* (1994) demonstrated that dominant-negative inhibitors of both ras and raf-1 could repress LPS-induced TNF- α production in RAW264 cells (Geppert, T. D. *et al.* (1994)). However, an accessory protein, MD-2, has been shown to be necessary for this activation (Yang, H. *et al.* (2000); Shimazu, R. *et al.* (1999)). Downstream targets of the MAPK cascade are extracellular regulated kinases 1/2 (ERK) and ets transcription factors. Parallel MAPK pathways that are induced by LPS are the MEKK (mitogen-activated and ERK kinase) leading to JNK (jun N-terminal kinase) and p38 activation, and activation of the transcription factors AP-1, ATF-1/2 and CREB (reviewed in Guha, M. *et al.* (2001)).

Figure 1. LPS-induced signal transduction

NF-κB regulation

Signals induced by many stimuli including LPS and other bacterial cell wall components, growth factors and cytokines converge in the activation of NF-κB. NF-κB activates gene transcription of numerous target genes (Table 3). The term NF-κB actually applies to a family of proteins, consisting of RelA (p65), NF-κB2 (p105 which is processed to p50), NF-κB1 (p100 which is processed to p52), cRel, RelB, and several isoforms of inhibitor proteins, IκBs. IκB proteins bind dimeric NF-κB proteins via a conserved ankryin repeat motif. The most common dimeric form of NF-κB is the p65:p50 heterodimer (reviewed in Rothwarf, D. M. *et al.* (1999); May, M. J. *et al.* (1998)). One of the most intriguing questions is how cells respond appropriately to these various stimuli when they commonly activate NF-κB. The answer seems to lie in the complexity of the levels of NF-κB activation, the participation of co-factors in NF-κB-dependent gene transcription, and the role of inhibitory proteins in releasing NF-κB from DNA (reviewed in Ghosh, S. *et al.* (2002)).

Table 3. NF-κB-inducible genes

Class of gene	NF-κB-dependent gene	
Cytokines/growth factors	TNF-α and β	IL-8
	IL-1-α and β	M-CSF
	IL-6	GM-CSF
	IL-2	G-CSF
	IL-12	IFN-β
Cytokine receptors	IL-2Rα	
Adhesion/regulatory molecules	IκBα	MHC class I
	ICAM-1	MHC class II
	VCAM-1	TCRα and β
	MadCAM-1	β ₂ microglobulin
	E-selectin	TAP1

Adapted from May & Ghosh 1998; Blackwell & Christman 1997

Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage stimulating factor; ICAM-1, intercellular adhesion molecule 1; MadCAM-1, mucosal addressin cell adhesion molecule 1; MHC, major histocompatibility complex; TAP1 transporter associated with antigen processing; TCR, T cell receptor; VCAM-1, vascular cell adhesion molecule 1.

Activation of NF- κ B by LPS involves release of NF- κ B p65:p50 from the inhibitory I κ B through a process of phosphorylation of I κ B (on serines 32 and 36 on I κ B α) which targets the inhibitor for ubiquitination and subsequent degradation by the 26S proteasome. Degradation of I κ B releases NF- κ B, which reveals the nuclear localization sequences (NLS) and allows nuclear translocation. Phosphorylation of I κ B is regulated by the I κ B kinase complex (IKK), which consists of IKK α , IKK β and IKK γ (NEMO) in addition to other protein kinases. IKK β is a more efficient kinase for I κ B α than IKK α , leading to speculation that IKK β is the kinase primarily responsible for phosphorylation of I κ B (reviewed in Ghosh, S. and Karin, M. (2002)). A potential role for IKK α is hypothesized to be the phosphorylation of p65. Transcriptional activity of NF- κ B is dramatically increased by phosphorylation of p65 on serines 276, 529 or 536, by the catalytic subunit of PKA (PKAc), which is also a component of IKK complex, and IKK α or IKK β , respectively (Sakurai, H. *et al.* (1999); Sizemore, N. *et al.* (2002); Zhong, H. *et al.* (1998)). Phosphorylation of p65 serves two possible purposes, enhancing DNA binding, and increasing the efficiency of interaction with coactivator proteins, CBP/p300 (Zhong, H. *et al.* (1998), Zhong, H. *et al.* (2002)).

Although the majority of research has focused on the role of I κ B α with respect to NF- κ B activation, recent reports indicate that I κ B β may play a more significant role in NF- κ B-dependent gene transcription (reviewed in Ghosh and Karin (2002); Malek, S. *et al.* (2001)). I κ B α binds p65:p50 heterodimers, and I κ B α -mediated NF- κ B transactivation is transient and rapid (Thompson, J. E. *et al.* (1995); SuYang, H. *et al.* (1996); Tran, K. *et al.* (1997)). I κ B α also contains a potent nuclear export sequence (NES), and has been shown to shuttle NF- κ B complexes between the nucleus and cytoplasm (Arenzana-Seisdedos, F. *et al.* (1995); Rodriguez, M. S. *et al.* (1999)). I κ B β contains no NES (Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001); Tam, W. F. *et al.*

(2001)). Additionally, NF- κ B induces transcription of I κ B α . Importantly, I κ B α and I κ B β are functionally redundant, with respect to binding and retention of NF- κ B heterodimers (Cheng, J. D. *et al.* (1998); Malek, S., *et al.* (2001)). The emerging picture is a complex regulation scheme in which I κ B β retains NF- κ B p65:p50 heterodimers in the cytoplasm of quiescent cells, and stimuli involved in NF- κ B activation result in degradation of I κ B β and subsequent nuclear translocation of the heterodimers. A plausible role for I κ B α is nuclear import of newly synthesized proteins (as a result of NF- κ B-dependent transcription of the I κ B α gene), binding of NF- κ B heterodimers leading to detachment from DNA, and export of the complexes out of the nucleus. This scenario implies I κ B β involvement in NF- κ B activation, and a requirement for I κ B α in cessation of NF- κ B-dependent transcription (reviewed in Ghosh, S. and Karin, M. (2002)).

Several signaling cascades converge at the activation of the IKK complex (reviewed in Ghosh, S. and Karin, M. (2002)). As mentioned, the complex contains proteins in addition to the IKKs. PKA, MEKK1, NF- κ B inducing kinase (NIK), and others have been identified as components of the complex (Nakano, H. *et al.* (1998); Nemoto, S. *et al.* (1998); Zhao, Q. *et al.* (1999)). Additionally, proteins which are capable of activating the complex as a result of stimuli other than LPS include TAK1, and PKC (reviewed in Ghosh, S. and Karin, M. (2002)). Interestingly, direct phosphorylation of p65 in the absence of IKK activity has been demonstrated by phosphatidylinositol-3 kinase (PI3K)-activated Akt and the small GTPase, Rho (Delhase, M. *et al.* (2000); Cammarano, M. S. *et al.* (2001); reviewed in Ye, R. D. (2001)).

Thus, although reports of NF- κ B activation in the absence of IKK activity exist, by and large the vast majority of inflammatory stimuli activate NF- κ B via IKK β . The IKK γ subunit is also indispensable to the process, and serves as the regulatory subunit for the complex (Chu, W. *et al.* (2000); Li, Z. W. *et al.* (1999); Schmidt-Supprian, M. *et al.* (2000); Senfleben, U. *et al.* (2001)).

The magnitude of NF- κ B activation, interaction with coactivators and histone deacetylases all seem to affect the transcriptional activity of NF- κ B (reviewed in Ghosh, S. and Karin, M. (2002)).

TNF- α regulation

TNF- α production is regulated transcriptionally, and post transcriptionally. The primary transcription factor required for TNF- α production is NF- κ B (reviewed in Jongeneel, C. V. (1994)). Numerous studies have shown that deletion or mutation of NF- κ B binding sites in the TNF- α promoter causes drastic inhibition (Collart, M. A. *et al.* (1990); Drouet, C. *et al.* (1991); Shakhov, A. N. *et al.* (1990); Trede, N. S. *et al.* (1995)). Both human and murine TNF- α promoters are responsive to NF- κ B, although there are differences in the NF- κ B binding sites (Kuprash, D. V. *et al.* (1999)). However, there is evidence for the contribution of other transcription factors to TNF- α transcription. As mentioned earlier, CBP/p300 is a powerful coactivator for NF- κ B-dependent transcription. In addition a role for cooperation with AP-1 transcription factors has been established for the TNF- α promoter (Tsai, E. Y. *et al.* (1996)). Also, MAPK kinase pathway terminal transcription factors, Egr, Elk and ets have been implicated as participants in TNF- α gene transcription (Pope, R. *et al.* (2000); Pope, R. M. *et al.* (1994); Sheppard, K. A. *et al.* (1999); Zagariya, A. *et al.* (1998))

TNF- α is also regulated post transcriptionally and translationally. The presence of a "UA" rich motif located in the 3' end of the message allows for the superinduction of the mRNAs under some circumstances, by increased message stability (Caput, D. *et al.* (1986); Sariban, E. *et al.* (1988)). In addition, PKC inhibitors reduce message stability of TNF- α mRNA, implying a role for the kinase in post transcriptional TNF- α regulation (Leitman, D. C. *et al.* (1992); Moreira, A. L. *et*

al. (1993)). Additionally, the TNF- α converting enzyme, a member of the IL-1R superfamily, processes pro-TNF- α to the mature secreted form (Solomon, K. A. *et al.* (1999)).

The regulation of TNF- α is complex, not unexpected given the potency of its effects. Transcriptional regulation is mediated primarily by NF- κ B, with additional roles for other transcription factors such as, AP-1, Elk and ets proteins. Translational regulation is mediated by presence of a UA-rich region of the message, and lastly, final maturation of TNF- α occurs as a result of cleavage by TACE for secretion of the mature cytokine.

Figure 2. Murine TNF- α promoter

III. Macrophage functions

Phagocytosis

Proper function of macrophages is critical for normal maintenance of homeostasis as well as immune regulation and tumor surveillance. Whereas phagocytosis of apoptotic cells is a normal homeostatic function of macrophages, phagocytosis and clearance of bacteria and viruses is a crucial function of innate immunity. The process of phagocytosis involves rearrangement of actin filaments and complex alterations of the cellular cytoskeleton (reviewed in Aderem, A. *et al.* (1999)). Although the complete signaling picture leading to macrophage phagocytosis is not clear, PI₃K is known to be required for, and regulate the process of, phagocytosis (Aderem, A. and Underhill, D. M. (1999); Gillooly, D. J. *et al.* (2001); Govoni, G. *et al.* (1998); Marshall, J. G. *et al.* (2001); Stephens, L. *et al.* (2002)). Tyrosine phosphorylation events leading to PI₃K activation are also important in the process of phagocytosis. Recently, Larsen and colleagues (2000) investigated the role of PKCs in phagocytosis and respiratory burst generation. They found differential requirement for PKC isoforms between these two critical macrophage functions; phagocytosis requiring PKC δ and PKC ϵ .

Respiratory burst

The macrophage respiratory burst is mediated by the formation of the NADPH oxidase, which produces superoxide ($\bullet\text{O}_2^-$) by oxidizing NADPH and transferring electrons to molecular oxygen. The functional NADPH oxidase is formed when the membrane complex consisting of three membrane localized proteins is activated by association of several additional cytosolic components forming the active *phagocyte oxidase* (phox) (reviewed in Dahlgren, C. *et al.* (1999)). The

membrane associated cytochrome b_{558} , (gp91^{phox}), flavoprotein (p22^{phox}) and Rap1A require the translocation of cytosolic p47^{phox}, p67^{phox}, p40^{phox} and rac1/2 to form the functional NADPH oxidase. The translocation of the cytosolic components has been shown to be a calcium-dependent event (Dusi, S. *et al.* (1993a)). A further role for calcium in NADPH oxidase function was demonstrated by Granfeldt and colleagues who determined that the capacitative calcium entry was necessary for activation of intracellular NADPH oxidase, but additional calcium signals were required for activation of plasma membrane localized NADPH oxidase (Granfeldt, D., *et al.* (2002)). However, whereas increased calcium was shown to be required for NADPH oxidase activation, it was not sufficient to cause translocation of the cytosolic phox proteins (Movitz, C. *et al.* (1997)). Park and Babior demonstrated that translocation of the cytosolic components to form the active NADPH oxidase was regulated by DAG and required G protein activity (Park, J. W. *et al.* (1992)). Additionally, the small GTPase, Rho, has been demonstrated to activate p21rac and allow its association with the oxidase (Abo, A. *et al.* (1994)).

Another level of NADPH oxidase regulation lies in phosphorylation of p47^{phox} and p67^{phox}, although the specific kinase responsible for this phosphorylation has not been identified (Dusi, S. *et al.* (1993b); Lal, A. S. *et al.* (1999)). The level of phosphorylated p67^{phox} was correlated with NADPH oxidase activity, and continuous phosphorylation of p67^{phox} was necessary to maintain NADPH activity at a high level (Dusi, S. and Rossi, F. (1993b)). Thus, the regulation of the NADPH oxidase is a complex, multistep process involving several signaling intermediates.

Disruptions in NADPH oxidase function leads to alterations in other cellular processes, since ROI, too can serve as second messengers. Specifically, inhibition of NADPH oxidase activity interferes with the normal signals induced by binding of growth factors to their receptors on normal fibroblasts (Ammendola, R. *et al.* (2002)). Additionally, disruption of NADPH oxidase activity

reduces NF- κ B activation, and alternatively, in some cells, NADPH oxidase activity enhances NF- κ B activation (Brar, S. S. *et al.* (2002); Koay, M. A. *et al.* (2001)).

Figure3. Diagram of the NADPH Oxidase

Bactericidal activity

Listeria monocytogenes is a facultative intracellular pathogen, which is widely used as a model organism for examining protective immune responses in mice. *Listeria* infects a number of cell types, including macrophages, hepatocytes, epithelial cells and fibroblasts. The bacterium escapes from the phagosome of phagocytic cells and multiplies in the cytoplasm. Cell-to-cell spread of *Listeria* occurs as the bacteria reorganize host cell actin filaments to propel them through the cytoplasm, and produce phospholipases which break down membranes, allowing the cells to invade adjacent cells thus avoiding immune effectors. Importantly, macrophages exposed to IFN- γ become intolerant to *Listeria*, retain them in phagosomes and kill the bacteria. Roles have also been established for TNF- α , IL-6, IL-1 and others (reviewed in Mocci *et al.*, 1997). In fact resistance to *Listeria* is quite complex, involving different cells at different stages of infection, numerous cytokines and free radicals (for reviews see Mocci *et al.*, 1997 and Unanue, 1997; Alvarez-Dominguez, *et al.* (2000); Endres, *et al.* (1997); Fehr, *et al.* (1997); Gregory & Wing (1993); Inoue, *et al.* (1995); Ohya, *et al.* (1998)).

It is clear that proper NADPH oxidase function contributes to the ability of phagocytes to effectively kill internalized bacteria. Contribution of ROI and RNI (reactive oxygen and nitrogen intermediates, respectively) to the destruction of *Listeria monocytogenes* has been debated in the literature. Dinanuer *et al.* showed that mice lacking NADPH oxidase activity, by gp91^{phox} deletion, were more susceptible to *Listeria* infection (Dinanuer, M. C. *et al.* (1997)). Alternatively, Endres *et al.* (1997) used a p47^{phox} ^{-/-} mouse model and demonstrated that these mice were relatively resistant to listeriosis. Therefore the overall contribution of ROI to *Listeria* killing is questionable. It is possible that mice lacking p47^{phox} somehow compensate for this defect, although previous

characterization of these mice confirm that they are unable to generate ROI (Jackson, S. H. *et al.* (1995)).

TNF- α has also been shown to be indispensable for resistance to *Listeria* infections *in vivo* (Havell, E. A. (1989)). Nakane *et al.* (1998) demonstrated that sublethal doses of *Listeria* were fatal in mice whose endogenous TNF- α production was neutralized by injection of anti-TNF- α antibodies. Nakane and colleagues went on to demonstrate that the roles of endogenous TNF- α and IFN γ differed in primary and secondary listeriosis, and that neutralization of either cytokine early in the infection was accompanied by suppression of antilisterial resistance (Nakane, A. *et al.* (1989)). Furthermore, antilisterial effects were augmented by addition of recombinant human TNF- α *in vivo*, and the TNF- α -treated macrophages showed enhanced listericidal abilities and superoxide production *in vitro* (Kato, K. *et al.* (1989)).

REFERENCES

- Abo, A., Webb, M. R., Grogan, A., and Segal, A. W. (1994). Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem.J.* **298 Pt 3**, 585-591.
- Adams, D. O. and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annu.Rev.Immunol.* **2**, 283-318.
- Aderem, A. and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu.Rev.Immunol.* **17**, 593-623.
- Alvarez-Dominguez, C., Carrasco-Marin, E., Lopez-Mato, P. and Leyva-Cobian, F. (2000). The contribution of both oxygen and nitrogen intermediates to the intracellular killing mechanisms of C1q-opsonized *Listeria monocytogenes* by the macrophage-like IC-21 cell line. *Immunol.* **101**, 83-89.
- Ammendola, R., Ruocchio, M. R., Chirico, G., Russo, L., De Felice, C., Esposito, F., Russo, T., and Cimino, F. (2002). Inhibition of NADH/NADPH oxidase affects signal transduction by growth factor receptors in normal fibroblasts. *Arch.Biochem.Biophys.* **397**, 253-257.

- Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1995). Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. *Mol.Cell Biol.* **15**, 2689-2696.
- Barnett, J. B. and Gandy, J. (1989). Effect of acute propanil exposure on the immune response of C57Bl/6 mice. *Fundam.Appl.Toxicol.* **12**, 757-764.
- Barnett, J. B., Gandy, J., Wilbourn, D., and Theus, S. A. (1992). Comparison of the immunotoxicity of propanil and its metabolite, 3,4-dichloroaniline, in C57Bl/6 mice. *Fundam.Appl.Toxicol.* **18**, 628-631.
- Bartha, R., and Pramer, D. (1970). Metabolism of acylanilide herbicides. *Adv.Appl. Microbiol.* **13**, 317-341.
- Berridge, M. J. (1997). The 1996 Massry Prize. Inositol trisphosphate and calcium: two interacting second messengers. *Am.J Nephrol.* **17**, 1-11.
- Berridge, M. J. and Dupont, G. (1994). Spatial and temporal signalling by calcium. *Curr.Opin.Cell Biol.* **6**, 267-274.
- Blyler, G., Landreth, K. S., Lillis, T., Schafer, R., Theus, S. A., Gandy, J. and Barnett, J. B. (1994). Selective myelotoxicity of propanil. *Fundam.Appl.Toxicol.* **22**, 505-510.

- Brar, S. S., Kennedy, T. P., Sturrock, A. B., Huecksteadt, T. P., Quinn, M. T., Murphy, T. M., Chitano, P., and Hoidal, J. R. (2002). NADPH oxidase promotes NF-kappaB activation and proliferation in human airway smooth muscle. *Am.J Physiol Lung Cell Mol.Physiol* **282**, L782-L795.
- Cammarano, M. S. and Minden, A. (2001). Dbl and the Rho GTPases activate NF kappa B by I kappa B kinase (IKK)-dependent and IKK-independent pathways. *J Biol.Chem.* **276**, 25876-25882.
- Cannon, S. B. (1978.) Epidemic kepone poisoning in chemical workers. *Am.J.Epidemiol.* **107**, 529-537.
- Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986). Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc.Natl.Acad.Sci.U.S.A* **83**, 1670-1674.
- Carter, A. B., Monick, M. M., and Hunninghake, G. W. (1998). Lipopolysaccharide-induced NF-kappaB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC- dependent. *Am.J.Respir.Cell Mol.Biol.* **18**, 384-391.
- Cheng, J. D., Ryseck, R. P., Attar, R. M., Dambach, D., and Bravo, R. (1998). Functional redundancy of the nuclear factor kappa B inhibitors I kappa B alpha and I kappa B beta. *J Exp.Med.* **188**, 1055-1062.

- Chisaka, H., Kearney, P. C. (1970). Metabolism of propanil in soils. *J Agric. Food Chem.* **18**, 854-858.
- Chu, W., Gong, X., Li, Z., Takabayashi, K., Ouyang, H., Chen, Y., Lois, A., Chen, D. J., Li, G. C., Karin, M., and Raz, E. (2000). DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909-918.
- Collart, M. A., Baeuerle, P., and Vassalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol.Cell Biol.* **10**, 1498-1506.
- Cuff, C. F., Zhao, W., Nukui, T., Schafer, R., and Barnett, J. B. (1996). 3,4-Dichloropropionanilide-induced atrophy of the thymus: mechanisms of toxicity and recovery. *Fundam.Appl.Toxicol.* **33**, 83-90.
- Dahchour, A., Bitton, G., Coste, C. M., and Bastide, J. (1986). Degradation of the herbicide propanil in distilled water. *Bull.Environ.Contam Toxicol.* **36**, 556-562.
- Dahlgren, C. and Karlsson, A. (1999). Respiratory burst in human neutrophils. *J.Immunol.Methods* **232**, 3-14.
- Delhase, M., Li, N., and Karin, M. (2000). Kinase regulation in inflammatory response. *Nature* **406**, 367-368.

- Dinauer, M. C., Deck, M. B., and Unanue, E. R. (1997). Mice lacking reduced nicotinamide adenine dinucleotide phosphate oxidase activity show increased susceptibility to early infection with *Listeria monocytogenes*. *J.Immunol.* **158**, 5581-5583.
- Drouet, C., Shakhov, A. N., and Jongeneel, C. V. (1991). Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor- α promoter in primary macrophages. *J.Immunol.* **147**, 1694-1700.
- Dusi, S., Della, B., V, Grzeskowiak, M., and Rossi, F. (1993a). Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca^{2+} -depleted human neutrophils. *Biochem.J.* **290**, 173-178.
- Dusi, S. and Rossi, F. (1993b). Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox. *Biochem.J.* **296**, 367-371.
- Endres, R., Luz, A., Schulze, H., Neubauer, H., Futterer, A., Holland, S. M., Wagner, H. and Pfeffer, K. (1997). Listeriosis in p47(phox $^{-/-}$) and TRp55 $^{-/-}$ mice: protection despite absence of ROI and susceptibility despite presence of RNI. *Immunity.* **7**, 419-432.
- Fabacher, D. L. and Chambers, H. (1971). A possible mechanism of insecticide resistance in mosquitofish. *Bull.Environ.Contam Toxicol.* **6**, 372-376.

- Fehr, T., Schoedon, G., Odermatt, B., Holtschke, T., Schneemann, M., Bachmann, M. F., Mak, T. W., Horak, I. and Zinkernagel, R. M. (1997). Crucial role of interferon consensus sequence binding protein, but neither of interferon regulatory factor 1 nor of nitric oxide synthesis for protection against murine listeriosis. *J.Exp.Med.* **185**, 921-931.
- Feuillard, J., Gouy, H., Bismuth, G., Lee, L. M., Debre, P., and Korner, M. (1991). NF-kappa B activation by tumor necrosis factor alpha in the Jurkat T cell line is independent of protein kinase A, protein kinase C, and Ca(2+)-regulated kinases. *Cytokine*. **3**, 257-265.
- Folland, D. S. (1978). Acute hemorrhagic cystitis: Industrial exposure to the pesticide chlordimeform. *J.Am.Med.Assoc.* **239**, 1052-1055.
- Geppert, T. D., Whitehurst, C. E., Thompson, P., and Beutler, B. (1994). Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. *Mol.Med.* **1**, 93-103.
- Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* **109**, S81-S96.
- Gillooly, D. J., Simonsen, A., and Stenmark, H. (2001). Phosphoinositides and phagocytosis. *J Cell Biol.* **155**, 15-17.
- Govoni, G. and Gros, P. (1998). Macrophage NRAMP1 and its role in resistance to microbial infections. *Inflamm.Res.* **47**, 277-284.

- Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002). Capacitative Ca²⁺ influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase. *J.Leukoc.Biol.* **71**, 611-617.
- Gregory, S. H. and Wing, E. J. (1993). IFN-gamma inhibits the replication of *Listeria monocytogenes* in hepatocytes. *J.Immunol.* **151**,1401-1409.
- Guha, M. and Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cell Signal.* **13**, 85-94.
- Havell, E. A. (1989). Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J.Immunol.* **143**, 2894-2899.
- Inoue, S., Itagaki, S. and Amano, F. (1995). Intracellular killing of *Listeria monocytogenes* in the J774.1 macrophage-like cell line and the lipopolysaccharide (LPS)-resistant mutant LPS1916 cell line defective in the generation of reactive oxygen intermediates after LPS treatment. *Infect.Immun.* **63**, 1876 – 1886.
- Jackson, S. H., Gallin, J. I., and Holland, S. M. (1995). The p47phox mouse knock-out model of chronic granulomatous disease. *J Exp.Med.* **182**, 751-758.
- Jiang, Q., Akashi, S., Miyake, K., and Petty, H. R. (2000). Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. *J Immunol* **165**, 3541-3544.

- Jonas, L., Schutt, C., Neels, P., Walzel, H., and Siegl, E. (1989). Electron microscopic visualization of receptor internalization induced by a monoclonal antibody recognizing the monocyte specific glycoprotein CD 14. *Acta Histochem.* **85**, 167-173.
- Jonas, L., Schutt, C., Neels, P., Walzel, H., and Siegl, E. (1990). Electron microscopic study of receptor mediated endocytosis of a monoclonal antibody (RoMo-1) against the surface marker CD 14 of human monocytes. *Acta Histochem.Suppl* **39**, 339-344.
- Jongeneel, C. V. (1994). Regulation of the TNF alpha gene. *Prog.Clin.Biol.Res.* **388**, 367-381.
- Kato, K., Nakane, A., Minagawa, T., Kasai, N., Yamamoto, K., Sato, N., and Tsuruoka, N. (1989). Human tumor necrosis factor increases the resistance against *Listeria* infection in mice. *Med.Microbiol.Immunol.(Berl)* **178**, 337-346.
- Kielian, T. L. and Blecha, F. (1995). CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology* **29**, 187-205.
- Kimbrough, R. D. (1980). Human health effects of selected pesticides, chloroaniline derivatives. *J Environ.Sci.Health B.* **15**, 977-992.
- Koay, M. A., Christman, J. W., Segal, B. H., Venkatakrishnan, A., Blackwell, T. R., Holland, S. M., and Blackwell, T. S. (2001). Impaired pulmonary NF-kappaB activation in response to lipopolysaccharide in NADPH oxidase-deficient mice. *Infect.Immun.* **69**, 5991-5996.

- Kosanke, G. J., Schwippert, W. W., and Beneke, T. W. (1988). The impairment of mobility and development in freshwater snails (*Physa fontinalis* and *Lymnaea stagnalis*) caused by herbicides. *Comp Biochem. Physiol C*. **90**, 373-379.
- Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R., and Nedospasov, S. A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol* **162**, 4045-4052.
- Lal, A. S., Parker, P. J., and Segal, A. W. (1999). Characterization and partial purification of a novel neutrophil membrane-associated kinase capable of phosphorylating the respiratory burst component p47phox. *Biochem.J* **338**, 359-366.
- Lanzilotta, R. P., and Pramer, D. (1970). Herbicide transformation. I. Studies with whole cells of *Fusarium solani*. *Appl. Microbiol.* **19**, 301-306.
- Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E., and Lennartz, M. R. (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J Immunol* **165**, 2809-2817.
- Leitman, D. C., Mackow, E. R., Williams, T., Baxter, J. D., and West, B. L. (1992). The core promoter region of the tumor necrosis factor alpha gene confers phorbol ester responsiveness to upstream transcriptional activators. *Mol. Cell Biol.* **12**, 1352-1356.

- Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp.Med.* **189**, 1839-1845.
- Liu, M. K., Herrera-Velit, P., Brownsey, R. W., and Reiner, N. E. (1994). CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. *J Immunol* **153**, 2642-2652.
- Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001). IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol.Chem.* **276**, 45225-45235.
- Malerba, I., Castoldi, A. F., Parent-Massin, D., and Gribaldo, L. (2002). In vitro myelotoxicity of propanil and 3,4-dichloroaniline on murine and human CFU-E/BFU-E progenitors. *Toxicol.Sci.* **69**, 433-438.
- Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T., and Grinstein, S. (2001). Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis. *J Cell Biol.* **153**, 1369-1380.
- Mateunaka, S. (1968). Propanil hydrolysis: inhibition in rice plants by insecticides. *Science* **160**, 1360-1361

- May, M. J. and Ghosh, S. (1998). Signal transduction through NF-kappa B. *Immunol Today* **19**, 80-88.
- McClure, G. Y., Helm, R. M., Stine, K., Burks, A. W., Jones, S. M., and Gandy, J. (2001). Evaluation of immune parameters in propanil-exposed farm families. *Arch.Environ.Contam Toxicol.* **41**, 104-111.
- McCorkle, F. M., Chambers, J. E., and Yarbrough, J. D. (1977). Acute toxicities of selected herbicides to fingerling channel catfish, *Ictalurus punctatus*. *Bull.Environ.Contam Toxicol.* **18**, 267-270.
- McMillan, D. C., McRae, T. A., and Hinson, J. A. (1990). Propanil-induced methemoglobinemia and hemoglobin binding in the rat. *Toxicol.Appl.Pharmacol.* **105**, 503-507.
- Mocci, S., Dairymple, S. A., Nishinakamura, R. and Murray, R. (1997). The cytokine stew and innate resistance to *L. monocytogenes*. *Immunol.Rev.* **158**,107-114.
- Moreira, A. L., Sampaio, E. P., Zmuidzinas, A., Frindt, P., Smith, K. A., and Kaplan, G. (1993). Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. *J Exp.Med.* **177**, 1675-1680.
- Morse, D. L., Baker, E. L., Jr., Kimbrough, R. D., and Wisseman, C. L., III (1979). Propanil-chloracne and methomyl toxicity in workers of a pesticide manufacturing plant. *Clin.Toxicol.* **15**, 13-21.

- Movitz, C., Sjolín, C., and Dahlgren, C. (1997). A rise in ionized calcium activates the neutrophil NADPH-oxidase but is not sufficient to directly translocate cytosolic p47phox or p67phox to b cytochrome containing membranes. *Inflammation* **21**, 531-540.
- Nakane, A., Minagawa, T., and Kato, K. (1988). Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. *Infect.Immun.* **56**, 2563-2569.
- Nakane, A., Minagawa, T., Kohanawa, M., Chen, Y., Sato, H., Moriyama, M., and Tsuruoka, N. (1989). Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. *Infect.Immun.* **57**, 3331-3337.
- Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc.Natl.Acad.Sci.U.S.A.* **95**, 3537-3542.
- Nemoto, S., DiDonato, J. A., and Lin, A. (1998). Coordinate regulation of IkappaB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-kappaB-inducing kinase. *Mol.Cell Biol.* **18**, 7336-7343.
- Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

- Ohya, S., Tanabe, Y., Makino, M., Nomura, T., Xiong, H., Arakawa, M., and Mitsuyama, M. (1998). The contributions of reactive oxygen intermediates and reactive nitrogen intermediates to listericidal mechanisms differ in macrophages activated pre- and postinfection. *Infect.Immun.* **66**,4043-4049.
- Park, J. W. and Babior, B. M. (1992). The translocation of respiratory burst oxidase components from cytosol to plasma membrane is regulated by guanine nucleotides and diacylglycerol. *J.Biol.Chem.* **267**, 19901-19906.
- Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-2088.
- Pope, R., Mungre, S., Liu, H., and Thimmapaya, B. (2000). Regulation of TNF-alpha expression in normal macrophages: the role of C/EBPbeta. *Cytokine* **12**, 1171-1181.
- Pope, R. M., Leutz, A., and Ness, S. A. (1994). C/EBP beta regulation of the tumor necrosis factor alpha gene. *J Clin.Invest* **94**, 1449-1455.
- Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999). Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp.Med.* **189**, 615-625.

- Reinmann, T., Buscher, D., Hipskind, R. A., Krautwald, S., Lohmann, M. M. and Baccarini, M. (1994). Lipopolysaccharide induces activation of the Far-1/MAPkinase pathway. A putative role for Raf-1 in the induction of the IL-1beta and the TNF-alpha genes. *J Immunol.* **153**, 5740-5749.
- Richards, S. M., McClure, G. Y., Lavy, T. L., Mattice, J. D., Keller, R. J., and Gandy, J. (2001). Propanil (3,4-dichloropropionanilide) particulate concentrations within and near the residences of families living adjacent to aerially sprayed rice fields. *Arch.Environ.Contam Toxicol.* **41**, 112-116.
- Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999). Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. *J Biol.Chem.* **274**, 9108-9115.
- Rothwarf, D. M. and Karin, M. (1999). The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci.STKE.* **1999**, RE1.
- Saijo, K., Mecklenbrauker, I., Santana, A., Leitger, M., Schmedt, C., and Tarakhovsky, A. (2002). Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha. *J Exp.Med.* **195**, 1647-1652.
- Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999). IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol.Chem.* **274**, 30353-30356.

- Santillo, M., Rippa, C., Morte, R. D., Villani, G. R. D, Santangelo, F., Staiano, N., and Mondola, P. (1995). Enhancement of tissue lipoperoxidation in propanil-treated rats. *Toxicol.Letters* **78**, 215-218.
- Sariban, E., Imamura, K., Luebbers, R., and Kufe, D. (1988). Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. *J Clin.Invest* **81**, 1506-1510.
- Schmidt-Suppran, M., Bloch, W., Courtois, G., Addicks, K., Israel, A., Rajewsky, K., and Pasparakis, M. (2000). NEMO/IKK gamma-deficient mice model incontinentia pigmenti. *Mol.Cell* **5**, 981-992.
- Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990). Structure and function of lipopolysaccharide binding protein. *Science* **249**, 1429-1431.
- Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., and Karin, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**, 1495-1499.
- Shaffer, A. L., Rosenwald, A., Hurt, E. M., Giltane, J. M., Lam, L. T., Pickeral, O. K., and Staudt, L. M. (2001). Signatures of the immune response. *Immunity*. **15**, 375-385.

- Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jongeneel, C. V. (1990). Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J.Exp.Med.* **171**, 35-47.
- Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999). Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol.Cell Biol.* **19**, 6367-6378.
- Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp.Med.* **189**, 1777-1782.
- Singleton, S. D. and Murphy, S. D. (1973). Propanil (3,4-dichloropropionanilide)-induced methemoglobin formation in mice in relation to acylamidase activity. *Toxicol.Appl.Pharmacol.* **25**, 20-29.
- Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., and Stark, G. R. (2002). Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. *J Biol.Chem.* **277**, 3863-3869.
- Solomon, K. A., Pesti, N., Wu, G., and Newton, R. C. (1999). Cutting edge: a dominant negative form of TNF-alpha converting enzyme inhibits proTNF and TNFRII secretion. *J Immunol* **163**, 4105-4108.

- Stefanova, I., Horejsi, V., Ansotegui, I. J., Knapp, W., and Stockinger, H. (1991). GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* **254**, 1016-1019.
- Stephens, L., Ellson, C., and Hawkins, P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr.Opin.Cell Biol.* **14**, 203-213.
- Still, C. C. (1968). Metabolism of 3,4-dichloropropionanilide in plants: the metabolic fate of the 3,4-dichloroaniline moiety. *Science* **159**, 992-993.
- Still, C. C. and Kuzirian, O. (1967). Enzyme detoxication of 3',4'-dichloropropionanilide in rice and barnyard grass, a factor in herbicide selectivity. *Nature* **216**, 799-800.
- SuYang, H., Phillips, R., Douglas, I., and Ghosh, S. (1996). Role of unphosphorylated, newly synthesized I kappa B beta in persistent activation of NF-kappa B. *Mol.Cell Biol.* **16**, 5444-5449.
- Sweet, M. J. and Hume, D. A. (1996). Endotoxin signal transduction in macrophages. *J Leukoc. Biol.* **60**, 8-26.
- Tam, W. F. and Sen, R. (2001). IkappaB family members function by different mechanisms. *J Biol.Chem.* **276**, 7701-7704.

- Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995). I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell* **80**, 573-582.
- Tran, K., Merika, M., and Thanos, D. (1997). Distinct functional properties of IkappaB alpha and IkappaB beta. *Mol.Cell Biol.* **17**, 5386-5399.
- Trede, N. S., Tsytsykova, A. V., Chatila, T., Goldfeld, A. E., and Geha, R. S. (1995). Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. *J Immunol* **155**, 902-908.
- Tsai, E. Y., Yie, J., Thanos, D., and Goldfeld, A. E. (1996). Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol.Cell Biol.* **16**, 5232-5244.
- Unanue, E. R. (1997). Why listeriosis? A perspective on cellular immunity to infection. *Immunol.Rev.* **158**, 5-9.
- Unanue, E. R. (1981). The regulatory role of macrophages in antigenic stimulation. Part Two: symbiotic relationship between lymphocytes and macrophages. *Adv.Immunol* **31**, 1-136.

- Vogel, S. N., Johnson, D., Perera, P. Y., Medvedev, A., Lariviere, L., Qureshi, S. T., and Malo, D. (1999). Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an Lpsn gene: in vivo evidence for a dominant negative mutation. *J.Immunol.* **162**, 5666-5670.
- Watson, V. A., Barnett, J. B., and Schafer, R. (2000). In vivo cytokine production and resistance to infection after acute exposure to 3,4-dichloropropionaniline. *J.Toxicol.EnvIRON.Health A.* **60**, 391-406.
- Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol.Appl.Pharmacol.* **145**, 184-191.
- Yang, H., Young, D. W., Gusovsky, F., and Chow, J. C. (2000). Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1. *J Biol.Chem.* **275**, 20861-20866.
- Ye, R. D. (2001). Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc.Biol.* **70**, 839-848.

- Zagariya, A., Mungre, S., Lovis, R., Birrer, M., Ness, S., Thimmapaya, B., and Pope, R. (1998). Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta-induced activation by c-Jun. *Mol.Cell Biol.* **18**, 2815-2824.
- Zeyer, J. and Kearney, P.C. (1984). Degradation of o-nitrophenol and m-nitrophenol by a *Pseudomonas putida*. *J. Agric. Food Chem.* **32**, 238-242.
- Zhao, Q. and Lee, F. S. (1999). Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J Biol.Chem.* **274**, 8355-8358.
- Zhao, W., Schafer, R., and Barnett, J. B. (1998). Cytokine production by C57BL/6 mouse spleen cells is selectively reduced by exposure to propanil. *J Toxicol.Environ.Health A.* **55**, 107-120.
- Zhao, W., Schafer, R., Cuff, C. F., Gandy, J., and Barnett, J. B. (1995). Changes in primary and secondary lymphoid organ T-cell subpopulations resulting from acute in vivo exposure to propanil. *J.Toxicol.Environ.Health* **46**, 171-181.
- Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol.Cell* **9**, 625-636.
- Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol.Cell* **1**, 661-671.

Chapter 1

Propanil inhibits tumor necrosis factor-alpha production by reducing nuclear levels of the transcription factor nuclear factor-kappa B in the macrophage cell line IC-21.

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ABSTRACT

Propanil inhibits tumor necrosis factor- α production by reducing nuclear levels of the transcription factor, nuclear factor-kappa B. **L.L. Frost, Y.X. Neeley, R. Schafer, L.F. Gibson, and J.B. Barnett, Toxicol. Applied Pharmacol.**

Lipopolysaccharide (LPS) stimulated macrophages produced less tumor necrosis factor- α (TNF- α) protein and message after treatment with the herbicide propanil (Xie et al., 1997). This effect was consistently observed with both native peritoneal macrophages and the macrophage cell line, IC-21 (Xie et al., 1997). Nuclear factor- κ B (NF- κ B) tightly regulates TNF- α transcription. Therefore, as a step toward understanding the mechanism of the effect of propanil on TNF- α transcription, IC-21 cells were transfected with a TNF- α promoter-luciferase construct and the effect of propanil on luciferase activity was measured. Cells transfected with promoter constructs containing a κ B site showed decreased luciferase activity relative to controls after propanil treatment. These observations implicate NF- κ B binding as an intracellular target of propanil. Further studies demonstrated a marked reduction in the nuclear levels of the stimulatory p65/p50 heterodimeric form of NF- κ B after propanil treatment; as measured by fluorescence confocal microscopy and Western blot analysis. The homodimeric p50/p50 form of NF- κ B, which exerts an inhibitory effect on TNF- α transcription, was not reduced after propanil exposure. Electrophoretic mobility gel shift assays (EMSA) showed significantly less DNA binding of p65/p50 NF- κ B to the TNF- α promoter of propanil-treated cells. The marked reduction in nuclear p65/p50 NF- κ B levels and TNF- α promoter binding in propanil-treated cells are consistent with reduced TNF- α levels induced by LPS.

Keywords: Propanil, Macrophage, IC-21 cells, Tumor Necrosis Factor- α , NF- κ B

INTRODUCTION

Propanil, 3,4-dichloropropionaniline, is a herbicide commonly used in the United States to control weeds in the production of rice. Immunotoxic effects of propanil include inhibition of T-dependent and T-independent antibody responses, reductions in mixed lymphocyte responses and reductions in cytokine production from splenocytes and a T cell line (Barnett and Gandy, 1989; Theus *et al.*, 1993; Zhao *et al.*, 1998; Zhao *et al.*, 1999). In macrophages, propanil reduces tumor necrosis factor - α (TNF- α) and interleukin (IL)-6 protein and message production (Xie *et al.*, 1997). These cytokine reductions were apparent in ex vivo-cultured spleen and peritoneal exudate (PE) cells (macrophages) seven days after in vivo exposure. Functionally identical effects were also noted following in vitro propanil exposure of harvested spleen and PE cells from non-treated animals as well as comparable T cell lines (Zhao *et al.*, 1999).

TNF- α production is tightly regulated at the transcriptional and translational levels. Gene expression is mainly regulated by binding of the transcription factor nuclear factor- κ B (NF- κ B). The TNF- α promoter contains four κ B binding sites, with the importance of NF- κ B in the transcription of TNF- α well documented (Albrecht *et al.*, 1995; Collart *et al.*, 1990; Drouet *et al.*, 1991; Shakhov *et al.*, 1990). The p65 and p50 proteins dimerize and bind DNA to affect transcription. The most common dimeric forms are p65/p50 and p50/p50. Heterodimers of p65/p50 are capable of activating the TNF- α promoter, whereas p50/p50 homodimers are inhibitory (Ziegler-Heitbrock, 1995; Baer *et al.*, 1998; Lui *et al.*, 2000). NF- κ B is retained in the cytoplasm of quiescent or non-stimulated cells by the binding of an inhibitory protein, I κ B, to NF- κ B which masks the nuclear localization signal (NLS). Stimulation of macrophages with bacterial lipopolysaccharide (LPS) results in rapid activation of an I κ B Kinase (IKK) complex, which mediates the phosphorylation of I κ B (reviewed in Rothwarf and Karin, 1999). Phosphorylation of I κ B targets it

for ubiquitination and degradation via the 26S proteasome. The degradation of I κ B unmasks the NLS on NF- κ B allowing transport into the nucleus. Compounds which inhibit I κ B phosphorylation or degradation have been shown to inhibit NF- κ B activation and TNF- α transcription (Kopp and Ghosh, 1994; Schow and Joly, 1997; Shackelford *et al.*, 1997).

NF- κ B was initially described as the prototypical p65/p50 heterodimer (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990). However, it is actually a family of proteins including RelA (p65); cRel; p105, which is processed to p50; and p100, which is processed to p52. The roles of two family members investigated in this report have been partially elucidated by gene deletion studies. Deletion of the p65 gene is lethal and p65 knockout mice die at day 16 of gestation (Beg *et al.* 1995). However, p105 knockouts are viable with few serious consequences (Sha *et al.* 1995). Mice deficient in p105, and hence the p50 cleavage product, exhibit defective antibody isotype switching and decreased levels of serum IgA, IgE and IgG1. These mice have increased susceptibility to bacterial infections, but increased resistance to viral infection. Thus, p50 seems to be important in regulating immune responses.

We focused on the effects of propanil on transcription of the TNF- α gene, and its regulation by NF- κ B. Our data show that propanil treated macrophages have reduced nuclear levels of NF- κ B as well as p65/p50 DNA binding, consistent with observed reductions in TNF- α production. Given the role of NF- κ B in transcription of numerous cytokine genes, the effects of propanil on NF- κ B may be a major mechanism of the immunotoxicity of the compound.

METHODS

Cell culture, stimulation and propanil treatment. The macrophage cell line, IC-21, was cultured in 5% CO₂ at 37°C as monolayers in RPMI 1640 (BioWhittaker, Walkerville, MD). The media was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), as well as L-

glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-mercaptoethanol (5×10^{-5} M) and HEPES buffer (10 mM); all obtained from BioWhittaker. Cells were stimulated for various times with 10 µg/ml LPS (Sigma Chemical, St. Louis, MO) in the presence of propanil or its vehicle, ethanol. Propanil (3,4 dichloropropionaniline), 99% pure, was purchased from ChemService, Inc. (West Chester, PA). Appropriate concentrations of propanil were dissolved in 95% ethanol, and added to cells at 0, 66, 167, 336, or 672 µM. The maximum dose chosen (672 µM) was one-half the concentration (1.3 mM) previously determined to significantly reduce cell viability as determined by the standard trypan blue exclusion assay (data not shown). All concentrations of propanil were determined by trypan blue exclusion to be nontoxic to cells and most experiments reported here used the lower doses of 66 or 167 µM. The final ethanol concentration added to all cultures was 0.33%; control cultures received equal concentrations of ethanol.

L929 assay for TNF. Because propanil interfered with the TNF ELISA, TNF levels in the IC-21 cells (Figure 1) was determined using a bioassay. TNF-sensitive L929 cells were plated in 96-well plates at a concentration of 5×10^5 in 100 µl. After 24 hours incubation at 37° C, 5% CO₂, the culture media was removed. To determine the quantity of TNF present by bioactivity, 50 µl of medium, together with 50 µl of either recombinant TNF-α (positive control) or sample was added to each well. Fifty microliters of actinomycin D solution (8 µg/ml) was also added to each well to stop the proliferation of L929 cells. The plates were incubated for 18 hours in a 37° C, 5% CO₂, humidified incubator. To measure TNF-induced cell death, supernatant from each well was then removed, and the cells washed once with 0.9% saline. Fifty microliters of 0.05% crystal violet in 20% ethanol was added to each well. The plates were incubated at room temperature for 10 minutes, thoroughly rinsed with cold tap water and then air dried for 2 hours. One hundred microliters of

100% methanol was added to each well. The plates were read immediately with a microtiter plate reader at an absorbance of 590 nm (BioTek Instruments, Winooski, VT).

Plasmids and transfections. IC-21 cells, at an initial density of 1×10^6 cells per well, were grown overnight in 6 well tissue culture plates (Costar, Corning, NY). The following day, they were transiently transfected with a control plasmid, PGL-3 (Promega, Madison, WS), TNF- α promoter-luciferase plasmids, or an NF- κ B-dependent plasmid (PBIIX) using FuGene6 (Boehringer Mannheim, Indianapolis, IN) at a 3:1 μ l FuGene6: μ l DNA ratio as per the manufacturer's protocol. Immediately after adding the FuGene6/DNA, plates were centrifuged for 3-5 minutes to facilitate transfection. The plates were then incubated at 37°C overnight. Cells were stimulated with 10 μ g/ml LPS in the presence or absence of 167 μ M propanil for the indicated times. Whole cell lysates were made using luciferase assay lysis buffer (Promega, Madison, WS), and luciferase activity was measured using luciferase assay reagent (Promega, Madison, WS) in a Berthold LB 9505C luminometer (Perkin Elmer, Gaithersburg, MD). Protein concentration of the lysates was determined using the bicinchoninic acid (BCA) protein determination method as per manufacturer's protocol (Pierce, Rockford, IL), and used to normalize luciferase activity. The NF- κ B-dependent reporter construct has been described previously (Kopp and Ghosh, 1994) and was a generous gift of Sankar Ghosh, Ph.D. of the Howard Hughes Medical Institute, Yale University. The TNF α promoter deletion constructs have been described previously (Baer *et al.*, 1998) and were a generous gift from Drs. Peter Johnson and Mark Baer of the National Cancer Institute, Frederick, MD.

Immunofluorescence. IC-21 macrophages were grown on coverslips overnight to approximately 70-90% confluence. The following day, cells were either not stimulated or stimulated with 10 μ g/ml LPS in the presence of either ethanol, 66 or 167 μ M propanil for 1-4 hours. Coverslips were rinsed 2 $\times$ in phosphate buffered saline (PBS), then the cells were fixed in a 1:1

methanol:acetone solution for 20 minutes, followed by blocking in PBS + 5% bovine serum albumin (BSA) for 30 minutes. Rabbit-anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), was added to the cells at 100 µg/ml in PBS and incubated at room temperature for 1 hour. Coverslips were washed 3× in PBS for 5 minutes each with gentle rotation. Goat-anti rabbit FITC, was added at 50 µg/ml in PBS for 1hour at room temperature and kept in the dark. Coverslips were washed 3× in PBS for 5 minutes each, then mounted to glass slides with Fluormount-G (Southern Biotechnology Associates, Inc, Birmingham, AL). Slides were kept in the dark until visualized by confocal microscopy (Zeiss LSM 510, Thornwood, NY, equipped with an argon laser).

Nuclear extracts. Nuclear extracts were prepared as previously described (Torgerson *et al.*, 1998), with minor adaptation. Briefly, 1×10^6 cells were stimulated with LPS and treated with propanil or ethanol for various times, as indicated. Cells were trypsinized, scraped from wells, placed in microcentrifuge tubes and pelleted by centrifugation for 15 seconds at 12,000×g. The cells were then washed once in PBS, and resuspended in 400 µl of a buffer consisting of 10 mM HEPES pH 7.9 at 4 °C, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol-*bis*(β-aminoethyl ether)N, N, N',N'-tetraacetic acid (EGTA), 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 1 µg/ml of protease inhibitor (leupeptin, aprotinin, antipain, chymostatin and pepstatin A) for 15 minutes on ice. The lysate was pelleted by centrifugation for 30 seconds, and the supernatant was collected and saved as the cytoplasmic extract. The pellet was resuspended in 55 µl of a 20 mM HEPES buffer containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT and 1µg/ml of the protease inhibitor (see above) for 15 minutes on ice. Debris was pelleted by centrifugation for 5 minutes, and the supernatant containing the nuclear fraction stored at -70°C. Total protein concentration was

determined for both cytoplasmic and nuclear extracts with Coomassie® Plus Protein Assay Reagent, as described by the manufacturer (Pierce, Rockford, IL).

Western Blotting. Nuclear extracts, 5 µg per treatment, were denatured in SDS/PAGE sample buffer (Laemmli, 1970) by boiling for 3 minutes. Samples were electrophoresed in 12% tris polyacrylamide gels with 4% stacking gels (BioRad, Hercules, CA) at 90 volts for 2 hours. Proteins were then transferred to PVDF membranes at 90 volts for 30 min. Western blotting was performed by incubating membranes with optimal concentrations of primary antibodies to NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) and p50, a generous gift from Dr. Nancy Rice, National Cancer Institute, Frederick, MD (Rice *et al.*, 1992) for 1 hour. Blots were then washed as follows: 1×10 minutes, 2×5 minutes, and 2×2.5 minutes in TBS+0.1% Tween 20. Blots were incubated for 1 hour with the optimal concentration of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then developed using Enhanced Chemiluminescence substrate (Amersham-Pharmacia Biotechnology, Piscataway, NJ) and visualized by exposing to X-Ray film (BioMax MR, Eastman Kodak Company, Rochester, NY) for 3 seconds to 10 minutes. Densitometric analysis was completed with Optimus software (Media Cybernetics, Silver Spring, MD). Statistical analyses between ethanol and propanil treatments for each time point were calculated by combining data from multiple experiments. Significance was established using the t test using SigmaStat software (SPSS Inc., Chicago, IL) with an alpha value of ≤0.05.

Electrophoretic Mobility Shift Assay (EMSA). An oligonucleotide corresponding to the κB3 sequence from the murine TNF-α promoter was labeled with γ-³²P (New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Amersham Pharmacia Biotechnology). The κB3 sequence from the TNF-α promoter is:

κB3: 5' GATCCACAGGGGGCTTTCCCTCCA
GTGTCCCCCGAAAGGGAGGTCTAG -3'

Nuclear extracts, 5 µg per sample, were incubated with 50,000 CPM of labeled probe for 30 minutes at room temperature in 1× binding buffer (5× stock: 250 mM NaCl, 50 mM Tris-Cl, 50% (v/v) glycerol, 5 mM DTT, 2.5 mM EDTA adjusted to pH 7.6, stored at –20 °C prior to use) to allow formation of band shift complexes. Supershift complexes were obtained by preincubating 5 µg of nuclear extracts in 1× binding buffer with NF-κB p65 specific antibody (Rockland Immunochemicals, Gilbertsville, PA) or p50 specific antibody (Santa Cruz Biotechnology) for 3 hours on ice before addition of the labeled probe for an additional 30 minutes at room temperature. Complexes were resolved from free probe in polyacrylamide gels (5% polyacrylamide, 1× TGE, 5% glycerol, 0.075% ammonium persulfate and 0.1% TEMED). Samples were electrophoresed at 150 – 200v for 1.5 – 2 hours, dried on Whatman 3MM paper and placed on PhosphorImage screens for analysis with a Molecular Dynamics PhosphorImager (Sunnyvale, CA) and X-Ray film (Eastman Kodak Company).

RESULTS

Effect of Propanil on TNF- α production by IC-21 cells. In vitro exposure to propanil causes the reduction of TNF production in peritoneal exudate cells (Xie et al., 1997). To confirm that the IC-21 macrophage cell line would react in a similar manner, LPS stimulated cells were exposed to 0, 84, 167, 336, and 672 μ M propanil in culture. Peak production of TNF by vehicle-treated stimulated IC-21 cells occurred at 24 hours and propanil did not alter the time of peak production (data not shown). Cells exposed to ≥ 167 μ M propanil showed significantly less TNF production (Figure 4). Thus, the reduction in TNF production noted with peritoneal exudate cells after propanil exposure (Xie et al., 1997) is reproduced in the IC-21 cell line.

Propanil reduces NF- κ B transcriptional activity. Transcriptional activity of NF- κ B was assessed using the PBIIX construct (Kopp and Ghosh, 1994, Zhong et al. 1997), which contains two κ B binding sites that drive the expression of a luciferase reporter gene. IC-21 cells were transfected with the construct then stimulated with LPS in the presence of either ethanol or propanil. Normalized luciferase activity was reduced significantly from 5747 (± 947) in ethanol controls to 3054 (± 1089) in propanil-treated cells (Figure 5). Reduced luciferase activity produced from this construct is indicative of reduced NF- κ B transcriptional activity.

Effect of Propanil on the TNF- α Promoter. The effect of propanil on the TNF- α promoter was evaluated to determine if specific transcription factors involved in TNF- α transcription were attenuated by propanil. IC-21 cells were transiently transfected with luciferase reporter plasmid constructs containing either the full-length TNF- α promoter or 5' deletions (Figure 6A). Cells were later stimulated with 10 μ g/ml of LPS in the presence of either 0 or 167 μ M propanil. Luciferase activity was measured 18-24 hours after stimulation and normalized to protein concentration for each sample. Significant reductions in normalized luciferase activity were observed from constructs,

which contained NF- κ B binding sites (Figure 6B). Luciferase activity from the PGL3 luciferase control vector was not changed in the presence of propanil. The full length TNF- α -luc construct was reduced from 22,233 ($\pm$ 4,561) in vehicle controls to 209 ($\pm$ 158) units in the presence of propanil. Propanil also caused reductions in normalized luciferase activity from the other constructs, which contain NF- κ B binding sites. The normalized luciferase activity for the -646 construct was 786 ($\pm$ 341) in ethanol controls compared to 104 ($\pm$ 45) in propanil-treated cells. Propanil caused reductions in normalized luciferase activity for the -527 construct from 720 ($\pm$ 247) to 131 ($\pm$ 22) units in vehicle- and propanil-treated cells, respectively. Luciferase production was not significantly reduced by propanil in the -514 and -210 constructs, which do not contain NF- κ B binding sites. Therefore, propanil caused reductions in promoter activity from those regions of the TNF- α promoter that contain NF- κ B binding sites.

Effect of Propanil on cellular localization of the p65 subunit of NF- κ B. NF- κ B is quickly activated and mobilized into the nucleus upon cellular stimulation. To determine if propanil caused reductions in nuclear translocation of NF- κ B p65, IC-21 cells were grown on cover slips, and either not stimulated or stimulated with 10 μ g/ml of LPS in the presence of 0, 66, or 167 μ M propanil. The cells were fixed and stained for the p65 subunit of NF- κ B. Confocal microscopy was used to visualize the patterns of NF- κ B p65 staining (Figure 7). The relative staining of nuclear regions of unstimulated cells was less intense than the staining of nuclear regions of stimulated cells treated with vehicle. Staining patterns of LPS-stimulated cells treated with either 66 or 167 μ M propanil was similar to that of the non-stimulated cells. The less intense nuclear p65 staining in propanil-treated cells suggests that there is less NF- κ B present in the nuclear region of these cells versus vehicle-treated cells.

Propanil reduces nuclear NF- κ B levels. A more quantitative way to evaluate the effect of propanil on NF- κ B translocation to the nucleus is to directly examine the nuclear protein levels by western blotting. Nuclear extracts were prepared from IC-21 cells treated for 1 to 4 hours with either 0 or 167 μ M propanil and western blotting completed as described (Figure 8A). Nuclear levels of NF- κ B p65, as determined by densitometric analysis, decreased to 84 ($\pm$ 19), 71 ($\pm$ 12), and 59 ($\pm$ 22) percent of vehicle controls after 1 hour, 2 hours, and 4 hours of propanil exposure, respectively (Figure 8B). The reductions at 2 and 4 hours were statistically significant. However, nuclear levels of NF- κ B p50 were not significantly changed in propanil-treated cells (Figure 9A); 110 ($\pm$ 32), 102 ($\pm$ 13), and 81 ($\pm$ 16) percent of respective vehicle controls at 1 hour, 2 hours, and 4 hours of propanil treatment, respectively (Figure 9B). These data indicate that propanil significantly reduces nuclear levels of NF- κ B p65.

EMSA analysis of NF- κ B. The effect of propanil on DNA binding abilities of NF- κ B was evaluated by EMSA. The p65/p50 heterodimers and p50/p50 homodimers were characterized by supershifting the complexes with antibodies specific for the p65 or p50 subunit (Figure 10A). Propanil caused reductions in DNA binding of both p65/p50 heterodimers and p50/p50 homodimers at each time point (Figure 10B). The most dramatic reduction of p65/p50 NF- κ B was seen at 4 hours of propanil treatment. EMSA analysis using a consensus κ B oligonucleotide also demonstrated reductions in NF- κ B binding (data not shown). Therefore, propanil causes significant reductions in NF- κ B DNA binding of the p65/p50 heterodimers and modest decreases in p50/p50 homodimers.

DISCUSSION

The studies reported here were done with the macrophage cell line, IC-21. These cells, although not identical to normal macrophages, have reduced cytokine production after exposure to propanil as previously demonstrated in peritoneal exudate cells (Xie et al., 1997). They have the distinct advantage of providing a consistent source of cells that can be easily maintained and manipulated. In this study, we showed that propanil reduced TNF- α promoter activity. Our findings indicate that propanil affects regions of the TNF- α promoter that contain NF- κ B binding sites. Immunofluorescence experiments detected less NF- κ B p65 in nuclear regions of propanil as compared to vehicle controls. This finding was supported by western blot analysis, which indicated reductions in nuclear levels of p65 in propanil-treated cells. The effects on nuclear p50 levels were less dramatic. Furthermore, EMSA analysis using the κ B3 site from the murine TNF- α promoter confirmed the western blot data, also showing reductions in nuclear NF- κ B levels. EMSA experiments demonstrated reductions in both NF- κ B p65/p50 heterodimers and the p50/p50 homodimers at each time point. Transcriptional activity of NF- κ B was also found to be reduced using an NF- κ B-dependent-luciferase reporter construct. Taken together, these data indicate that propanil causes reductions in NF- κ B nuclear levels, NF- κ B DNA binding and NF- κ B-dependent gene transcription.

LPS-induced TNF- α transcription is NF- κ B dependent (Albrecht *et al.*, 1995; Drouet *et al.*, 1991; Shakhov *et al.*, 1990; Swantek *et al.*, 1999, Lui et al., 2000). The murine TNF- α promoter contains four κ B sites (Shakhov *et al.*, 1990). Compounds that block NF- κ B activation also inhibit LPS-induced TNF- α production. Aspirin, originally shown to inhibit NF- κ B activation by Kopp and Ghosh (1994), was further shown to inhibit LPS-induced TNF- α production by inhibiting NF- κ B activation (Osnes et al., 1996; Shackelford *et al.*, 1997; Yin et al., 1998). Aspirin inhibits NF- κ B

activation by binding to IKK β , which phosphorylates I κ B leading to its degradation and the translocation of NF- κ B (Yin et al., 1998). However, our preliminary data (not shown) do not indicate any change in I κ B degradation pattern in propanil treated cells, thus, it is probable that propanil exerts its effect through a different mechanism. Proteasome inhibitors, such as N-acetyl-leuciny-leuciny-norleucinal (ALLN), also prevent I κ B degradation, and therefore, interfere with NF- κ B activation and subsequently suppress TNF- α production (Schow and Joly, 1997). We are currently determining the effects of propanil on proteasome activity. However, as the I κ B degradation does not appear to differ in propanil treated cells (preliminary data; not shown), we do not anticipate that propanil will affect proteasome activity. Speculatively, it is possible that propanil interferes with the nuclear membrane transport mechanism (reviewed in Nakielnny and Dreyfuss, 1999), which could inhibit NF- κ B transport into the nucleus in spite of the normal release of NF- κ B from the I κ B:NF- κ B complex. Thus, our data provides further evidence that NF- κ B is critical for TNF- α promoter activation in macrophages, however, how propanil prevents NF- κ B translocation is still in question.

The murine κ B3 site, used in our EMSA experiments, was shown to strongly bind both NF- κ B p65/p50 heterodimers and p50/p50 homodimers (Baer *et al.*, 1998). Our EMSA data (Figure 10) demonstrated that propanil caused a significant decrease in DNA binding of p65/p50 heterodimers and modest decreases of p50/p50 homodimers. The p50/p50 form of NF- κ B is a transcriptional inhibitor (Baer *et al.*, 1998; Ziegler-Heitbrock, 1995; Liu et al., 2000) and has been speculated to be important in the down regulation of transcription (Liu et al., 2000). Liu et al. (2000) noted that as TNF- α transcription is initiated, the stimulatory p65/p50 heterodimeric form is the predominant binding species, however, as TNF- α transcription is down regulated (presumably to control the possible detrimental effects of over production of TNF- α), then the p50/p50 homodimer is the

predominant form. Our finding of a significant reduction in DNA binding levels of the p65/p50 form of NF- κ B may indicate that propanil may also interfere with the normal mechanisms to control transcription.

Our data demonstrate that propanil causes reductions in TNF- α promoter activity, specifically from those regions of the promoter that contain NF- κ B binding sites. Nuclear levels of NF- κ B and DNA binding are also repressed by propanil. Furthermore additional studies showed that the transcriptional activity of NF- κ B is also reduced. Therefore, the reduction of TNF- α production by murine macrophages after exposure to propanil may be due to its effects on the critical transcription factor, NF- κ B.

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Figure 4.

Effect of propanil on TNF production by IC-21 cells. IC-21 cells were plated at 3×10^6 cells/ml for TNF determinations. Stock solutions of propanil in 99% ethanol were added to cultures for a final concentration of 0, 24, 167, 336, or 672 μ M propanil. Cells were stimulated with 10 μ g/ml LPS. After 24 hours of culture, supernatants were removed from the culture and assayed for TNF content as described in the Materials and Methods. Unstimulated cells did not produce TNF. Statistical differences at $p < 0.05$ are indicated by an asterisk.

Figure 1.

↑
Up

Figure 5.

Propanil reduces luciferase production from an NF- κ B-dependant reporter construct. IC-21 cells were transfected with PBIIIX (see Methods), then stimulated with LPS in the presence of either ETOH or PRN. Cells were lysed and luciferase activity was measured using Luciferase Assay Reagent. Luciferase activity was normalized to protein concentration.

Figure 2

↑
Up

Figure 6.

Effect of propanil on the TNF- α promoter. A. Diagram of full length and 5' truncated TNF- α promoter constructs. B. TNF- α promoter constructs or a constitutive luciferase control (PGL3) were transiently transfected into IC-21 cells. After 24 hours to recover from transfection, cells were stimulated with 10 μ g/ml LPS and treated with 167 μ M propanil or vehicle for 24 hours. Whole cell lysates were prepared and assayed for luciferase production as described in Materials and Methods. Luciferase production was normalized to protein concentration. Statistical differences ($p < 0.05$) in normalized luciferase activity in propanil-treated lysates from the respective vehicle control are indicated with an asterisk.

Figure 3

Figure 7.

Immunofluorescence staining of NF- κ B p65. IC-21 cells were grown on coverslips overnight, then stimulated with 10 μ g/ml LPS and treated with 0, 66 or 167 μ M propanil for 1 hour. NF- κ B p65 was detected with a specific monoclonal antibody followed by a FITC-conjugated secondary antibody. Confocal microscopy was used for visualization as described in the Materials and Methods.

Figure 4

Figure 8.

Western blot analysis of NF- κ B p65. A. Nuclear extracts were prepared from IC-21 cells that were stimulated with 10 μ g/ml LPS and either 0 (-) or 167 μ M propanil (+) for the times indicated. B. X-Ray autographs were analyzed with Optimus software to determine the density of each band. Statistical analyses were done for vehicle and propanil treatments for each time point as previously described. The percent of the respective vehicle control for each time point is shown and significant differences ($P < 0.05$) are indicated with an asterisk.

Figure 5

Figure 9.

Western blot analysis of NF- κ B p50. A. Nuclear extracts were prepared from IC-21 cells that were stimulated with 10 μ g/ml LPS and either 0 (-) or 167 μ M propanil (+) for the times indicated. Western blots were performed as described in the Materials and Methods. B. Optimus software was used to determine the optical densities of bands corresponding to NF- κ B p50. Statistical analyses were done to compare vehicle and propanil treatments for each time point. The percent of the respective vehicle control for each time point was graphed; differences were not significant at any time point.

Figure 6.

A

B.

Figure 10.

EMSA analysis for NF- κ B. A. NF- κ B binding was determined by incubating 5 μ g of IC-21 nuclear extract with a 32 P-labeled dsDNA oligonucleotide probe corresponding to the κ B3 site of the murine TNF- α promoter (lane 1). Nuclear extracts were pre-incubated with antibodies specific for p65 or p50 for supershifts of p65/p50 heterodimers and p50/p50 homodimers (lanes 2 and 3, respectively). Nonspecific binding by the p65 and p50 antibodies was determined by incubating them with probe in the absence of nuclear proteins (lanes 4 and 5, respectively). B. Nuclear extracts were prepared from cells stimulated with LPS and treated with either 0 or 167 μ M propanil for the indicated times. EMSA analysis was performed as described in Methods.

Figure 7.

A.

B.

REFERENCES

- Albrecht, H., Schook, L.B., and Jongeneel, C.V.(1995). Nuclear migration of NF-kappa B correlates with TNF-alpha mRNA accumulation. *J.Inflamm.* **45**, 64-71.
- Baer, M., Dillner, A., Schwartz, R.C., Sedon, C., Nedospasov, S., and Johnson, P.F.(1998). Tumor necrosis factor alpha transcription in macrophages is attenuated by an autocrine factor that preferentially induces NF-kappaB p50. *Mol.Cell Biol.* **18**, 5678-5689.
- Baeuerle, P.A. and Baltimore, D.(1989). A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. *Genes Dev.* **3**, 1689-1698.
- Barnett, J.B. and Gandy, J.(1989). Effect of acute propanil exposure on the immune response of C57Bl/6 mice. *Fundam.Appl.Toxicol.* **12**, 757-764.
- Collart, M.A., Baeuerle, P., and Vassalli, P.(1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol.Cell Biol.* **10**, 1498-1506.
- Drouet, C., Shakhov, A.N., and Jongeneel, C.V.(1991). Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-alpha promoter in primary macrophages. *J.Immunol.* **147**, 1694-1700.
- Kopp, E. and S. Ghosh. 1994. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**, 956-959.
- Ghosh, S. and Baltimore, D.(1990). Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* **344**, 678-682.

- Laemmli, U.K.(1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Liu, H., P. Sidiropoulos, G. Song, L. J. Pagliari, M. J. Birrer, B. Stein, J. Anrather, and R. M. Pope. 2000. TNF-alpha gene expression in macrophages: regulation by NF-kappaB is independent of c-Jun or C/EBPbeta [In Process Citation]. *J Immunol.* **164**, 4277-4285.
- Nakielnny, S. and G. Dreyfuss. (1999). Transport of proteins and RNAs in and out of the nucleus. *Cell* **99**, 677-690.
- Osnes, L. T., K. B. Foss, G. B. Joo, C. Okkenhaug, A. B. Westvik, R. Ovstebo, and P. Kierulf. 1996. Acetylsalicylic acid and sodium salicylate inhibit LPS-induced NF-kappa B/c-Rel nuclear translocation, and synthesis of tissue factor (TF) and tumor necrosis factor alfa (TNF-alpha) in human monocytes. *Thromb.Haemost.* **76**, 970-976.
- Rice, N.R., MacKichan, M.L., and Israel, A.(1992). The precursor of NF-kappa B p50 has I kappa B-like functions. *Cell* **71**, 243-253.
- Schow, S.R. and Joly, A.(1997). N-acetyl-leuciny-leuciny-norleucinal inhibits lipopolysaccharide-induced NF-kappaB activation and prevents TNF and IL-6 synthesis in vivo. *Cell Immunol.* **175**, 199-202.
- Shackelford, R.E., Alford, P.B., Xue, Y., Thai, S.F., Adams, D.O., and Pizzo, S.(1997). Aspirin inhibits tumor necrosis factoralpha gene expression in murine tissue macrophages. *Mol.Pharmacol.* **52**, 421-429.

Shakhov, A.N., Collart, M.A., Vassalli, P., Nedospasov, S.A., and Jongeneel, C.V.(1990).

Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J.Exp.Med.* **171**, 35-47.

Swantek, J.L., Christerson, L., and Cobb, M.H.(1999). Lipopolysaccharide-induced tumor necrosis factor-alpha promoter activity is inhibitor of nuclear factor-kappaB kinase-dependent. *J.Biol.Chem.* **274**, 11667-11671.

Theus, S.A., Tabor, D.R., Gandy, J., and Barnett, J.B.(1993). Alteration of macrophage cytotoxicity through endogenous interferon and tumor necrosis factor alpha induction by propanil. *Toxicol.Appl.Pharmacol.* **118**, 46-52.

Torgerson, T.R., Colosia, A.D., Donahue, J.P., Lin, Y.Z., and Hawiger, J.(1998). Regulation of NF-kappa B, AP-1, NFAT, and STAT1 nuclear import in T lymphocytes by noninvasive delivery of peptide carrying the nuclear localization sequence of NF-kappa B p50. *J.Immunol.* **161**, 6084-6092.

Wadgaonkar, R., Phelps, K.M., Haque, Z., Williams, A.J., Silverman, E.S., and Collins, T.(1999). CREB-binding protein is a nuclear integrator of nuclear factor-kappaB and p53 signaling. *J.Biol.Chem.* **274**, 1879-1882.

Yin, M. J., Y. Yamamoto, and R. B. Gaynor. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* **396**,77-80.

- Xie, Y.C., Schafer, R., and Barnett, J.B.(1997). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol.Appl.Pharmacol.* **145**, 184-191.
- Zhao, W., Schafer, R., and Barnett, J.B.(1998). Cytokine Production by C57BL/6 Mouse Spleen Cells is Selectively Reduced by Exposure to Propanil. *J.Toxicol.Environ.Health* 55:107-120
- Zhao, W., Schafer, R., and Barnett, J.B.(1999). Propanil affects transcriptional and posttranscriptional regulation of IL-2 expression in activated EL-4 cells. *Toxicol.Appl.Pharmacol.* **154**, 153-159.
- Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S.(1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89**, 413-424.
- Ziegler-Heitbrock, H.W.(1995). Molecular mechanism in tolerance to lipopolysaccharide. *J.Inflamm.* **45**, 13-26.

**Effects of propanil on NF- κ B and I κ B activation in a human macrophage cell line,
THP-1**

Addendum to:

Frost, L. L., Neeley, Y. X., Schafer, R., Gibson, L. F., and Barnett, J. B. (2001). Propanil inhibits tumor necrosis factor- α production by reducing nuclear levels of the transcription factor nuclear factor-kappaB in the macrophage cell line ic-21. *Toxicol.Appl.Pharmacol.* 172, 186-193.

ABSTRACT

The herbicide propanil has been shown to alter macrophage cytokine production and the ability of macrophages to function normally. The effects of propanil on cytokine production, however, have been evaluated exclusively in murine macrophages. Herein we extend the evaluation of the effects of propanil into human monocytic cells using the cell line, THP-1. Similar to PEC and the murine macrophage cell line, IC-21, propanil also reduced TNF- α protein production from THP-1 cells. Nuclear levels of NF- κ B were also decreased in propanil-treated cells. However, analysis of I κ B α showed no difference in degradation between the propanil-treated and ethanol-treated control cells. Additionally, propanil treatment did not alter the levels of phosphorylated I κ B α as compared to ethanol control treatments. Therefore, although propanil causes decreased TNF- α production and NF- κ B nuclear translocation in both murine macrophage cell lines as well as in the human monocytic cell lines, THP-1, the mechanism for this alteration remains unknown.

INTRODUCTION

LPS is thought to activate the prototypical NF- κ B p65/p50 heterodimer in macrophages through a complex paradigm. The overall result of LPS-induced signaling is phosphorylation and ubiquitination of cytoplasmic inhibitors, I κ B proteins, which targets them for proteasome degradation. Degradation of I κ B reveals the nuclear localization sequence (NLS) of the p65/p50 NF- κ B heterodimer allowing its cytoplasmic to nuclear translocation. Current information indicates that the signal induced by LPS is initiated by the formation of a cell-surface complex consisting of Toll-like receptor 4 (TLR4), CD-14 and a protein associated with the extracellular domain of TLR4 called, MD-2. The signal then generated by the intracellular signaling domain of TLR4 travels through the intermediate proteins MyD88 (myeloid differentiation protein), IRAK (IL-1 receptor-associated kinase), TRAF6 (TNF receptor associated protein 6), TAB2, and TAK1 (transforming growth factor β -associated kinase 1) (reviewed in Guha, M. *et al.* (2001); Silverman, N. *et al.* (2001)). Ultimately, in the case of LPS signaling, the IKK complex consisting of at least IKK α , IKK β and NEMO (NF- κ B essential modulator), and probably several other kinases capable of activating the IKK activity, is activated which leads to the phosphorylation of I κ B α on serine 32 (S32) and S36, probably by IKK β (May, M. J. *et al.* (1999); Beg, A. A. *et al.* (1995)). Additional control of NF- κ B exists by the phosphorylation of the p65 subunit. Potential candidates to carry out this phosphorylation include IKK β , which has been shown to phosphorylate p65 on S536 (Sakurai, H. *et al.* (1999)), and NEMO, which is complexed with the regulatory subunit of protein kinase A (PKA), and phosphorylates p65 on S276 (Zhong, H. *et al.* (1997)). These phosphorylation events likely occur in the cytoplasm before nuclear translocation, and may allow efficient

binding to the coactivator protein CBP/p300 (Zhong, H. *et al.* (1998); Zhong, H. *et al.* (2002)).

Given that propanil down regulates TNF- α production in a variety of experimental paradigms Frost, L. L. *et al.* (2001); Xie, Y. C. *et al.* (1997b); Xie, Y. C. *et al.* (1997a)) and the critical role of NF- κ B in the induction of this important cytokine, we were interested in the effects of propanil on I κ B activation. Inasmuch as we have previously demonstrated that propanil alters NF- κ B nuclear translocation in murine IC-21 cells, we chose to extend our initial studies (Frost, L. L., *et al.*, (2001)), first by confirming our previous observations on NF- κ B activation using a human T cell line (THP-1), and second, by evaluating the effect of propanil on I κ B activation in this cell line.

MATERIALS AND METHODS

Cell line culture and maintenance

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in RPMI 1640 media (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 10mM HEPES, 100 U/ml penicillin-100 μ g/ml streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (all obtained from Sigma, St. Louis, MO) (complete media), and incubated at 37°C in an atmosphere of 5% CO₂.

Nuclear extraction and Western blotting

Nuclear extracts were prepared as previously described (Torgerson, T. R. *et al.* (1998)), with minor adaptations. Briefly, 1×10^6 THP-1 cells were treated with propanil or ethanol and stimulated with 1 μ g/ml LPS for various times, as indicated. Cells were pelleted by

centrifugation for 15 seconds at $12,000 \times g$. The cells were then washed once in PBS, and resuspended in 400 μ l of a buffer consisting of 10mM HEPES pH 7.9 @ 4 C, 10 mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1.0 mM PMSF, 1.0 mM DTT, and 1 μ g/ml of the protease inhibitors: leupeptin, aprotinin, antipain, chymostatin and pepstatin A for 15 minutes on ice. The lysate was pelleted by centrifugation for 30 seconds, and the supernatant was collected and saved as the cytoplasmic extract. The pellet was resuspended in 55 μ l of a 20 mM HEPES buffer containing 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT and 1 μ g/ml of the protease inhibitors: leupeptin, aprotinin, antipain, chymostatin, and pepstatin A for 15 minutes on ice (all from Sigma). Debris was pelleted by centrifugation for 5 minutes at $12,000 \times g$, and the supernatant was collected as the nuclear extract and stored at -70°C . Total protein concentration was determined in both cytoplasmic and nuclear extracts by the Coomassie blue protein determination, as described by the manufacturer (Pierce, Rockford, IL).

Nuclear extracts, 5 μ g per treatment, or 35 μ g per treatment of cytoplasmic extract, were denatured in SDS/PAGE sample buffer (Laemmli, U. K. (1970)) by boiling for 3 minutes. Samples were electrophoresed in 12% tris polyacrylamide gels with 4% stacking gels (BioRad, Hercules, CA) at 90 volts for 2 hours. Proteins were then transferred to PVDF membranes at 90 volts for 30 min. Western blotting was performed by incubating membranes for 1 hour with primary antibodies to NF- κ B p65, murine and human (1:500 in TBS + 0.1% Tween 20 (TBST)) (Santa Cruz Biotechnology, Santa Cruz, CA), or human I κ B α (1:1000 in TBST + 5% milk) (Cell Signaling Technology, formerly of New England Bio Labs, Beverly, MA). Blots were then washed as follows: 1×10 minutes, 2×5 minutes, and 2×2.5 minutes in TBST. Blots were incubated for 1 hour with horseradish peroxidase-

conjugated secondary antibody (Santa Cruz Biotechnology), 1:2000. Blots were then developed using Enhanced Chemiluminescence substrate (Amersham-Pharmacia, Piscataway, NJ) and visualized by exposing to X-Ray film for 3 seconds to 10 minutes.

RESULTS

Propanil suppressed TNF- α production by THP-1 cells

Production of the cytokine TNF- α is reduced in murine macrophages treated both *in vitro* and *in vivo* (Frost, L. L., *et al.*, (2001); Xie, Y. C *et al.*, (1997b); Xie, Y. C., *et al.*, (1997a)). To determine the effects of propanil on TNF- α production in human monocytes, we used the human cell line THP-1. The effect on TNF- α production paralleled that seen in murine cells. Propanil reduced TNF- α production from 468, 276, and 161 pg/ml in ethanol control cells at 2, 4 and 8 hours respectively, to 440, 160 and 211 pg/ml in cells treated with 25 μ M propanil, 402, 123, and 166 pg/ml in cells treated with 50 μ M propanil, and 236, 125 and 84 pg/ml in cells treated with 100 μ M propanil at the same time points (Figure 11). The peak TNF- α production in THP-1 cells occurred at 2 hours for all of the treatments (Figure 11).

Propanil reduced nuclear NF- κ B in THP-1 cells

TNF- α production is regulated by NF- κ B, therefore, we were interested in the effects of propanil on NF- κ B activation. Nuclear levels of NF- κ B p65 were measured in THP-1 cells. The cells were stimulated with 1 μ g/mL LPS and treated with either ethanol, as a control, or 100 μ M propanil. Nuclear extracts were prepared at 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. 5 μ g of each was separated by SDS-PAGE. Propanil caused a decrease in NF- κ B p65 at all time points (Figure 12a). p65 was determined to be decreased by 22, 28, 9, 25 and 44 percent at 15

minutes, 30 minutes, 1 hour, 2 hours, and 4 hours, respectively (Figure 12b). ANOVA analysis of the log of the optical densities of the p65 bands demonstrated statistically significant differences at all time points ($p < 0.05$).

Additionally, the dose-dependent response of NF- κ B p65 levels to increasing concentrations of propanil was determined. Figure 13 shows a representative Western blot depicting the decrease in p65 by 25 μ M, 50 μ M and 100 μ M propanil.

I κ B α is degraded normally in propanil-treated macrophages

To determine if NF- κ B is remaining sequestered in the cytoplasm by I κ B α , we analyzed degradation of I κ B α by Western blot. Cytoplasmic extracts were prepared from THP-1 cells stimulated with 1 μ g/mL LPS and either ethanol or 100 μ M propanil. 35 μ g of each extract was separated by SDS-PAGE and immunoblotted for I κ B α . Figure 14a shows normal degradation of I κ B α in both propanil-treated and ethanol control THP-1 cells.

Analysis of the phosphorylated form of I κ B α was performed to confirm that propanil did not alter I κ B α degradation (Fig. 14b). The percent of the total I κ B α that was phosphorylated in ethanol control cells was 30%, 77%, 45%, 29% and 58% at 0, 10, 20, 30 and 60 minutes, respectively, following LPS stimulation. Exposure of the cells to 5 μ M, 25 μ M or 100 μ M did not cause significant alterations in the percent phosphorylation of I κ B α . Phosphorylated levels of I κ B α from cells treated with 5 μ M propanil were 65%, 34%, 41%, and 40%, 25 μ M propanil resulted in 65%, 41%, 28% and 36%, and 100 μ M propanil resulted in 86%, 29%, 25% and 41% phosphorylation at 10, 20, 30, and 60 minutes, respectively.

DISCUSSION

Previously we reported that exposure of murine macrophages to propanil *in vitro* suppressed NF- κ B nuclear transport. The experimental data presented herein extend those findings and demonstrate that exposure *in vivo* has the same effect. Using an established treatment regime for propanil exposure and harvesting of PEC, our data demonstrate that *in vivo* propanil exposure led to reduced NF- κ B nuclear localization upon *ex vivo* LPS stimulation. Additionally, we also demonstrated that human macrophages react similarly to propanil; we show that propanil reduced nuclear NF- κ B levels in human monocytes using the human monocytic cell line, THP-1. We also show that degradation kinetics of I κ B- α are similar in propanil-treated and ethanol control cells. These results suggest that propanil does not interrupt LPS signaling upstream of I κ B- α , but rather inhibits nuclear translocation of NF- κ B.

Previously published data demonstrate that propanil inhibits TNF- α protein and mRNA production (Xie, Y. C., *et al.*, (1997b); Frost, L. L., *et al.*, (2001)). We have also shown that nuclear NF- κ B is reduced in a murine macrophage cell line, IC-21 (Frost, L. L., *et al.*, (2001)). Here we demonstrate that NF- κ B nuclear translocation is reduced in *ex vivo* stimulated PEC that have been exposed to propanil *in vivo*, which likely contributes to the suppression of TNF- α protein and message production from these cells. Likewise, NF- κ B is also reduced in the human macrophage cell line, THP-1.

Although both human and murine TNF- α genes require NF- κ B for maximal activation, the human TNF- α gene may rely on other additional factors for NF- κ B activation as well. Additional protein interactions with AP-1, SP-1 and C/EBP β influence the promoter of the human TNF- α gene (Zagariya, A. *et al.* (1998); Pope, R. *et al.* (2000); Pope,

R. M. *et al.* (1994); Tsai, E. Y. *et al.* (2000)). We have only studied the effect of propanil on NF- κ B in human monocytes, however, unpublished results suggest that propanil also affects AP-1 and c-jun in human Jurkat T-cells. We believe that propanil may alter nuclear levels of various proteins involved in transcription of several cytokine genes.

Transcriptional activation of NF- κ B can be enhanced by interaction with several factors. Specifically, cyclicAMP response element binding protein (CREB) binding protein (CBP) and p300 have been shown to increase the ability of NF- κ B to bind to DNA (Zhong, H., *et al.*, (1998); Zhong, H., *et al.*, (2002)). Additionally, recently a nucleocytoplasmic shuttling protein, hnRNPA1, was shown to be required for maximal NF- κ B transcriptional activity (Hay, D. C. *et al.* (2001)). Although propanil reduces nuclear levels of NF- κ B, additional effects on binding partners may serve to further reduce NF- κ B transcriptional activity.

Previous studies have demonstrated that propanil alters the normal calcium dynamics upon LPS stimulation (Xie, Y. C., *et al.*, (1997a)). The disruption of calcium is expected to affect several layers of downstream signaling events, including but not limited to, decreased activation of PKC isoforms and decreased activation of NADPH oxidase. Signaling induced by LPS has been shown to include PKC activation (DeFranco, A. L. *et al.* (1998)). Although considerable overlap exists in NF- κ B activation paradigms, there is evidence that suggests that both calcium and PKC contribute to activation of the I κ B kinase complex and subsequent NF- κ B activation (Chen, B. C. *et al.* (2001); Saijo, K. *et al.* (2002)). Calcium is also involved in respiratory burst activation (Foyouzi-Youssefi, R. *et al.* (1997); Granfeldt, D. *et al.* (2002); Larsen, E. C. *et al.* (2000)). Furthermore, there is some evidence that there

is crosstalk between reactive oxygen species generated through respiratory burst activation and NF- κ B activation (Kaul, N. *et al.* (1996)).

Herein we have shown that propanil decreases nuclear NF- κ B translocation, but does not significantly alter degradation of I κ B α . Therefore, the mechanism by which propanil inhibits NF- κ B nuclear translocation remains elusive. Recently, much research has focused on the roles of various I κ B species in NF- κ B activation (reviewed in Ghosh, S. *et al.* (2002); Malek, S. *et al.* (2001)). Specifically, I κ B β , when bound to the prototypical NF- κ B heterodimer conceals the nuclear localization signal (NLS) on both the p65 and p50 subunits, and is functionally identical to I κ B α , thus is more likely the primary inhibitory protein (Malek, S., *et al.*, (2001)); Cheng, J. D. *et al.* (1998)). I κ B α , contains a potent nuclear export signal (NES), and only conceals the NLS on the p65 subunit (Malek, S., *et al.*, (2001)); Tam, W. F. *et al.* (2001)). Therefore, I κ B α has been implicated in the nucleocytoplasmic shuttling of NF- κ B (reviewed in Ghosh, S. and Karin, M. (2002); Malek, S., *et al.*, (2001) Huang, T. T. *et al.* (2000); Johnson, C. *et al.* (1999)). Herein we demonstrate that propanil does not substantially affect I κ B α phosphorylation or degradation, however the effects on I κ B β are not explored in this manuscript. The research into the effects of propanil on other I κ B species is currently the focus of continuing investigations in our lab. Preliminary data, however, suggests that propanil also does not alter I κ B β degradation upon LPS stimulation in murine IC-21 cells.

In summary, we have shown that propanil reduces nuclear NF- κ B levels in human THP-1 cells. However, I κ B- α was normally degraded in THP-1 and IC-21 cells, indicating

that the LPS signal was intact. These data lead us to speculate that propanil may affect other I κ B species or nuclear transport of NF- κ B at the level of the nuclear membrane.

Figure 11

Propanil reduces TNF- α protein production from THP-1 cells. THP-1 cells were stimulated with 1 μ g/ml LPS for 2, 4 and 8 hours in the presence of ethanol or 25, 50 or 100 μ M propanil. Supernatants were collected and TNF- α protein was measured using a specific ELISA.

Figure 12.

Propanil reduces nuclear levels of NF- κ B p65 in THP-1 cells. **A.** A representative NF- κ B p65 Western blot from THP-1 cells that were stimulated with 1 μ g/ml LPS in the presence of ethanol or 167 μ M propanil for 0 (*lane 11*), 15 (*lanes 1 and 2*), 30 (*lanes 3 and 4*), 60 (*lanes 5 and 6*), 120 (*lanes 7 and 8*), or 240 (*lanes 9 and 10*) minutes. **B.** Densitometric analysis of NF- κ B p65 Western blots from 3 separate experiments, expressed as percent of the ethanol control. (* = $p < 0.05$)

A.

B.

Figure 13.

Propanil reduces nuclear NF- κ B p65 in a dose-dependent manner in THP-1 cells.

Nuclear extracts were prepared and analyzed for NF- κ B p65 from THP-1 cells that were stimulated with 1 μ g/ml LPS (*lane 5*), in the presence of ethanol (*lane 1*), or 25 μ M (*lane 2*), 50 μ M (*lane 3*), or 100 μ M propanil (*lane 4*), for 1 hour. The unstimulated control is represented in lane 6.

Figure 14.

Effects of propanil on I κ B α in THP-1 cells. **A.** Western blot analysis of I κ B α from THP-1 cells stimulated with 1 μ g/ml LPS in the presence of ethanol or 167 μ M propanil for 15 (*lanes 1 and 2*), 30 (*lanes 3 and 4*), 60 (*lanes 5 and 6*), 120 (*lanes 7 and 8*), and 240 (*lanes 9 and 10*), minutes. The unstimulated (no LPS) control is shown in lane 11. **B.** Cytoplasmic extracts were prepared from THP-1 cells that had been stimulated with 1 μ g/ml LPS for 0, 15, 30, 45 or 60 minutes in the presence of ethanol or 5, 25 or 100 μ M propanil. Levels of total I κ B α and phosphorylated I κ B α were determined. The graph of a representative experiment depicts the ratio of phosphorylated to total I κ B α .

A.

B.

REFERENCES

- Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* **376**, 167-170.
- Chen, B. C. and Lin, W. W. (2001). PKC- and ERK-dependent activation of I kappa B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation. *Br.J Pharmacol.* **134**, 1055-1065.
- Cheng, J. D., Ryseck, R. P., Attar, R. M., Dambach, D., and Bravo, R. (1998). Functional redundancy of the nuclear factor kappa B inhibitors I kappa B alpha and I kappa B beta. *J Exp.Med.* **188**, 1055-1062.
- DeFranco, A. L., Crowley, M. T., Finn, A., Hambleton, J., and Weinstein, S. L. (1998). The role of tyrosine kinases and map kinases in LPS-induced signaling. *Prog.Clin.Biol.Res.* **397:119-36.**, 119-136.
- Foyouzi-Youssefi, R., Petersson, F., Lew, D. P., Krause, K. H., and Nüsse, O. (1997). Chemoattractant-induced respiratory burst: increases in cytosolic Ca²⁺ concentrations are essential and synergize with a kinetically distinct second signal. *Biochem.J.* **322**, 709-718.

- Frost, L. L., Neeley, Y. X., Schafer, R., Gibson, L. F., and Barnett, J. B. (2001). Propanil inhibits tumor necrosis factor- α production by reducing nuclear levels of the transcription factor nuclear factor- κ B in the macrophage cell line ic-21. *Toxicol.Appl.Pharmacol.* **172**, 186-193.
- Ghosh, S. and Karin, M. (2002). Missing pieces in the NF- κ B puzzle. *Cell* **109**, S81-S96.
- Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002). Capacitative Ca^{2+} influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase. *J.Leukoc.Biol.* **71**, 611-617.
- Guha, M. and Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cell Signal.* **13**, 85-94.
- Hay, D. C., Kemp, G. D., Dargemont, C., and Hay, R. T. (2001). Interaction between hnRNPA1 and I κ B α is required for maximal activation of NF- κ B-dependent transcription. *Mol.Cell Biol.* **21**, 3482-3490.
- Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000). A nuclear export signal in the N-terminal regulatory domain of I κ B α controls cytoplasmic localization of inactive NF- κ B/I κ B α complexes. *Proc.Natl.Acad.Sci.U.S.A* **97**, 1014-1019.

- Johnson, C., Van Antwerp, D., and Hope, T. J. (1999). An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha. *EMBO J* **18**, 6682-6693.
- Kaul, N. and Forman, H. J. (1996). Activation of NF kappa B by the respiratory burst of macrophages. *Free Radic.Biol.Med.* **21** , 401-405.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E., and Lennartz, M. R. (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J Immunol* **165**, 2809-2817.
- Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001). IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol.Chem.* **276**, 45225-45235.
- May, M. J. and Ghosh, S. (1999). IkappaB kinases: kinsmen with different crafts. *Science* **284**, 271-273.

- Pope, R., Mungre, S., Liu, H., and Thimmapaya, B. (2000). Regulation of TNF- α expression in normal macrophages: the role of C/EBP β . *Cytokine* **12**, 1171-1181.
- Pope, R. M., Leutz, A., and Ness, S. A. (1994). C/EBP β regulation of the tumor necrosis factor α gene. *J Clin. Invest* **94**, 1449-1455.
- Saijo, K., Mecklenbrauker, I., Santana, A., Leitger, M., Schmedt, C., and Tarakhovsky, A. (2002). Protein kinase C β controls nuclear factor κ B activation in B cells through selective regulation of the I κ B kinase α . *J Exp. Med.* **195**, 1647-1652.
- Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999). I κ B kinases phosphorylate NF- κ B p65 subunit on serine 536 in the transactivation domain. *J Biol. Chem.* **274**, 30353-30356.
- Silverman, N. and Maniatis, T. (2001). NF- κ B signaling pathways in mammalian and insect innate immunity. *Genes Dev.* **15**, 2321-2342.
- Tam, W. F. and Sen, R. (2001). I κ B family members function by different mechanisms. *J Biol. Chem.* **276**, 7701-7704.
- Torgerson, T. R., Colosia, A. D., Donahue, J. P., Lin, Y. Z., and Hawiger, J. (1998). Regulation of NF- κ B, AP-1, NFAT, and STAT1 nuclear import in T lymphocytes by noninvasive delivery of peptide carrying the nuclear localization sequence of NF- κ B p50. *J. Immunol.* **161**, 6084-6092.

- Tsai, E. Y., Falvo, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000). A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol.Cell Biol.* **20**, 6084-6094.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997a). Inhibitory effect of 3,4-dichloropropionaniline on cytokine production by macrophages is associated with LPS-mediated signal transduction. *J.Leukoc.Biol.* **61**, 745-752.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997b). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol.Appl.Pharmacol.* **145**, 184-191.
- Zagariya, A., Mungre, S., Lovis, R., Birrer, M., Ness, S., Thimmapaya, B., and Pope, R. (1998). Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta-induced activation by c-Jun. *Mol.Cell Biol.* **18**, 2815-2824.
- Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol.Cell* **9**, 625-636.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89**, 413-424.

Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol.Cell* **1**, 661-671.

Chapter 2

Propanil suppresses normal macrophage function

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ABSTRACT

Macrophages are a critical part of the innate immune response and natural surveillance mechanisms. As such, proper macrophage function is crucial for immune functions and maintenance of normal homeostasis. Propanil is a post emergent herbicide that has been shown to suppress several immune parameters, including TNF- α production and NF- κ B nuclear translocation in macrophages. The goal of this study was to evaluate the effects of propanil on macrophage functions, including phagocytosis, respiratory burst activation, bactericidal activity and MHC class II expression in both murine and human cells. We used primary peritoneal exudate cells (PEC) and a human monocytic cell line, THP-1, for these experiments. Our results indicate that propanil decreases the ability of both types of macrophages to phagocytize beads, and also inhibits respiratory burst activation. Analysis of bacterial killing by *Listeria monocytogenes*-infected cells demonstrated that propanil decreased bactericidal activity of both murine and human cells. Although a trend towards decreased MHC class II (HLA-DR) expression was observed in propanil-treated THP-1 cells, the differences were not statistically significant. These results demonstrate that propanil has profound effects on macrophage function, and provide insight into the potential mechanisms of immunosuppression by propanil.

INTRODUCTION

Macrophages are critical for normal maintenance of homeostasis as well as immune regulation and tumor surveillance. Whereas phagocytosis of apoptotic cells is a normal homeostatic function of macrophages, phagocytosis and clearance of bacteria and viruses is a crucial function of innate immunity. Through cytokine and chemokine production, direct cellular contacts and antigen presentation, macrophages orchestrate initiation of adaptive immune responses (Adams and Hamilton, 1984; Ganz, 1993; Celada and Nathan, 1994). Clearly, alterations in any of the normal functions of these cells can lead to serious, even fatal consequences.

Defects in normal macrophage function such as in chronic granulomatous disease (CGD), result in altered immunity to bacterial and fungal pathogens. CGD patients suffer from a malfunction in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase caused by one of four known genetic mutations. The phenotype of two of these mutations is a complete lack of the cytochrome b portion of the enzyme, and a more severe manifestation of the disease (Segal et al., 2000). NADPH oxidase is a functional complex consisting of several cytoplasmic proteins that translocate to the membrane-associated cytochrome core. This complex functions by catalyzing a one electron reduction of molecular oxygen to superoxide using NADPH as the electron donor (reviewed in (Jones et al., 2000). NADPH oxidase also functions in regulation of cellular membrane potentials through activation of voltage-gated proton pumps (DeCoursey et al., 2001; Geiszt et al., 2001).

NADPH oxidase activity is a critical component of macrophage bactericidal mechanisms against facultative intracellular pathogens, such as *Listeria monocytogenes* (Ohya et al., 1998; Alvarez-Dominguez et al., 2000). Listericidal mechanisms include the

production of reactive oxygen intermediates, hydrogen peroxide, superoxide ($\text{O}_2^{\bullet-}$), reactive nitrogen intermediates (RNI), nitric oxide ($\text{NO}^{\bullet}$), and peroxynitrite (the reaction product of superoxide and nitric oxide) by activated macrophages (Ohya et al., 1998; Ouadrhiri et al., 1999; Muller et al., 1999; Alvarez-Dominguez et al., 2000). Priming of macrophages, which is necessary for clearance of *L. monocytogenes*, requires interferon- γ (IFN- γ) activation of macrophages and production of tumor necrosis factor- α (TNF- α) (Kiderlen et al., 1984; Buchmeier and Schreiber, 1985; Nakane et al., 1988; Nakane et al., 1989). Therefore, alterations in macrophage cytokine production or respiratory burst activity result in increased susceptibility to *L. monocytogenes* infection.

Propanil is an herbicide known to alter several macrophage functions, including TNF- α and IL-6 production, IP₃-mediated calcium release and nuclear NF- κ B translocation (Xie et al., 1997a; Xie et al., 1997b; Frost et al., 2001). We were interested in the direct effects of propanil on several aspects of macrophage function, and in this report we demonstrate that propanil reduced phagocytic ability, respiratory burst and listericidal activity of macrophages, and altered MHC class II expression.

MATERIALS AND METHODS

Animals

C57Bl/6 mice were purchased from Charles River Farms (Wilmington, DE). Animals were allowed to acclimate for 7 days before experimental procedures were initiated. Food and water were provided ad libitum. Experimental procedures were conducted between the ages of 7 and 12 weeks.

Cell line culture and maintenance

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 media with phenol red (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT), 10mM HEPES, 100 U/ml penicillin-100 µg/ml streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (all obtained from Sigma, St. Louis, MO) (complete media), and incubated at 37°C in an atmosphere of 5% CO₂.

Macrophage harvest and preparation

Mice were injected intraperitoneally (i.p.) with 1.5 ml of aged 4% sterile thioglycollate broth four days before macrophage harvest. Elicited macrophages (PEC, peritoneal exudate cells) were harvested as described (Xie et al., 1997a). Briefly, 9 ml sterile PBS (BioWhittaker, Walkersville MD) containing 10 U/ml heparin (Sigma, St. Louis, MO) was injected into the exposed peritonea of mice and syringe aspirated. Resulting cell suspensions were washed twice in complete media, plated at a density of $1-3 \times 10^6$ cells/ml in 35 mm or 100 mm tissue culture dishes and allowed to adhere for 2 hours at 37°C in 5% CO₂. Nonadherent cells were removed by addition of two bursts of warm PBS and vacuum aspiration. Viability was monitored by trypan blue exclusion and was routinely $\geq 95\%$.

Exposure of cells to propanil

Primary cells and the THP-1 cells were treated with 5, 25, 50, or 100 µM propanil (3,4-dichloropropionanilide, 99% purity, ChemServices, West Chester, PA) diluted in absolute ethanol or an equivalent amount of absolute ethanol only (vehicle control) (AAPER Alcohol and Chemical Company, Shelbyville, KY). The final concentration of ethanol was 0.10%. Viability was routinely checked and was $>90\%$ for all groups and never showed a statistical difference between the groups.

TNF- α specific enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from THP-1 cells that had been plated at 1×10^6 cell/ml in 4 ml in 6 well plates and stimulated with $1 \mu\text{g/ml}$ bacterial lipopolysaccharide (LPS, Sigma St. Louis, MO) in the presence of propanil or an equal volume of ethanol (vehicle) for the indicated times. ELISA compatible anti-TNF- α antibodies were purchased in pairs from BD PharMingen (San Diego, CA). Capture anti-TNF- α antibody was added to wells of a 96 well, enhanced protein-binding plate in a coating buffer (0.1M Na_2HPO_4 , pH 9.0) and incubated at 4°C overnight. Plates were washed 4 times with PBS/0.05% Tween 20, and blocked with PBS containing 3% bovine serum albumin (BSA, Sigma St. Louis, MO) for 2 hours. Plates were again washed 4 times before addition of standards, rhTNF- α (BD PharMingen), and samples at appropriate dilutions (in PBS with 1% BSA and 0.05% Tween 20) and incubated at 4° overnight. Plates were washed four times with PBS with 0.05% Tween 20 followed by addition of the secondary biotinylated anti-TNF- α antibody. The plates were incubated at room temperature for 1 hour then washed 6 times. Avidin-peroxidase was added for 30 minutes at room temperature before addition of ABTS substrate (2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonic acid]) with H_2O_2 . The plates were then read at an optical density of 405 nm on a μ -Quant plate reader (BioTek Instruments, Inc. Winooski, VT).

Phagocytosis

THP-1 cells were differentiated by addition of 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma St. Louis, MO) for 24 hours. Differentiation of the cells led to adherence to tissue culture dishes. Monolayers were washed vigorously with PBS and cultured in complete media for 72 hours. On the day of the phagocytosis assay,

differentiated THP-1 cells or PEC (previously prepared and allowed to adhere overnight) were trypsinized, washed in PBS, seeded in 5 ml snap-cap tubes at 1×10^6 cells/ml in PBS +1% FBS. Phagocytosis assays were conducted as described with minor modifications (Antonini et al., 2000). Briefly, carboxylate-modified, 2.0 μ m yellow-green FluoroSpheres (Molecular Probes, Eugene, OR) were added at a concentration of approximately 30 beads/cell. LPS, propanil and ethanol treatments were added as indicated. Cells were incubated on a rocker in constant motion at 37°C in 5% CO₂ for 2 hours. Cells were pelleted by centrifugation at 500 g, washed twice to remove any free beads and allowed to adhere to sterile cover slips in 6 well plates for two hours. Adherent cells were washed twice with warm PBS, fixed with 2% paraformaldehyde for 30 minutes, stained with the fluorochrome Nile red (0.1 μ g/ml; Molecular Probes, Eugene, OR) for 5 minutes and washed with PBS. Coverslips were then mounted to glass slides and stored in the dark (a maximum of 48 hours before analysis). Confocal microscopy using a Zeiss LSM 510 microscope equipped with an argon laser (Zeiss, Thornwood, NY) was performed to evaluate the cells which were scored as having 0, 1-2, 3-4, ≥ 5 beads/cell. A weighted phagocytic index was calculated by multiplying the number of cells with 0 beads by 0; the number of cells with 1-2 beads/cell by 1; the number of cells with 3-4 beads/cell by 2; and the number of cells with 5 or more beads/cell by 4, summing these products, and dividing by 200 (the total number of cells counted) (Antonini et al., 2000).

Respiratory burst

Luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione (Sigma) was used to amplify chemiluminescence (CL) signals generated by activation of the NADPH-oxidase (Allen and Loose, 1976; Dahlgren and Karlsson, 1999). The CL signal generated by luminol occurs as

the compound is oxidized by peroxynitrite as it returns to ground state. To measure respiratory burst, we differentiated THP-1 cells, or harvested PEC as previously described. All cells were plated at 5×10^5 cells/ml in 35 mm tissue culture dishes. Cells were washed with PBS to eliminate the phenol red before elicitation of the respiratory burst. In a total reaction volume of 2 ml, respiratory burst activity was elicited by addition of 100 μ M PMA simultaneously with PBS, 5 μ M luminol, and indicated concentrations of LPS, and either ethanol or propanil. Dishes were placed at 34 C in a pre-warmed Xglux FB-12 dish luminometer (Berthold, Co., Wilbad, Germany) and CL readings were taken every minute for 20 minutes. Data shown represent integrated CL curves generated from these readings over time extending to peak of the luminescence.

Bacteria

Virulent *Listeria monocytogenes*, strain EGD, were grown overnight in 10 ml of brain-heart infusion broth (Difco Laboratories, Detroit, MI) with agitation at 37°C. On the day of the experiment 1 ml of the overnight culture was used to inoculate a 50 ml LB culture, which was grown to an OD₆₀₀ of approximately 0.3-0.5.

Determination of Listericidal activity

THP-1 or PEC (5×10^5 cells/ml) were activated with 100 U/ml rhIFN- γ (BD PharMingen) or rmIFN- γ (BD PharMingen), respectively, for 24 hours in RPMI 1640 media containing 10% FBS (no antibiotics). This activation did not cause adhesion of THP-1 cells, which continued to grow in loose suspension as described (Ouadrhiri et al., 1999). THP-1 cells were washed with PBS, resuspended in 1 ml PBS containing 1% FBS and infected with 2.5×10^6 *Listeria monocytogenes*, strain EGD/ml. Activated PEC were trypsinized, washed in PBS, resuspended in PBS containing 1% FBS, and also infected with 2.5×10^6 *L.*

monocytogenes, strain EGD/ml. LPS stimulation and ethanol and propanil treatments were initiated at the same time as initial infection. Cells were transferred to 5 ml snap-cap tubes and incubated at 37°C in 5% CO₂ on a rocker for 1 hour to allow for infection. Cells were then centrifuged at 500 g and washed twice with PBS. The final pellet was resuspended in 0.5 ml of PBS containing 25 µg/ml gentamicin. Initial infection levels (1 hour) were obtained after gentamicin treatment by plating 50 µl of the resulting lysate from cells lysed in 500 µl of ice-cold, sterile, distilled water. Cell lysates were also prepared at 3 and 5 hours post infection. Lysates were plated on TSA plates (Trypticase Soy Agar; Difco Laboratories, Detroit, MI) using an Autoplate 4000 (Spiral Biotech, Bethesda, MD) in triplicate. Plates were incubated at 37°C for 24 hours, and colonies were counted using a CASBA 4 plate scanner and CIA-BEN colony imaging and analysis software (Spiral Biotech). Resulting CFU/ml *L. monocytogenes* were calculated and compared across treatments.

Measurement of MHC class II surface expression

THP-1 cells were seeded in 24 well plates at 5×10^5 cells/ml and activated with 100 U/ml rhIFN- γ for 48 hours. Cells were stimulated with 1 µg/ml LPS in the presence or absence of ethanol or propanil, at the indicated concentrations, for the final 24 or 4 hours. Cells were centrifuged in the 24 well plates at 500 g for 8 minutes, supernatants discarded, and cells were resuspended in 100 µl of PBS containing 2% FBS, and 0.02% Na-azide (FACS media), and transferred to 96 well V-bottom plates. Cells were washed twice in FACS media, the final pellet was resuspended in 25 µl of PBS containing 10% human AB serum (to block FC receptors) (US Biologicals, Swampscott, MA), and incubated on ice for 25 minutes. 100 µl of FACS media was added to the wells, cells were pelleted and washed

twice more in FACS media. Surface expression of HLA-DR was detected by incubation with a FITC (fluorescein isothiocyanate) conjugated anti-HLA-DR antibody (BD PharMingen) diluted to 1:10 in FACS media, on ice, in the dark, for 25 minutes, followed by two washes in FACS media. The final pellet was resuspended in 100 μ l of 0.04% paraformaldehyde; plates were sealed, covered and stored at 4°C overnight. The following day, cells were washed twice in FACS media, and transferred to 5 ml tubes, in a total volume of 1 ml of FACS media, for analysis with a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). Data were analyzed using WinMDI software (Version 2.8).

Statistical Analysis

Data were analyzed using JMP (SAS, Inc, Belmont, CA) and SigmaStat (SPSS, Inc. Chicago, IL) statistical analysis software. Significance of interactions between treatment groups was assessed using an analysis of variance (ANOVA). For all analyses, the minimum criterion of significance was set at $p < 0.05$. Each experiment was repeated at least twice with similar results. Data shown represent means of at least three experiments or representative experiments, as indicated. Error bars represent standard deviation unless otherwise noted to represent standard error, where appropriate.

RESULTS

Propanil suppresses TNF- α production

TNF- α , a key inflammatory mediator produced by activated macrophages, is reduced when murine macrophages are exposed to propanil (Xie et al., 1997b; Frost et al., 2001). To control for possible species specific effects, a human cell line was used throughout this study. To confirm that propanil caused a similar reduction in TNF- α in human cells, we

measured TNF- α production from the human monocytic cell line, THP-1. Cells were stimulated with 1 μ g/ml LPS and either 100 μ M propanil or an equal volume of ethanol, as a vehicle control, for the indicated times. Peak protein production occurred at 2 hours; propanil, however, reduced TNF- α production from THP-1 cells at all time points tested. In the experiment shown, propanil caused a statistically significant reduction in TNF- α production ($p < 0.001$). TNF- α protein was reduced from 892 pg/ml in the ethanol control cells to 327 pg/ml in the 100 μ M propanil-treated cells at 2 hours, a 63% reduction (Figure 15).

***In vitro* exposure to propanil reduces phagocytic capabilities**

A major function of macrophages *in vivo* is phagocytosis of organisms and particulates. To determine if propanil affected the ability of PMA-differentiated THP-1 cells and PEC to phagocytize fluorescent 2.0 μ m beads a weighted phagocytic index was measured. Propanil caused significant reductions ($p < 0.05$) in the ability of THP-1 and PEC cells to phagocytize beads. The weighted phagocytic index for THP-1 cells was reduced from 0.730 (± 0.303) in the ethanol control to 0.267 (± 0.068) in the 100 μ M propanil group, a 63% decrease in phagocytosis (Figure 16A). Significant differences ($p < 0.05$) were evident with both the 50 μ M and 100 μ M propanil treatments in the PEC, from an average of 1.08 (± 0.11) in the ethanol control group to 0.72 (± 0.07) and 0.53 (± 0.07) in the 50 μ M and 100 μ M treatments, respectively (Figure 16B).

Propanil exposure results in alterations in the ability to generate a respiratory burst

Functional aspects of macrophage biology include destruction of phagocytosed organisms via generation of reactive oxidants and nitrogen species. We have employed a respiratory burst detection method utilizing luminol to amplify CL generated by PMA-

stimulated THP-1 cells and PEC (Dahlgren and Karlsson, 1999; Faldt et al., 1999). Our results indicate that 100 μ M propanil virtually shuts down the respiratory burst activity of both cell types, whereas 50 μ M propanil gave an intermediate response (Figure 17A and B). The response of THP-1 cells treated with 25 μ M propanil was similar to that given by ethanol control cells (Figure 17A). The respiratory burst of PEC was more sensitive to the effects of propanil, resulting in significantly different dose-dependent decreases in integrated relative light units upon exposure to 5, 50 and 100 μ M propanil ($p < 0.05$, from 3-5 minutes; $p < 0.001$ from 6-12 minutes) (Figure 17B).

Listericidal activity of macrophages is reduced by propanil exposure

We measured the effects of propanil on the ability of macrophages to kill *L. monocytogenes* as a tangible indicator of the potential biological consequences. THP-1 cells were cultured in antibiotic-free media for 48 hours and activated with 100 U/ml rhIFN γ for 24 hours before simultaneous addition of 2.5×10^6 *L. monocytogenes*, 1 μ g/ml LPS and ethanol or propanil as indicated. Initial infection levels were determined after one hour by briefly incubating cells in a gentamicin solution, to kill bacteria that had not been internalized, then lysing the macrophages and plating 50 μ l of the lysate to determine the CFU/ml that successfully infected the cells. Resulting CFU/ml at 3 and 5 hours were compared to the initial infection level for each treatment. Whereas IFN- γ , alone and in combination with LPS and ethanol, resulted in significant reductions ($p < 0.05$) in *L. monocytogenes* CFU/ml, 100 μ M propanil treatments abrogated the listericidal abilities of both THP-1 cells and PEC (Figure 18A and B, respectively). IFN- γ , LPS and ethanol treatments in both cell types exhibited sufficient listericidal abilities to result in significant bacterial reductions by 5 hours ($p < 0.05$) (Figure 18A and B). Furthermore, significantly

fewer bacteria were initially phagocytosed by THP-1 cells treated with 100 μ M propanil than by cells treated with either IFN- γ alone, or in combination with ethanol ($p < 0.05$) (Figure 4A; 1 hour). Similarly, 100 μ M propanil-treated PEC also demonstrated decreased phagocytosis of *L. monocytogenes* ($p < 0.05$) as compared to cells treated with IFN- γ alone, and in combination with LPS, ethanol and 25 μ M propanil (Figure 4B; 1 hour). These results demonstrate that propanil causes inhibition of listericidal abilities of macrophages, and further support the earlier findings regarding the effects of propanil on phagocytosis.

Analysis of MHC class II surface expression

Another hallmark of macrophage activation is up regulation of MHC class II molecules. We examined the effects of propanil on HLA-DR expression by THP-1 cells (Figure 19A and B). Histograms from a representative THP-1 experiment show that 100 μ M propanil exposure (for either 24 or 4 hours) caused a 2 fold decrease in HLA-DR expression from ethanol controls (Figure 19A). Similarly, the average of three experiments demonstrated that 24 hours of exposure to 25, 50 and 100 μ M propanil decreases HLA-DR expression from ethanol controls by 17%, 26% and 49%, respectively (Figure 19B). The same propanil doses for only 4 hours caused similar decreases from ethanol control cells of 15%, 28% and 36%, respectively (Figure 19B). Conversely, propanil-treatment of PEC for 24 hours did not alter I-A^b expression (data not shown). We therefore incubated PEC in the presence of propanil or ethanol treatments for the entire 48 hours of IFN- γ activation. Surface expression of I-A^b remained unaltered under these conditions (data not shown).

DISCUSSION

Appropriate macrophage function is critical for normal immune functions and homeostasis, as macrophages participate in various aspects of innate and specific immunity as well as tumor surveillance. Propanil has previously been shown to decrease TNF- α production by murine macrophages (Xie et al., 1997b; Frost et al., 2001). Here we report that propanil also reduced TNF- α production from the human THP-1 cell line. We also demonstrate that propanil caused reductions in phagocytic capabilities, respiratory burst and listericidal activity in both the THP-1 cell line and primary murine PEC.

The THP-1 cell line is a human monocytic cell line that has been extensively characterized (Tsuchiya et al., 1980; Tsuchiya et al., 1982). These cells have been shown to mobilize NF- κ B into the nucleus upon stimulation with LPS and produce cytokines typical of activated human macrophages (Steer et al., 2000; Gatanaga et al., 1991). Further differentiation of the cells with PMA leads to a mature, differentiated macrophage phenotype (Tsuchiya et al., 1982). We wanted to demonstrate the effects of propanil in both murine and human models. Primary PEC were used for murine studies because we had previously established the effects of propanil in macrophages on cytokine production, and other immune parameters with these cells (Xie et al., 1997a; Xie et al., 1997b, Frost et al., manuscript in preparation). THP-1 cells were chosen for a human model due to the existing body of literature regarding their monocytic phenotype, cytokine response, respiratory burst activity, and phagocytic abilities (Tsuchiya et al., 1980; Gatanaga et al., 1991; Condio-Neto et al, 1998; Steer et al., 2000; Delbosc et al., 2002). The use of THP-1 cells also eliminated the restrictions of utilizing primary human monocytes.

Propanil has also been previously shown to ablate the IP₃-mediated calcium release in macrophages despite the generation of IP₃ levels equivalent to control cells (Xie et al., 1997a). Although the mechanism by which propanil alters the [Ca⁺⁺]_i release remains unclear, we speculate that the ablation of the initial calcium release also alters the subsequent flow of calcium over the plasma membrane, termed the capacitative calcium influx (reviewed in (Putney and McKay, 1999). An extensive body of literature has established a critical role for calcium in various aspects of the generation of a respiratory burst in macrophages and neutrophils (Dahlgren et al., 1992; Dusi et al., 1993; Movitz et al., 1997; Zhou et al., 1997; Foyouzi-Youssefi et al., 1997; Geiszt et al., 1999; Granfeldt et al., 2002). Calcium has specifically been implicated to play a role in the translocation of the p47^{phox} cytosolic component of the NADPH oxidase to the membrane-bound cytochrome b portion (Zhou et al., 1997). Recently, Granfeldt et al. (2002) further evaluated the differential activation of plasma membrane- and granule-localized NADPH oxidase, showing that intracellular NADPH oxidase activity could be activated by an increase in [Ca⁺⁺]_i via the capacitative Ca⁺⁺ entry, whereas plasma membrane-localized NADPH oxidase activity required the capacitative Ca⁺⁺ influx in addition to other signaling events. Our results showing reductions in respiratory burst activity have been generated using luminol, which can cross the plasma membrane (Dahlgren and Karlsson, 1999), as the chemiluminescent detector, therefore our measurements may reflect a decrease in activity of either intracellular or plasma membrane-localized NADPH oxidase activity, or both. This decrease in NADPH oxidase activity is potentially a result of the altered calcium dynamics induced by propanil.

The effects of propanil on respiratory burst activity may also affect the listericidal capability of macrophages. Contributions of the various phagocyte oxidase (phox) proteins to the listericidal capacity of macrophages have been investigated using mice with targeted gene deletions (Endres et al., 1997; Dinauer et al., 1997; Shiloh et al., 1999). Neither the loss of gp91^{phox} nor p47^{phox} significantly altered the ability of mice to clear *L. monocytogenes* infections (Endres et al., 1997; Shiloh et al., 1999), however macrophages harvested from gp91^{phox-/-} mice were substantially defective in their ability to kill *L. monocytogenes in vitro* (Shiloh et al., 1999). Dinauer *et al.* (1997), also using gp91^{phox-/-} mice, suggested that neutrophils and ROI control early *in vivo* *Listeria* infection, whereas macrophages and RNI production may participate in later stages of infection.

A debate over the importance of ROI and RNI in *L. monocytogenes* killing *in vivo* and *in vitro* has persisted in the literature (Lepay et al., 1985; Peck, 1989; Beckerman et al., 1993; Gregory et al., 1993; Boockvar et al., 1994; Leenen et al., 1994; Inoue et al., 1995; Samsom et al., 1996; Ohya et al., 1998; Muller et al., 1999). Some of the differences between these studies are likely due to the cells, animals, stimuli and methods used to elicit and measure ROI and RNI. We used luminol chemiluminescence to detect the occurrence of a respiratory burst generated by addition of PMA. In this system, the reaction of luminol with peroxynitrite (ONOO⁻) is the main light-generating source; (Radi et al., 1993; Catz et al., 1995; Castro et al., 1996; Xia and Zweier, 1997; Gross et al., 1998). Production of peroxynitrite occurs as a result of the spontaneous reaction of $\bullet\text{O}_2^-$ and $\bullet\text{NO}$, produced by NADPH oxidase and nitric oxide synthase (iNOS), respectively. Therefore, our measurements reflect activity of both ROI and RNI, and we are unable to distinguish between the relative importance of either in listericidal activity of THP-1 cells or

thioglycollate elicited PEC. However, peroxynitrite alone is capable of efficiently killing *L. monocytogenes* in a dose-dependant manner, and may be the ultimate effector molecule of macrophages (Muller et al., 1999). Interest in the effects and measurement of ONOO⁻ has sparked a growing body of literature and the speculation that many previous studies employing luminol CL were in fact measuring ONOO⁻ (Radi et al., 1993; Antonini et al., 1994; Catz et al., 1995; Castro et al., 1996; Gross et al., 1998). Our results provide indirect support to the findings of Muller *et al.* (1999) implicating ONOO⁻ as the anti-*Listeria* effector molecule.

The oxidative burst in activated macrophages, such as THP-1 or peritoneal exudate cells (PEC), results the production of superoxide from activated NADPH oxidase (reviewed in Johnson and Kitagawa 1985; Sasada *et al.* 1983). In chronic disease states the production of superoxide used to generate peroxynitrite is likely from mitochondria, while in acute inflammatory disease it may be generated by a combination of mitochondrial and other activated NADPH oxidase sources (Ostrakhovitch, E.A. and Afanas'ev I.B. 2001; Miesel R., *et al.*, 1996).

The main oxidative killing mechanism of macrophages is peroxide peroxynitrite or (OONO-) (Darrah, P. A. *et al.*, 2000; Hickman-Davis, J., *et al.*, 1999; Nozaki, Y., *et al.*, 1997; Misra, A., *et al.*, 1996). The nitric oxide portion of the peroxynitrite is derived from the enzyme NO synthase II or inducible NOS using L-arginine and oxygen as substrates producing nitric oxide free radical and citrulline. When the nitric oxide radical and superoxide radical react together with high affinity, peroxynitrite is formed. Therefore, the production of peroxynitrite could be lessened by inhibiting the enzymes which form

superoxide or nitric oxide, or annihilating the superoxide or nitric oxide once formed. Peroxynitrite toxicity could be controlled by destroying the peroxide directly with antioxidants with which it reacts (Ostrakhovitch, E.A. and Afanas'ev I.B. 2001; Ferdinandy, P., *et al.*, 2000; Sies, H and de Groot, H. 1992).

Since peroxynitrite reacts with luminol to produce luminescence, this reaction can be used to measure peroxynitrite inside or outside cells because luminol is membrane permeable. In vitro experiments conducted in 35 mm dishes where PEC or THP-1 cells were stimulated with LPS revealed production of peroxynitrite which was inhibited by propanil in a dose-response relative to drug concentration (Figure 17).

To determine whether the propanil interfered with the formation of the reactants used to produce peroxynitrite or with the peroxynitrite-luminol reaction itself, we elicited the help of the SIN-1 reaction. Disintegration of SIN-1 generates superoxide and nitric oxide simultaneously over time which then react to form peroxynitrite. In the presence of luminol, the SIN-1 reaction produces light over a 30 minute time period. Therefore, we ran this reaction with 25, 50, 100 or 200 μ M propanil to test for interferences with the chemistry associated with formation of oxidative activity of peroxynitrite. Propanil exerted no measurable effect on peroxynitrite-based luminol luminescence at any dose tested (data not shown). This lack of interference with luminol dependent luminescence indicates that cellular studies with luminol and propanil (Figure 3), stems from a direct or indirect inhibition of cellular NO synthase II or NADPH oxidase/mitochondrial generation of superoxide.

Propanil decreased phagocytosis and phagocytosis in macrophages is regulated, to some extent by activation of the signaling proteins syk and phosphoinositol-3kinase (PI-3K)

(Aderem and Underhill, 1999). Activation of PI-3K leads to the downstream activation of protein kinase C (PKC), protein kinase B (Akt) and NF- κ B. It has also been shown that the Internalin B (Inl B) protein of *L. monocytogenes*, which mediates internalization of the bacterium, also requires activation of PI-3K (Ireton et al., 1996). InlB also ultimately leads to NF- κ B activation via PI-3K and Akt (Mansell et al., 2001). We have previously shown that propanil alters NF- κ B activation (Frost et al., 2001) and further studies in our lab have shown that propanil also affects AKT activity (unpublished data). Studies examining the effects of propanil on the upstream mediator PI-3K are ongoing. We speculate that the alterations in signaling induced by propanil exposure affect several pathways that culminate in the observed reductions in phagocytosis.

Previous studies using a *Listeria monocytogenes* model of infection in propanil-treated mice showed no difference in *Listeria* infection from vehicle control mice (Watson *et al.*, 2000). Watson *et al.* observed an increase in serum IL-6 levels from mice infected 2 days post-propanil-treatment, and sacrificed 3 days post-infection. Watson *et al.* (2000), observed no difference in IL-6 levels in mice that were infected with *Listeria* 7 days post propanil-treatment, then sacrificed three days post-infection. The peak neutrophil-dependent phase of *Listeria* infections in mice is approximately 2 days (Dinauer, M. C. *et al.*, 1997; Dalrymple, S. A., *et al.*, 1995). Also, IL-6 has been implicated as playing a major role in the early, neutrophil-dependent phase of *Listeria* clearance (Mocci, S. *et al.*, 1997; Dalrymple, S. A., *et al.*, 1995). Effects on IL-6 production by propanil have been evaluated in splenocytes and macrophages, but not neutrophils (Zhao W. *et al.*, 1998; Xie, X. C. *et al.*, 1997). It is possible that propanil-treated mice exhibit increased neutrophil activity, perhaps to compensate for decreased macrophage/monocyte function, resulting in resistance to

Listeria 3 days post-infection. Furthermore, unpublished results suggest that propanil-treated mice experience an increased flux of neutrophils in the spleen by 7 days post treatment (de la Rosa, P. Schafer, R., unpublished observations). Additionally, the effects of propanil treatment on secondary immune responses to *Listeria* have not been characterized.

The data herein demonstrate that propanil diminishes several macrophage functions. We have shown that propanil exposure results in decreased TNF- α production, phagocytosis, respiratory burst and listericidal activity. We have also shown that exposure of THP-1 cells to propanil resulted in a trend of less HLA-DR expression on cells with increasing doses, however the differences were not statistically significant. Other intriguing questions that remain to be answered are the effects of propanil on respiratory burst function and cytokine production by neutrophils.

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Figure 15

TNF- α production by THP-1 cells is decreased by propanil. THP-1 cells were plated at 1×10^6 cells/ in 6 well plates and stimulated with $1 \mu\text{g}$ / LPS in the presence of ethanol or $100 \mu\text{M}$ propanil. Supernatants were collected over a 24 hour time course, and TNF- α protein was measured by specific ELISA. A representative experiment is shown, depicting peak TNF- α production at 2 hours. The experiment was performed 4 times with similar results.

Figure 16

Phagocytosis is reduced by propanil. PMA-differentiated THP-1 cells (A) or PEC (B) were activated by IFN- γ for 24 hours before addition of LPS with either ethanol or indicated concentrations of propanil. Phagocytosis of 2 μ m fluorescent beads was determined by confocal microscopy. A weighted phagocytic index was calculated as described in the Materials. Experiments were performed 3 times with similar results. The mean phagocytic index $\pm$ SD is shown.

Figure 17

Propanil reduces respiratory burst activity. Generation of respiratory burst was measured from PMA-differentiated THP-1 cells (a) and PEC (b). PMA was used to elicit respiratory burst from cells simultaneously treated with LPS, and either ethanol or indicated concentrations of propanil, in the presence of luminol. CL was detected by a luminometer and expressed as relative light units. Experiments were performed twice in THP-1 cells and 3 times in PEC with similar results. A representative THP-1 experiment (A) is shown due to variability in the level of luminescence detected from the THP-1 cells in experiments conducted on different days. Mean relative light units $\pm$ SD are shown for PEC (B). There are significant differences by ANOVA between each treatment group from the 3 minute time point until the experiment was terminated.

A

Figure 18

Propanil reduces listericidal activity of macrophages. Mean *Listeria* CFU $\pm$ SD from IFN- γ activated THP-1 (A) or PEC (B) following *Listeria* infection at 1, 3 or 5 hours after infection. The initial infection levels at 1 hour (■), i.e., without gentamicin present in the media. The 3 and 5 hour treatments values, (▤) and (□), respectively, represent intracellular killing activity (as described in Methods). Samples were plated in triplicate. A representative experiment is shown and the mean $\pm$ SEM of PEC from 4 individual mice. a, b, c and d - represent significant differences from the IFN- γ , LPS, ethanol, and 25 μ M propanil treatments, respectively, within the same time point (i.e. 1, 3 or 5 hours).

Figure 19

Effects of propanil on HLA-DR expression on THP-1 cells. A. Histograms from a representative experiment showing surface HLA-DR expression in THP-1 cells activated with IFN- γ (48 hours) and stimulated with LPS in the presence of ethanol or the indicated concentrations of propanil for the final 24 or 4 hours. The Nil control shows HLA-DR expression on THP-1 cells that have not been activated with IFN- γ .

B. Mean THP-1 HLA-DR expression $\pm$ SD from 3 experiments.

REFERENCES

- Adams, D. O. and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annu. Rev. Immunol.* **2**, 283-318.
- Aderem, A. and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* **17**, 593-623.
- Allen, R. C. and Loose, L. D. (1976). Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **69**, 245-252.
- Alvarez-Dominguez, C., Carrasco-Marin, E., Lopez-Mato, P., and Leyva-Cobian, F. (2000). The contribution of both oxygen and nitrogen intermediates to the intracellular killing mechanisms of C1q-opsonized *Listeria monocytogenes* by the macrophage-like IC-21 cell line. *Immunology* **101**, 83-89.
- Antonini, J. M., Van Dyke, K., Ye, Z., DiMatteo, M., and Reasor, M. J. (1994). Introduction of luminol-dependent chemiluminescence as a method to study silica inflammation in the tissue and phagocytic cells of rat lung. *Environ. Health Perspect.* **102**, 37-42.
- Antonini, J. M., Yang, H. M., Ma, J. Y., Roberts, J. R., Barger, M. W., Butterworth, L., Charron, T. G., and Castranova, V. (2000). Subchronic silica exposure enhances respiratory defense mechanisms and the pulmonary clearance of *Listeria monocytogenes* in rats. *Inhal. Toxicol.* **12**, 1017-1036.

- Barbaro, A. L., Tosi, G., Frumento, G., Bruschi, E., D'Agostino, A., Valle, M. T., Manca, F., and Accolla, R. S. (2002). Block of Stat-1 activation in macrophages phagocytosing bacteria causes reduced transcription of CIITA and consequent impaired antigen presentation. *Eur. J Immunol* **32**, 1309-1318.
- Beckerman, K. P., Rogers, H. W., Corbett, J. A., Schreiber, R. D., McDaniel, M. L., and Unanue, E. R. (1993). Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to *Listeria monocytogenes*. *J. Immunol.* **150**, 888-895.
- Boockvar, K. S., Granger, D. L., Poston, R. M., Maybodi, M., Washington, M. K., Hibbs, J. B., Jr., and Kurlander, R. L. (1994). Nitric oxide produced during murine listeriosis is protective. *Infect. Immun.* **62**, 1089-1100.
- Buchmeier, N. A. and Schreiber, R. D. (1985). Requirement of endogenous interferon-gamma production for resolution of *Listeria monocytogenes* infection. *Proc. Natl. Acad. Sci. U. S. A* **82**, 7404-7408.
- Castro, L., Alvarez, M. N., and Radi, R. (1996). Modulatory role of nitric oxide on superoxide-dependent luminol chemiluminescence. *Arch. Biochem. Biophys.* **333**, 179-188.
- Catz, S. D., Carreras, M. C., and Poderoso, J. J. (1995). Nitric oxide synthase inhibitors decrease human polymorphonuclear leukocyte luminol-dependent chemiluminescence. *Free Radic. Biol. Med.* **19**, 741-748.

- Celada, A. and Nathan, C. (1994). Macrophage activation revisited. *Immunol. Today* **15**, 100-102.
- Dahlgren, C., Johansson, A., Lundqvist, H., Bjerrum, O. W., and Borregaard, N. (1992). Activation of the oxygen-radical-generating system in granules of intact human neutrophils by a calcium ionophore (ionomycin). *Biochim. Biophys. Acta* **1137**, 182-188.
- Dahlgren, C. and Karlsson, A. (1999). Respiratory burst in human neutrophils. *J. Immunol. Methods* **232**, 3-14.
- Dalrymple, S. A., Lucian, L. A., Slattery, R., McNeil, T., Aud, D. M., Fuchino, S., Lee, F., and Murray, R. (1995). Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. *Infect Immun.* **63**, 2262-8.
- Darrah, P. A., Hondalus, M. K., Chen, Q., Ischiropoulos, H., and Mosser, D. M. (2000). Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. *Infect Immun.* **68**, 3587-93.
- DeCoursey, T. E., Cherny, V. V., Morgan, D., Katz, B. Z., and Dinauer, M. C. (2001). The gp91phox component of NADPH oxidase is not the voltage-gated proton channel in phagocytes, but it helps. *J. Biol. Chem.* **276**, 36063-36066.

- Dinauer, M. C., Deck, M. B., and Unanue, E. R. (1997). Mice lacking reduced nicotinamide adenine dinucleotide phosphate oxidase activity show increased susceptibility to early infection with *Listeria monocytogenes*. *J. Immunol.* **158**, 5581-5583.
- Dusi, S., Della, B., V, Grzeskowiak, M., and Rossi, F. (1993). Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca(2+)-depleted human neutrophils. *Biochem. J.* **290**, 173-178.
- Endres, R., Luz, A., Schulze, H., Neubauer, H., Futterer, A., Holland, S. M., Wagner, H., and Pfeffer, K. (1997). Listeriosis in p47(phox-/-) and TRp55-/- mice: protection despite absence of ROI and susceptibility despite presence of RNI. *Immunity.* **7**, 419-432.
- Falder, J., Ridell, M., Karlsson, A., and Dahlgren, C. (1999). The phagocyte chemiluminescence paradox: luminol can act as an inhibitor of neutrophil NADPH-oxidase activity. *Luminescence.* **14**, 153-160.
- Ferdinandy, P., Danial, H., Ambrus, I., Rothery, R. A., and Schulz, R. (2000). Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res.* **87**, 241-7.
- Foyouzi-Youssefi, R., Petersson, F., Lew, D. P., Krause, K. H., and Nüsse, O. (1997). Chemoattractant-induced respiratory burst: increases in cytosolic Ca²⁺ concentrations are essential and synergize with a kinetically distinct second signal. *Biochem. J.* **322**, 709-718.

- Frost, L. L., Neeley, Y. X., Schafer, R., Gibson, L. F., and Barnett, J. B. (2001). Propanil inhibits tumor necrosis factor- α production by reducing nuclear levels of the transcription factor nuclear factor- κ B in the macrophage cell line ic-21. *Toxicol. Appl. Pharmacol.* **172**, 186-193.
- Ganz, T. (1993). Macrophage function. *New Horiz.* **1**, 23-27.
- Geiszt, M., Kapus, A., and Ligeti, E. (2001). Chronic granulomatous disease: more than the lack of superoxide? *J. Leukoc. Biol.* **69**, 191-196.
- Geiszt, M., Szeberenyi, J. B., Kaldi, K., and Ligeti, E. (1999). Role of different Ca^{2+} sources in the superoxide production of human neutrophil granulocytes. *Free Radic. Biol. Med.* **26**, 1092-1099.
- Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002). Capacitative Ca^{2+} influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase. *J. Leukoc. Biol.* **71**, 611-617.
- Gregory, S. H., Wing, E. J., Hoffman, R. A., and Simmons, R. L. (1993). Reactive nitrogen intermediates suppress the primary immunologic response to *Listeria*. *J. Immunol.* **150**, 2901-2909.
- Grimm, M., Spiecker, M., De Caterina, R., Shin, W. S., and Liao, J. K. (2002). Inhibition of MHC class II gene transcription by nitric oxide and antioxidants. *J Biol. Chem.* **277**, 26460-26469.

- Gross, A., Dugas, N., Spiesser, S., Vouldoukis, I., Damais, C., Kolb, J. P., Dugas, B., and Dornand, J. (1998). Nitric oxide production in human macrophagic cells phagocytizing opsonized zymosan: direct characterization by measurement of the luminol dependent chemiluminescence. *Free Radic. Res.* **28**, 179-191.
- Harton, J. A. and Ting, J. P. (2000). Class II transactivator: mastering the art of major histocompatibility complex expression. *Mol. Cell Biol.* **20**, 6185-6194.
- Havell, E. A. (1989). Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J. Immunol.* **143**, 2894-2899.
- Hickman-Davis, J., Gibbs-Erwin, J., Lindsey, J. R., and Matalon, S. (1999). Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages by production of peroxynitrite. *Proc Natl Acad Sci U S A.* **96**, 4953-8.
- Inoue, S., Itagaki, S., and Amano, F. (1995). Intracellular killing of *Listeria monocytogenes* in the J774.1 macrophage-like cell line and the lipopolysaccharide (LPS)-resistant mutant LPS1916 cell line defective in the generation of reactive oxygen intermediates after LPS treatment. *Infect. Immun.* **63**, 1876-1886.
- Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., and Cossart, P. (1996). A role for phosphoinositide 3-kinase in bacterial invasion. *Science* **274**, 780-782.
- Jones, R. D., Hancock, J. T., and Morice, A. H. (2000). NADPH oxidase: a universal oxygen sensor? *Free Radic. Biol. Med.* **29**, 416-424.

- Johnston, R. B., Jr, and Kitagawa, S. (1985). Molecular basis for the enhanced respiratory burst of activated macrophages. *Fed Proc.* **44**, 2927-32.
- Kiderlen, A. F., Kaufmann, S. H., and Lohmann-Matthes, M. L. (1984). Protection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. *Eur. J. Immunol.* **14**, 964-967.
- Leenen, P. J., Canono, B. P., Drevets, D. A., Voerman, J. S., and Campbell, P. A. (1994). TNF-alpha and IFN-gamma stimulate a macrophage precursor cell line to kill *Listeria monocytogenes* in a nitric oxide-independent manner. *J. Immunol.* **153**, 5141-5147.
- Lepay, D. A., Steinman, R. M., Nathan, C. F., Murray, H. W., and Cohn, Z. A. (1985). Liver macrophages in murine listeriosis. Cell-mediated immunity is correlated with an influx of macrophages capable of generating reactive oxygen intermediates. *J. Exp. Med.* **161**, 1503-1512.
- Mansell, A., Khelef, N., Cossart, P., and O'Neill, L. A. (2001). Internalin B activates nuclear factor-kappa B via Ras, phosphoinositide 3-kinase, and Akt. *J. Biol. Chem.* **276**, 43597-43603.
- Miesel R., Murphy, M.P., and Kroger, H. (1996). Enhanced mitochondrial radical production in patients with rheumatoid arthritis correlates with elevated levels of tumor necrosis factor alpha in plasma. *Free Radic Res.* **25**, 161-9.

- Misra, A., Mukerjee, R., and Chaturvedi, U. C (1996). Release of reactive oxygen intermediates by dengue virus-induced macrophage cytotoxin. *Int J Exp Pathol.* **77**, 37-42.
- Mocci, S., Dalrymple, S. A., Nishinakamura, R., and Murray, R. (1997). The cytokine stew and innate resistance to *L. monocytogenes*. *Immunol Rev.* **158**, 107-14.
- Morris, A. C., Beresford, G. W., Mooney, M. R., and Boss, J. M. (2002). Kinetics of a Gamma Interferon Response: Expression and Assembly of CIITA Promoter IV and Inhibition by Methylation. *Mol. Cell Biol.* **22**, 4781-4791.
- Movitz, C., Sjolín, C., and Dahlgren, C. (1997). A rise in ionized calcium activates the neutrophil NADPH-oxidase but is not sufficient to directly translocate cytosolic p47phox or p67phox to b cytochrome containing membranes. *Inflammation* **21**, 531-540.
- Muller, M., Althaus, R., Frohlich, D., Frei, K., and Eugster, H. P. (1999). Reduced antilisterial activity of TNF-deficient bone marrow-derived macrophages is due to impaired superoxide production. *Eur. J. Immunol.* **29**, 3089-3097.
- Nakane, A., Minagawa, T., and Kato, K. (1988). Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. *Infect. Immun.* **56**, 2563-2569.

- Nakane ,A., Minagawa, T., Kohanawa, M., Chen, Y., Sato, H., Moriyama, M., and Tsuruoka, N. (1989). Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. *Infect. Immun.* **57**, 3331-3337.
- Nozaki, Y., Hasegawa, Y., Ichiyama, S., Nakashima, I., and Shimokata, K. (1997). Mechanism of nitric oxide-dependent killing of *Mycobacterium bovis* BCG in human alveolar macrophages. *Infect Immun.* **65**, 3644-7.
- Ohya, S., Tanabe, Y., Makino, M., Nomura, T., Xiong, H., Arakawa, M., and Mitsuyama, M. (1998). The contributions of reactive oxygen intermediates and reactive nitrogen intermediates to listericidal mechanisms differ in macrophages activated pre- and postinfection. *Infect. Immun.* **66**, 4043-4049.
- Ostrakhovitch, E. A., Afanas'ev, I. B. (2001). Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators. *Biochem Pharmacol.* **62**, 743-6.
- Ouadrhiri, Y., Scoreaux, B., Sibille, Y., and Tulkens, P. M. (1999). Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by gamma interferon. *Antimicrob. Agents Chemother.* **43**, 1242-1251.
- Peck, R. (1989). Gamma interferon induces monocyte killing of *Listeria monocytogenes* by an oxygen-dependent pathway; alpha- or beta-interferons by oxygen-independent pathways. *J. Leukoc. Biol.* **46**, 434-440.

- Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* **73**, 457-467.
- Putney, J. W., Jr. and McKay, R. R. (1999). Capacitative calcium entry channels. *Bioessays* **21**, 38-46.
- Radi, R., Cosgrove, T. P., Beckman, J. S., and Freeman, B. A. (1993). Peroxynitrite-induced luminol chemiluminescence. *Biochem. J.* **290**, 51-57.
- Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* **364**, 798-802.
- Samsom, J. N., Langermans, J. A., Groeneveld, P. H., and van Furth, R. (1996). Acquired resistance against a secondary infection with *Listeria monocytogenes* in mice is not dependent on reactive nitrogen intermediates. *Infect. Immun.* **64**, 1197-1202.
- Sasada, M., Pabst, M. J., and Johnston, R. B., Jr. (1983). Activation of mouse peritoneal macrophages by lipopolysaccharide alters the kinetic parameters of the superoxide-producing NADPH oxidase. *J Biol Chem.* **258**, 9631-5.

- Segal, B. H., Leto, T. L., Gallin, J. I., Malech, H. L., and Holland, S. M. (2000). Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* **79**, 170-200.
- Sies, H., and de Groot, H. (1992). Role of reactive oxygen species in cell toxicity. *Toxicol Lett.* **64-65**, 547-51.
- Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinaker, M., and Nathan, C. (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity.* **10**, 29-38.
- Ting, J. P. and Trowsdale, J. (2002). Genetic control of MHC class II expression. *Cell* **109**, S21-S33.
- Watson, V.A., Barnett, J. B., and Schafer, R. (2000). In vivo cytokine production and resistance to infection after acute exposure to 3,4-dichloropropionaniline. *J Toxicol Environ Health A.* **60**, 391-406.
- Wojciechowski, W., DeSanctis, J., Skamene, E., and Radzioch, D. (1999). Attenuation of MHC class II expression in macrophages infected with *Mycobacterium bovis* bacillus Calmette-Guerin involves class II transactivator and depends on the *Nramp1* gene. *J. Immunol.* **163**, 2688-2696.
- Xia, Y. and Zweier, J.L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. U. S. A* **94**, 6954-6958.

- Xie, Y.C., Schafer, R., and Barnett, J.B. (1997a). Inhibitory effect of 3,4-dichloro-propionaniline on cytokine production by macrophages is associated with LPS-mediated signal transduction. *J. Leukoc. Biol.* **61**, 745-752.
- Xie, Y.C., Schafer, R., and Barnett, J.B. (1997b). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol. Appl. Pharmacol.* **145**, 184-191.
- Zhou, H., Duncan, R. F., Robison, T. W., Gao, L., and Forman, H .J. (1997). Ca^{2+} -dependent p47phox translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. *Am. J. Physiol* **273**, L1042-L1047.

Chapter 3

In vivo exposure of C57Bl/6 mice to propanil reduces NF- κ B activation of their peritoneal exudate cells.

ABSTRACT

The herbicide, propanil, has been shown to alter macrophage cytokine production and the ability of macrophages to function normally. Previous studies have documented the reductions in cytokine production from murine peritoneal exudate cells (PEC) harvested from mice treated with propanil *in vivo*. In this study, the effect of *in vivo* propanil treatment on NF- κ B activation was evaluated. The results demonstrated that PEC exposed to propanil *in vivo* exhibited diminished nuclear levels of NF- κ B upon *ex vivo* stimulation with bacterial lipopolysaccharide (LPS).

INTRODUCTION

Given that propanil down regulates TNF- α production in a variety of experimental paradigms (Frost, L. L., *et al.*, (2001); Xie, X. C., *et al.* (1997a); Xie, X. C., *et al.*, (1997b)) and the critical role of NF- κ B in the induction of this important cytokine, we were interested in the effects of propanil on NF- κ B activation. Propanil has previously been shown to reduce TNF- α and nuclear NF- κ B concentrations in a murine macrophage cell line, IC-21, and a human monocytic cell line, THP-1. We were interested in determining if the nuclear NF- κ B levels in PEC treated with propanil *in vivo* paralleled those observed in monocyte/macrophage cell lines treated *in vitro*.

MATERIALS AND METHODS

Animals

C57Bl/6 mice were purchased from Charles River Farms (Wilmington, DE) and allowed to acclimate for 7 days before experimental procedures were initiated. Food and water were provided *ad libitum*. Experimental procedures were conducted on mice that ranged in age between 7 and 12 weeks.

In vivo exposure of mice to propanil, and macrophage harvest and preparation

Mice were injected intraperitoneally (i.p.) with an appropriate volume of propanil (ChemService, West Chester, PA; >98% purity) (diluted in corn oil) to achieve a concentration of 200 mg/kg, or corn oil alone. Three days post propanil treatment, PEC were elicited by injecting mice i.p. with 1.5 ml of 4% sterile aged thioglycollate. On day 7, PEC were harvested by sacrificing mice and exposing the peritonea with a midline surgical incision. Nine ml of sterile phosphate-buffered saline (PBS; BioWhittaker, Walkersville,

MD) supplemented with 10U/ml heparin (Sigma, St. Louis, MO) was injected into the peritoneal cavity followed by syringe aspiration. Total cells were centrifuged and washed with PBS. Red blood cells were lysed and remaining cells counted. Macrophages were allowed to adhere to plastic dishes; nonadherent cells were removed by addition of two bursts of warm PBS and vacuum aspiration. Viability was monitored by trypan blue exclusion and was routinely $\geq 95\%$.

RESULTS

Propanil reduced nuclear NF- κ B PEC treated *in vivo*

Nuclear levels of NF- κ B p65 were also analyzed in nuclear extracts prepared from PEC harvested from mice treated with 200 mg/kg propanil or the vehicle, corn oil, were stimulated with 10 μ g/ml LPS for 15 minutes to 4 hours. 5 μ g of each extract was separated by SDS-PAGE and immunoblotted for the p65 subunit of NF- κ B (Figure 20A). Densitometric analysis of p65 bands from PEC harvested from propanil-treated mice showed an average decrease of 9, 23, 24, 29 and 44 percent as compared to the corn oil control at 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours, respectively (Figure 20B). ANOVA analysis of the optical densities of the NF- κ B p65 bands demonstrated that the decrease in p65 in propanil-treated PEC at 4 hours was significantly different ($P < 0.05$).

DISCUSSION

Previously we reported that exposure of murine macrophages to propanil *in vitro* suppressed NF- κ B nuclear transport. The experimental data presented herein extend those findings and demonstrate that exposure *in vivo* has the same effect. Using an established treatment

regime for propanil exposure and harvesting of PEC, our data demonstrate that *in vivo* propanil exposure led to reduced NF- κ B nuclear localization upon *ex vivo* LPS stimulation.

Previously published data demonstrate that propanil inhibits TNF- α protein and mRNA production (Xie, X. C., *et al.*, (1997); Frost, L. L., *et al.* (2001)). We have also shown that nuclear NF- κ B is reduced in a murine macrophage cell line, IC-21, after *in vitro* exposure as well as in peritoneal exudate cells exposed to propanil *in vitro* (Frost, L. L., *et al.*, (2001)). Here we demonstrate that NF- κ B nuclear translocation is reduced in *ex vivo* stimulated PEC that have been exposed to propanil *in vivo*. This reduced NF- κ B activation likely contributes to the suppression of TNF- α protein and message production from these cells.

Figure 20

Propanil reduces nuclear levels of NF- κ B p65 from *in vivo*-treated PEC. C57Bl/6 mice were treated with 200 mg/kg propanil or corn oil, as a vehicle control. Mice were sacrificed on day 7, and PEC were harvested. The cells were allowed to adhere for 2 hours, then stimulated with 10 μ g/ml LPS. **A.** a representative NF- κ B p65 Western blot in which nuclear extracts were prepared from LPS-stimulated PEC at 0 (*lanes 6 and 12*), 15 (*lanes 1 and 7*), 30 (*lanes 2 and 8*), 60 (*lanes 3 and 9*), 120 (*lanes 4 and 10*) and 240 (*lanes 5 and 11*) minutes. **B.** Densitometric analysis of NF- κ B p65 Western blots from 3 separate experiments, expressed as percent of the ethanol control. (* = $p < 0.05$)

A

B

REFERENCES

- Frost, L. L., Neeley, Y. X., Schafer, R., Gibson, L. F., and Barnett, J. B. (2001). Propanil inhibits tumor necrosis factor-alpha production by reducing nuclear levels of the transcription factor nuclear factor-kappaB in the macrophage cell line ic-21. *Toxicol.Appl.Pharmacol.* **172**, 186-193.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997a). Inhibitory effect of 3,4-dichloro-propionaniline on cytokine production by macrophages is associated with LPS-mediated signal transduction. *J.Leukoc.Biol.* **61**, 745-752.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997b). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol.Appl.Pharmacol.*

GENERAL DISCUSSION

The immunotoxic effects of propanil have been explored with regard to effects on humoral immunity, cellular immunity and hematopoiesis. Ongoing investigations into the mechanisms of immune dysfunctions include the effects of propanil on B and T cell development and alterations in signaling in T cells and macrophages. Additional studies are providing insight into the immunotoxic consequences of other herbicides in mixtures with propanil. This dissertation focuses on 1) alterations in NF- κ B signaling caused by propanil in macrophages of both murine and human origin and 2) the effects of propanil on critical macrophage functions.

I. Both *In Vivo* and *In Vitro* Propanil Exposure Decrease Nuclear Levels of NF- κ B in Macrophages.

Propanil has previously been shown to decrease production of TNF- α in murine macrophages treated *in vivo* or *in vitro*. Both mRNA and protein levels of TNF- α were reduced by propanil exposure (Xie, Y. C. *et al.* (1997b)). TNF- α production, in response to a variety of stimuli, is strongly dependent on the transcription factor NF- κ B (Trede, N. S. *et al.* (1995); Frost, L. L. *et al.* (2001); Shackelford, R. E. *et al.* (1997); Schow, S. R. *et al.* (1997); Shakhov, A. N. *et al.* (1990b); Jongeneel, C. V. (1994)). Our investigations into the effects of propanil on NF- κ B revealed that nuclear levels of the transcription factor were reduced by exposure to the herbicide *in vitro* or *in vivo*. The murine peritoneal macrophage cell line, IC-21 and the human monocytic cell line, THP-1 both showed decreased nuclear levels of NF- κ B when treated with 167 μ M propanil. Kuprash *et al.* (1999) demonstrated that both the human and murine TNF- α promoters rely strongly on NF- κ B elements for

transcriptional activity, while other elements are required for maximal activation Kuprash, D. V. *et al.* (1999). Deletion or mutation of distal κ B binding elements in both promoters resulted in dramatic suppression of transcriptional activity (Collart, M. A. *et al.* (1990); Drouet, C. *et al.* (1991); Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R., and Nedospasov, S. A. (1999); Liu, H. *et al.* (2000); Shakhov, A. N. *et al.* (1990a); Udalova, I. A. *et al.* (2001); Oeth, P. *et al.* (1997)). Additional transcriptional elements shown to cooperate with NF- κ B to induce maximal transcriptional activity differ with cell type (T cells, B cells and monocytes/macrophages) and stimulus (LPS, PMA, ionophores, cytokines) Tsai, E. Y. *et al.* (2000); Yao, *et al.*, (1997); Guha, M. *et al.* (2001); Means, T. K. *et al.* (2000); Pope, R. M. *et al.* (1994); Tsai, E. Y. *et al.* (1996); Tsai, E. Y., *et al.*, (2000).

In the context of TNF- α regulation, several nuclear cofactors have been shown to either directly bind the TNF- α promoter, or alternatively bind to or cooperate with NF- κ B thereby altering its DNA transactivating potential. Cooperation between AP-1 and NF- κ B has been demonstrated in human monocytes and T cells (Zagariya, A. *et al.* (1998); Adcock, I. M. (1997); Matt, T. (2002); Oeth, P., *et al.*, (1997); Udalova, I. A. and Kwiatkowski, D. (2001)). Additionally, others have shown that maximal TNF- α transcription or stimulus-specific transcription, by ionomycin, virus infection or LPS requires participation of the transcription factors NFAT, Elk-1 and Ets proteins (Tsai, E. Y., *et al.*, (1996)). Also implicated in the regulation of the TNF- α gene is the CCAAT/enhancer binding protein (C/EBP) (Pope, R. *et al.* (2000); Pope, R. M., *et al.*, (1994)). However, the most important events in NF- κ B-dependent transcription seem to be specific phosphorylation of the p65 subunit of NF- κ B on S276, by the catalytic subunit of PKA which is associated with the

IKK complex, and the subsequent interaction with CBP/p300 (reviewed in Ghosh, S. *et al.* (2002); Zhong, H. *et al.* (1997); Zhong, H. *et al.* (1998); Zhong, H. *et al.* (2002)). Association with CBP/p300 increases NF- κ B transcriptional efficiency (Sheppard, K. A. *et al.* (1999)).

Our experiments to determine the nature of NF- κ B inhibition by propanil led to I κ B α analysis. Evaluation of I κ B α degradation, however, showed no difference in propanil-treated cells from the ethanol controls. Functional redundancy between I κ B β and I κ B α in NF- κ B activation necessitates that the effects of propanil on I κ B β also be explored. The experiments to determine the effects of propanil on I κ B β are currently ongoing. Their results will provide great insight into the possible mechanism of NF- κ B suppression by propanil. However, if degradation of I κ B β is also not altered by propanil, the implication will be that propanil likely acts at the level of nuclear transport.

There is evidence to support specific inhibition of NF- κ B nuclear entry. Recently, Hippenstiel *et al.* (2002) demonstrated that specific inhibition of the small GTPase, Rho, in human endothelial cells lead to inhibition of NF- κ B nuclear transport despite normal degradation of I κ B in response to TNF- α . Furthermore, recruitment of activated Rac1/PI3K to TLR2, followed by NF- κ B activation in a manner independent of I κ B α degradation was demonstrated in THP-1 cells upon bacterial stimulation (Lee, J. *et al.* (2000)). Other anti-inflammatory compounds, dehydroxymethylepoxyquinomicin and dimethylfumerate, have also been shown to inhibit NF- κ B nuclear translocation independent of release from I κ B (Lee, J., Mira-Arbibe, L., and Ulevitch, R. J. (2000); Loewe, *et al.*, (2002)).

We have only begun to address the effects of propanil on NF- κ B activation. Many questions remain unanswered: 1) does propanil alter the composition of nuclear co-

activating proteins; 2) does propanil alter phosphorylation of p65, and does that affect nuclear translocation; and 3) does propanil affect the GTPases Rho or Ran (classically involved in macromolecular nuclear import), thus altering nuclear translocation of NF- κ B?

II. Propanil Alters Several Critical Macrophage Functions.

Although the *in vivo* toxicity of propanil has been addressed, and shown to involve depression of several levels of immune parameters, and *in vitro* propanil has been shown to reduce production of several cytokines from macrophages (Xie, Y. C., *et al.*, (1997b); Xie, Y. C. *et al.* (1997a); Cuff, C. F. *et al.* (1996); Zhao, W. *et al.* (1995); Barnett, J. B. *et al.* (1992); Theus, S. A. *et al.* (1992); Singleton, S. D. *et al.* (1973); Barnett, J. B. *et al.* (1989); Theus, S. A. *et al.* (1993); Zhao, W. *et al.* (1998)), this was the first study to specifically evaluate macrophage functions. We have demonstrated that propanil caused inhibition of phagocytosis, respiratory burst activation and listericidal abilities of macrophages (Frost *et al.*, manuscript in preparation).

Previous studies in macrophages have shown that propanil abrogates the initial IP₃-mediated calcium release (Xie, Y. C., *et al.*, (1997b)). The disruption in calcium dynamics likely contributes to many of the observed alterations in macrophage function and signaling. The process of phagocytosis has been shown to be associated with transient increases in intracellular free calcium in both neutrophils and monocytes (Geiszt, M. *et al.* (2001); Larsen, E. C. *et al.* (2000)). Furthermore, calcium fluxes play a critical role in the activation of the NADPH oxidase (Dusi, S. *et al.* (1993); Foyouzi-Youssefi, R. *et al.* (1997); Geiszt, M. *et al.* (1999); Granfeldt, D. *et al.* (2002); Zhou, H. *et al.* (1997)). NADPH oxidase function requires the translocation of cytoplasmic factors p47^{phox}, p67^{phox}, p21Rac (a Rho-

family GTPase), p40 and RAP1A (reviewed in Geiszt, M., *et al.*, (2001); Wientjes, F. B. *et al.* (1995)). Translocation of the *phox* proteins, p47 and p67 are reliant on increased intracellular calcium, most likely due to the initial IP₃-mediated calcium release (Dusi, S., *et al.*, (1993); Zhou, H., *et al.*, (1997)). We speculate that altered calcium dynamics in macrophages that result from propanil exposure are critical events in the observed reduction in phagocytosis and NADPH oxidase activity.

Reduced listericidal activity of propanil-treated macrophages is also likely a result of the inability of the macrophages to generate a respiratory burst. The ability to clear *in vivo* *Listeria monocytogenes* infections has been shown to require NADPH oxidase activity (Dinauer, M. C. *et al.* (1997); Shiloh, M. U. *et al.* (1999)), in addition to endogenous TNF- α production (Havell, E. A. (1989); Kato, K. *et al.* (1989); Leenen, P. J. *et al.* (1994); Muller, M. *et al.* (1999); Nakane, A. *et al.* (1988); Rothe, J. *et al.* (1993)). The fact that propanil-treated macrophages demonstrate reduced listericidal activity is not surprising given the reductions in both TNF- α production and NADPH oxidase activity.

Previous studies using a *Listeria monocytogenes* model of infection in propanil-treated mice showed no difference in *Listeria* infection from vehicle control mice (Watson *et al.*, 2000). Watson *et al.* observed an increase in serum IL-6 levels from mice infected 2 days post-propanil-treatment, and sacrificed 3 days post-infection. Watson *et al.* (2000), observed no difference in IL-6 levels in mice that were infected with *Listeria* 7 days post propanil-treatment, then sacrificed three days post-infection. The peak neutrophil-dependent phase of *Listeria* infections in mice is approximately 2 days (Dinauer, M. C. *et al.*, (1997); Dalrymple, S. A., *et al.*, (1995)). Also, IL-6 has been implicated as playing a major role in the early, neutrophil-dependent phase of *Listeria* clearance (Mocci, S. *et al.*, (1997); Dalrymple, S. A., *et al.*, (1995)). Effects on IL-6 production by propanil have been evaluated in splenocytes and macrophages, but not neutrophils (Zhao W. *et al.*, (1998); Xie, X. C. *et*

al., (1997b)). It is possible that propanil-treated mice exhibit increased neutrophil activity, perhaps to compensate for decreased macrophage/monocyte function, resulting in resistance to *Listeria* 3 days post-infection. Furthermore, unpublished results suggest that propanil-treated mice experience an increased flux of neutrophils in the spleen by 7 days post treatment (de la Rosa, P. Schafer, R., unpublished observations). Additionally, the effects of propanil treatment on secondary immune responses to *Listeria* have not been characterized.

In summary, a common factor to many of the observed effects of propanil on macrophages lies within the Rho family of GTPases. As mentioned above, NADPH oxidase activation requires translocation of the Rho family GTPase, p21Rac (Abo, A. *et al.*, (1994)). Interestingly, in addition to the role Rho plays in NF- κ B activation and nuclear import (independent of I κ B degradation), it is also involved in activation of calcium fluxes and phagocytosis (Cammarano, M. S. *et al.*, (2001); Hehner, S. P. *et al.*, (2000)); Stephens, L. *et al.*, (2002)). Therefore, evaluating the effects of propanil on Rho GTPases may provide a critical link to many of the observed defects in macrophages resulting from propanil treatment.

REFERENCES

- Abo, A., Webb, M. R., Grogan, A., and Segal, A. W. (1994). Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem.J.* **298**, 585-591.
- Adcock, I. M. (1997). Transcription factors as activators of gene transcription: AP-1 and NF-kappa B. *Monaldi.Arch.Chest Dis.* **52**, 178-186.
- Barnett, J. B. and Gandy, J. (1989). Effect of acute propanil exposure on the immune response of C57Bl/6 mice. *Fundam.Appl.Toxicol.* **12**, 757-764.
- Barnett, J. B., Gandy, J., Wilbourn, D., and Theus, S. A. (1992). Comparison of the immunotoxicity of propanil and its metabolite, 3,4-dichloroaniline, in C57Bl/6 mice. *Fundam.Appl.Toxicol.* **18**, 628-631.
- Cammarano, M. S. and Minden, A. (2001). Dbl and the Rho GTPases activate NF kappa B by I kappa B kinase (IKK)-dependent and IKK-independent pathways. *J Biol.Chem.* **276**, 25876-25882.
- Collart, M. A., Baeuerle, P., and Vassalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol.Cell Biol.* **10**, 1498-1506.
- Cuff, C. F., Zhao, W., Nukui, T., Schafer, R., and Barnett, J. B. (1996). 3,4-Dichloropropionanilide-induced atrophy of the thymus: mechanisms of toxicity and recovery. *Fundam.Appl.Toxicol.* **33**, 83-90.

- Dinauer, M. C., Deck, M. B., and Unanue, E. R. (1997). Mice lacking reduced nicotinamide adenine dinucleotide phosphate oxidase activity show increased susceptibility to early infection with *Listeria monocytogenes*. *J.Immunol.* **158**, 5581-5583.
- Drouet, C., Shakhov, A. N., and Jongeneel, C. V. (1991). Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor- α promoter in primary macrophages. *J.Immunol.* **147**, 1694-1700.
- Dalrymple, S. A., Lucian, L. A., Slattery, R., McNeil, T., Aud, D. M., Fuchino, S., Lee, F., and Murray, R. (1995). Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. *Infect Immun.* **63**, 2262-8.
- Dusi, S., Della, B., V, Grzeskowiak, M., and Rossi, F. (1993). Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca(2+)-depleted human neutrophils. *Biochem.J.* **290**, 173-178.
- Foyouzi-Youssefi, R., Petersson, F., Lew, D. P., Krause, K. H., and Nüsse, O. (1997). Chemoattractant-induced respiratory burst: increases in cytosolic Ca²⁺ concentrations are essential and synergize with a kinetically distinct second signal. *Biochem.J.* **322**, 709-718.
- Frost, L. L., Neeley, Y. X., Schafer, R., Gibson, L. F., and Barnett, J. B. (2001). Propanil inhibits tumor necrosis factor- α production by reducing nuclear levels of the transcription factor nuclear factor- κ B in the macrophage cell line ic-21. *Toxicol.Appl.Pharmacol.* **172**, 186-193.
- Geiszt, M., Kapus, A., and Ligeti, E. (2001). Chronic granulomatous disease: more than the lack of superoxide? *J.Leukoc.Biol.* **69**, 191-196.

- Geiszt, M., Szeberenyi, J. B., Kaldi, K., and Ligeti, E. (1999). Role of different Ca²⁺ sources in the superoxide production of human neutrophil granulocytes. *Free Radic.Biol.Med.* **26**, 1092-1099.
- Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* **109**, S81-S96.
- Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002). Capacitative Ca²⁺ influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase. *J.Leukoc.Biol.* **71**, 611-617.
- Guha, M., O'Connell, M. A., Pawlinski, R., Hollis, A., McGovern, P., Yan, S. F., Stern, D., and Mackman, N. (2001). Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor alpha expression by inducing Elk-1 phosphorylation and Egr-1 expression. *Blood* **98**, 1429-1439.
- Havell, E. A. (1989). Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J.Immunol.* **143**, 2894-2899.
- Hehner, S. P., Hofmann, T. G., Ushmorov, A., Dienz, O., Wing-Lan, L., I, Lassam, N., Scheidereit, C., Droge, W., and Schmitz, M. L. (2000). Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex. *Mol.Cell Biol.* **20**, 2556-2568.
- Hippenstiel, S., Schmeck, B., Seybold, J., Krull, M., Eichel-Streiber, C., and Suttorp, N. (2002) Reduction of tumor necrosis factor-alpha (TNF-alpha) related nuclear factor-kappaB (NF-kappaB) translocation but not inhibitor kappa-B (Ikappa-B)-degradation by Rho protein inhibition in human endothelial cells. *Biochem. Pharmacol.* **64**, 971-7.

- Jongeneel, C. V. (1994). Regulation of the TNF alpha gene. *Prog.Clin.Biol.Res.* **388**, 367-381.
- Kato, K., Nakane, A., Minagawa, T., Kasai, N., Yamamoto, K., Sato, N., and Tsuruoka, N. (1989). Human tumor necrosis factor increases the resistance against *Listeria* infection in mice. *Med.Microbiol.Immunol.(Berl)* **178**, 337-346.
- Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R., and Nedospasov, S. A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol* **162**, 4045-4052.
- Larsen, E. C., DiGennaro, J. A., Saito, N., Mehta, S., Loegering, D. J., Mazurkiewicz, J. E., and Lennartz, M. R. (2000). Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J Immunol* **165**, 2809-2817.
- Lee, J., Mira-Arbibe, L., and Ulevitch, R. J. (2000). TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. *J Leukoc.Biol.* **68**, 909-915.
- Leenen, P. J., Canono, B. P., Drevets, D. A., Voerman, J. S., and Campbell, P. A. (1994). TNF-alpha and IFN-gamma stimulate a macrophage precursor cell line to kill *Listeria* monocytogenes in a nitric oxide-independent manner. *J.Immunol.* **153**, 5141-5147.
- Liu, H., Sidiropoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pope, R. M. (2000). TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J Immunol* **164**, 4277-4285.

- Loewe, R., Holnthoner, W., Groger, M., Pillinger, M., Gruber, F., Mechtcheriakova, D., Hofer E, Wolff, K., and Petzelbauer, P. (2002). Dimethylfumarate inhibits TNF-induced nuclear entry of NF-kappa B/p65 in human endothelial cells. *J. Immunol.* **168**, 4781-7.
- Matt, T. (2002). Transcriptional control of the inflammatory response: a role for the CREB-binding protein (CBP). *Acta Med.Austriaca* **29**, 77-79.
- Means, T. K., Pavlovich, R. P., Roca, D., Vermeulen, M. W., and Fenton, M. J. (2000). Activation of TNF-alpha transcription utilizes distinct MAP kinase pathways in different macrophage populations. *J.Leukoc.Biol.* **67**, 885-893.
- Mocci, S., Dalrymple, S. A., Nishinakamura, R., and Murray, R. (1997). The cytokine stew and innate resistance to *L. monocytogenes*. *Immunol. Rev.* **158**, 107-14.
- Muller, M., Althaus, R., Frohlich, D., Frei, K., and Eugster, H. P. (1999). Reduced antilisterial activity of TNF-deficient bone marrow-derived macrophages is due to impaired superoxide production. *Eur.J.Immunol.* **29**, 3089-3097.
- Nakane, A., Minagawa, T., and Kato, K. (1988). Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. *Infect.Immun.* **56**, 2563-2569.
- Oeth, P., Parry, G. C., and Mackman, N. (1997). Regulation of the tissue factor gene in human monocytic cells. Role of AP-1, NF-kappa B/Rel, and Sp1 proteins in uninduced and lipopolysaccharide-induced expression. *Arterioscler.Thromb.Vasc.Biol.* **17**, 365-374.
- Pope, R., Mungre, S., Liu, H., and Thimmapaya, B. (2000). Regulation of TNF-alpha expression in normal macrophages: the role of C/EBPbeta. *Cytokine* **12**, 1171-1181.

- Pope, R. M., Leutz, A., and Ness, S. A. (1994). C/EBP beta regulation of the tumor necrosis factor alpha gene. *J Clin. Invest* **94**, 1449-1455.
- Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* **364**, 798-802.
- Schow, S. R. and Joly, A. (1997). N-acetyl-leuciny-leuciny-norleucinal inhibits lipopolysaccharide-induced NF-kappaB activation and prevents TNF and IL-6 synthesis in vivo. *Cell Immunol.* **175**, 199-202.
- Shackelford, R. E., Alford, P. B., Xue, Y., Thai, S. F., Adams, D. O., and Pizzo, S. (1997). Aspirin inhibits tumor necrosis factor alpha gene expression in murine tissue macrophages. *Mol. Pharmacol.* **52**, 421-429.
- Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jongeneel, C. V. (1990a). Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp. Med.* **171**, 35-47.
- Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jongeneel, C. V. (1990b). Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J. Exp. Med.* **171**, 35-47.
- Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999). Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol. Cell Biol.* **19**, 6367-6378.

- Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinanuer, M., and Nathan, C. (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity*. **10**, 29-38.
- Singleton, S. D. and Murphy, S. D. (1973). Propanil (3,4-dichloropropionanilide)-induced methemoglobin formation in mice in relation to acylamidase activity. *Toxicol.Appl.Pharmacol.* **25**, 20-29.
- Stephens, L., Ellson, C., and Hawkins, P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr.Opin.Cell Biol.* **14**, 203-213.
- Theus, S. A., Lau, K. A., Tabor, D. R., Soderberg, L. S., and Barnett, J. B. (1992). In vivo prenatal chlordane exposure induces development of endogenous inflammatory macrophages. *J.Leukoc.Biol.* **51**, 366-372.
- Theus, S. A., Tabor, D. R., Gandy, J., and Barnett, J. B. (1993). Alteration of macrophage cytotoxicity through endogenous interferon and tumor necrosis factor alpha induction by propanil. *Toxicol.Appl.Pharmacol.* **118**, 46-52.
- Trede, N. S., Tsytsykova, A. V., Chatila, T., Goldfeld, A. E., and Geha, R. S. (1995). Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. *J Immunol* **155**, 902-908.
- Tsai, E. Y., Falvo, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000). A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol.Cell Biol.* **20**, 6084-6094.

- Tsai, E. Y., Yie, J., Thanos, D., and Goldfeld, A. E. (1996). Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol.Cell Biol.* **16**, 5232-5244.
- Udalova, I. A. and Kwiatkowski, D. (2001). Interaction of AP-1 with a cluster of NF-kappa B binding elements in the human TNF promoter region. *Biochem.Biophys.Res.Comm.* **289**, 25-33.
- Watson, V.A., Barnett, J. B., and Schafer, R. (2000). In vivo cytokine production and resistance to infection after acute exposure to 3,4-dichloropropionaniline. *J Toxicol. Environ. Health A.* **60**, 391-406.
- Wientjes, F. B. and Segal, A. W. (1995). NADPH oxidase and the respiratory burst. *Semin.Cell Biol.* **6**, 357-365.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997a). Inhibitory effect of 3,4-dichloropropionaniline on cytokine production by macrophages is associated with LPS-mediated signal transduction. *J.Leukoc.Biol.* **61**, 745-752.
- Xie, Y. C., Schafer, R., and Barnett, J. B. (1997b). The immunomodulatory effects of the herbicide propanil on murine macrophage interleukin-6 and tumor necrosis factor-alpha production. *Toxicol.Appl.Pharmacol.* **145**, 184-191.
- Yao, J., Mackman, N., Edgington, T. S., and Fan, S. T. (1997). Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J. Biol. Chem.* **272**, 17795-801.

Zagariya, A., Mungre, S., Lovis, R., Birrer, M., Ness, S., Thimmapaya, B., and Pope, R. (1998). Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta-induced activation by c-Jun. *Mol.Cell Biol.* **18**, 2815-2824.

Zhao, W., Schafer, R., and Barnett, J. B. (1998). Cytokine Production by C57BL/6 Mouse Spleen Cells is Selectively Reduced by Exposure to Propanil. *J.Toxicol.EnvIRON.Health.*

Zhao, W., Schafer, R., Cuff, C. F., Gandy, J., and Barnett, J. B. (1995). Changes in primary and secondary lymphoid organ T-cell subpopulations resulting from acute in vivo exposure to propanil. *J.Toxicol.EnvIRON.Health* **46**, 171-181.

Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol.Cell* **9**, 625-636.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89**, 413-424.

Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol.Cell* **1**, 661-671.

Zhou, H., Duncan, R. F., Robison, T. W., Gao, L., and Forman, H. J. (1997). Ca(2+)-dependent p47phox translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. *Am.J.Physiol* **273**, L1042-L1047.