December 1993

Nitric Oxide Production: A Mechanism for Inhibition of Chlamydia Trachomatis Replication

Bojun Chen
East Tennessee State University

Order Number 9412223

Nitric oxide production: A mechanism for inhibition of *Chlamydia trachomatis* replication

Chen, Bojun, Ph.D.

East Tennessee State University, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

NITRIC OXIDE PRODUCTION: A MECHANISM FOR INHIBITION OF *CHLAMYDIA TRACHOMATIS* REPLICATION

A Dissertation

presented to

the Faculty of the Department of Microbiology

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

---

by Bojun Chen

December 1993

APPROVAL

This is to certify that the Graduate committee of

Bojun Chen

met on the

27TH day of August, 1993

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of
the Graduate Council

Associate Vice-President for Research
and Dean of the Graduate School

Nitric Oxide Production: A Mechanism for Inhibition of *Chlamydia trachomatis* Replication

by

Bojun Chen

Chlamydia trachomatis (CT) replicates in macrophages, but is inhibited by IFN-γ or LPS. IFN-γ and/or LPS induced nitrite production in mouse peritoneal macrophages, macrophage cell lines (RAW264.7 and J774A.1) and McCoy cells. Kinetic studies indicated that peak production occurred 48 hours post-treatment. CT infection itself was insufficient to induce nitrite production, but resulted in enhancement of nitrite production in IFN-γ-treated cells. Treatment with IFN-γ or LPS resulted in significant inhibition of CT replication in these cells. Strong correlation between nitrite production and inhibition of CT replication was observed in RAW264.7 and J774A.1 cells (correlation coefficients: -0.93 and -0.94, $p<0.001$). N^g-monomethyl-L-arginine (L-NMMA) specifically inhibited nitrite production and partially reversed inhibition of CT replication in macrophage cell lines. NOS mRNA was measured in RAW264.7 cells by Northern blot and Dot blot hybridization. Strong correlation between NOS mRNA expression and inhibition of CT replication (correlation coefficient: -0.97, $p<0.05$) was observed. Anti-TNF-α antibody completely neutralized the biological activity of TNF-α secreted by LPS-treated RAW264.7 cells, yet the antibody neither reduced nitrite production nor restored CT replication. Combination of the antibody and L-NMMA significantly enhanced restoration of CT replication. In peritoneal macrophages, inhibition of CT replication induced by IFN-γ was partially restored by L-NMMA or anti-TNF-α antibody. In McCoy cells, inhibition of CT replication induced by IFN-γ and LPS was not significantly restored by L-NMMA. Great restoration of CT replication by 1 mM L-NMMA was observed in LPS-treated J774A.1 cells (31%), but not in IFN-γ-treated cells (5%). Our data indicate that (1) NO production is one of the mechanisms for inhibition of CT replication in IFN-γ-activated peritoneal macrophages and RAW264.7 cells; (2) NO plays a significant role in CT inhibition in LPS-treated macrophage cell lines, but not peritoneal macrophages; (3) TNF-α may be associated with inhibition, but the mechanism(s) may not involve NO production; (4) NO production may not be the mechanism for CT inhibition in McCoy cells treated with IFN-γ and LPS.

DEDICATION

This dissertation is dedicated to the memory of my loving mother.

ACKNOWLEDGMENTS

I am pleased to have this opportunity to thank all those who helped me during the five years of my graduate study. First I wish to express my deepest gratitude to my major advisor and friend, Dr. William Campbell, without whose support, guidance and great help, the completion of this dissertation would not have been possible. I would like to thank the other members of my committee, Dr. Robert Stout, Dr. David Chi, Dr. Jill Suttles and Dr. Phillip Musich for their invaluable time and advice, whose guidance directed my research along the right path. I would also like to thank my research colleagues and friends, Tove, Kim, Xiang, Yining, Chuanfu and Betty for their unselfish assistance. I want to thank Dr. William Mayberry for his help with the computer, Janette Taylor for her help in preparing this manuscript and Barbara Stokes for scheduling my committee meetings. I do want to thank my mother-in-law, who took care of Nan-Nan and household during this "difficult" period. Finally, I would like to thank my lovely wife, Dr. He Wang for her endless support and encouragement, without whom this dream would never been realized.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

Ab	antibody
AIDS	acquired immune deficiency syndrome.
ATCC	American Type Culture Collection.
BCG	bacillus of Calmette and Guerin.
CDC	Center for Disease Control.
CH	cycloheximide.
CMI	cell-mediated immune.
con A	concanavalin A.
CSF-GM	colony-stimulating factor-granulocyte/macrophage.
CSF-M	colony-stimulating factor-macrophage.
CT	*Chlamydia trachomatis.*
CTL	cytotoxic T lymphocyte.
DEPC	diethylpyrocarbonate.
DMEM	Dulbecco's Modified Eagle's medium.
DMSO	dimethyl sulfoxide.
DPBS	Dulbecco's phosphate buffered asline.
EB	elementary body.
FBS	fetal bovine serum.
GPIC	guinea pig inclusion conjunctivitis.
hr	hour
IFA	immunofluorescence assay.

IFN	interferon.
IFU	inclusion forming unit.
IL	interleukin.
LB medium	Luria-Bertani medium.
LGV	lymphogranuloma venereum.
L-NMMA	N^g-monomethyl-L-arginine.
LPS	lipopolysaccharide.
LSD	the Least Significant Difference
MDMϕ	monocyte-derived macrophages.
MEM	Eagle's minimum essential medium.
MHC	major histocompatibility.
MOI	multiplicity of infection.
MOMP	major outer membrane protein.
MoPn	CT biovar mouse pneumonitis.
MOPS	3-N-morpholinopropanesulfonic acid.
NO	nitric oxide.
NOS	nitric oxide synthase.
PGE_2	prostaglandin E_2.
pi	post-inoculation.
PMA	phorbol myristate acetate.
PPD	purified protein derivative of old tuberculin.
pt	post-treatment.

RB	reticulate body.
rIFN-γ	recombinant interferon-γ.
rTNF-α	recombinant tumor necrosis factor-α.
TGF-β	transforming growth factor-β.
Th	helper T lymphocyte.
Thio-macrophage	thioglycollate-elicited peritoneal macrophage.
TNF	tumor necrosis factor.
TNF-R	TNF-α receptor.

CHAPTER 1

Introduction

Epidemiology of Chlamydial Genital Infection

Chlamydia trachomatis (CT) is a major cause of sexually transmitted disease with more than 4,000,000 new cases diagnosed annually in the United States (CDC 1985, Holmes 1981, Schachter 1978). Genital CT infection in men can cause urethritis which accounts for 50-60% of nongonococcal urethritis (Bowie 1984, Thelin et al. 1980). Genital CT infection in women can progress to serious diseases such as mucopurulent cervicitis, acute pelvic inflammatory disease, peritonitis, perihepatitis and salpingitis. Although 60-70% of the women were clinically asymptomatic at the time of diagnosis (Campbell and Dodson 1990, Lipkin et al. 1986, CDC 1985), genital CT infection can result in ectopic pregnancy and tubal infertility (Chow et al. 1990, Henry-Suchet 1988, Walters et al. 1988). Infants delivered from infected mothers have a high incidence of inclusion conjunctivitis and pneumonia (Schaefer et al. 1985).

General Characteristics of *Chlamydia trachomatis*

CT is a species of the genus *Chlamydia* which includes two other species: *Chlamydia psittaci* and *Chlamydia pneumoniae* (Mardh 1989). Although the degree of genomic DNA relatedness among the three species is low (ranging from less than 10% to almost 30%, Mamat et al 1992), they share many biological and biochemical

properties such as obligate intracellular multiplication, an unique developmental cycle, LPS and inclusion formation in infected host cells. They also can be differentiated on the basis of inclusion type and sensitivity to sulfonamides. CT inclusions contain glycogen whereas *C. psittaci* and *C. pneumoniae* do not. CT is more sensitive to sulfonamides than the others (Mardh 1989). Moreover, CT can be subdivided into three biovars: trachoma, lymphogranuloma venereum (LGV) and mouse pneumonitis (MoPn). The biovar trachoma consists of 14 serovars (A-K, Ba, Da and Ia). The serovars A, B, Ba and C are associated with endemic trachoma, a leading cause of preventable blindness in the world. The remainder are primarily associated with genital and neonatal infection (Freeman 1985, Wang and Grayston 1991). Four serovars (L1, L2, L2a and L3) in the biovar LGV cause lymphogranuloma venereum in tropical or subtropical areas as well as in the United States (Schachter 1978). MoPn generally causes pneumonia in mice, but has been used in animals as a model for genital CT infection (Rank et al. 1985).

CT is a small, gram-negative eubacterium (Weisburg et al. 1986), containing chromosomal and plasmid DNA as well as 21S, 16S and 4S rRNA (Newhall 1988). Because of its small genome (600-850 kilobase pairs), CT can not encode all enzymes necessary to generate ATP, and is entirely dependent upon host cells for energy (Moulder 1988, Becker 1978). As other gram-negative bacteria, CT contains lipopolysaccharide (LPS) which consists of the typical components of enterobacterial LPS (Hearn and McNavv 1991). However, the endotoxin of chlamydial LPS seems to be much less active than that of gram-negative enterobacteria (Leinonen 1992).

Chlamydial LPS has been found in lysosomes and on the surface of the plasma membrane of infected cells (Hearn and McNabb 1991).

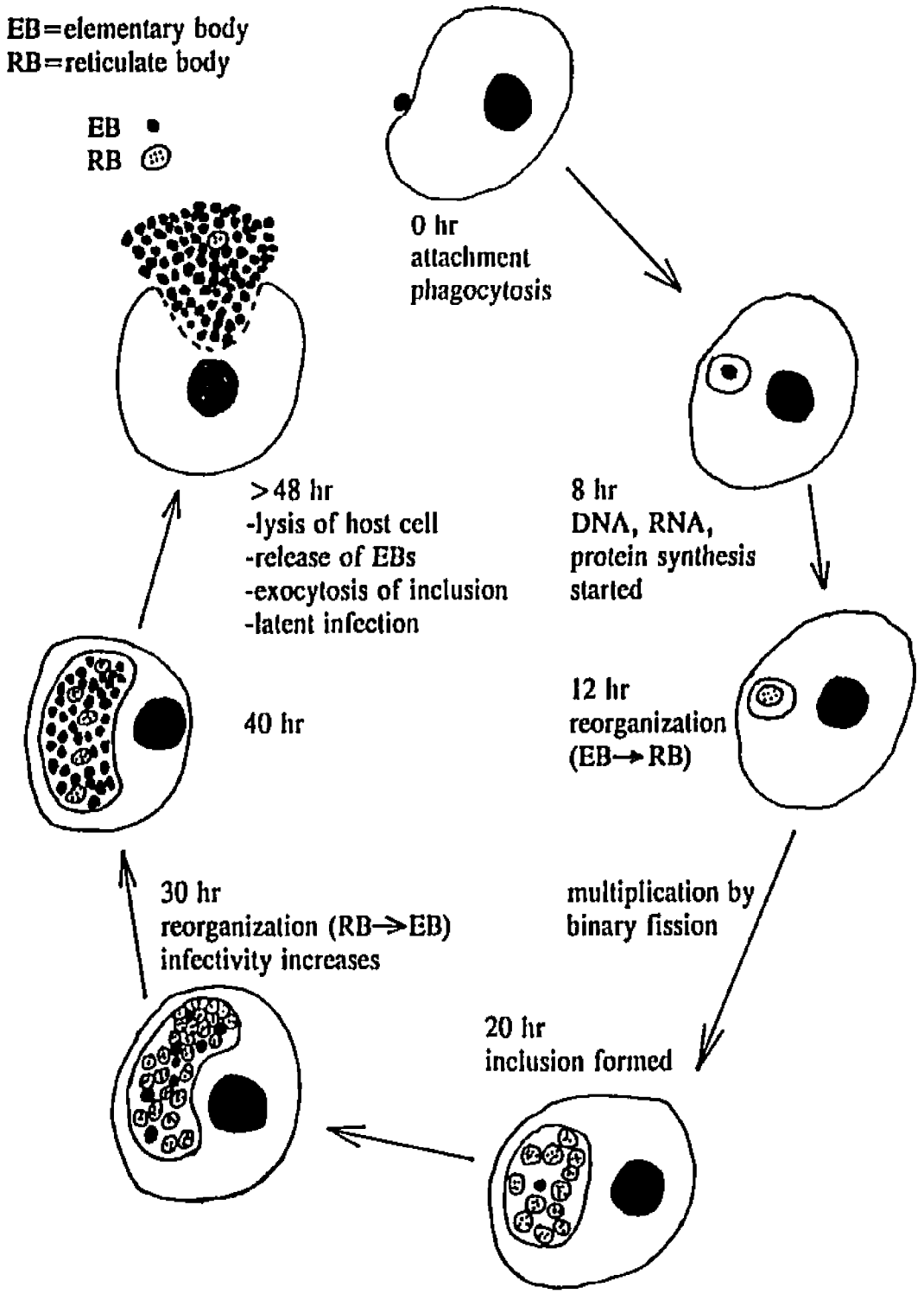
Developmental Cycle of CT

CT has a unique developmental cycle involving alternation between two forms: the elementary body (EB) and the reticulate body (RB). The EB, an infectious particle (400-600 nm in diameter) is adapted to extracellular survival, whereas the RB, a metabolically active particle (800-1200 nm in diameter) is a labile, noninfectious form that exists only intracellularly. Both EBs and RBs contain approximately equal amounts of a major outer membrane protein (MOMP) with a molecular mass of about 40 kDa. However, EBs have lesser amounts of the 13, 59 and 62 kDa outer membrane proteins, but have a cysteine-rich 60-kDa outer membrane protein which is not found in RBs (Moulder 1991, Newhall 1987, Hatch 1988, Hatch et al. 1984). EBs also have a greater extent of disulfide bond cross-linking than RBs (Hatch et al. 1984) and a dense nucleus at an eccentric region (Matsumoto 1988).

The chlamydial growth cycle is initiated by the adherence of the EB to host cells as shown in Figure 1. After attachment, the EB enters the host cell by receptor-mediated endocytosis (Hodinka et al. 1988, Wyrick et al. 1989). The endosome, which develops into a inclusion, exists in the cytoplasm, escaping from fusion with lysosome (Moulder 1991). In the endosome, the EB reorganizes into an RB which begins to divide by binary fission. Within 18 to 24 hours after attachment, RBs begin to undergo maturation culminating in the formation of EBs. Approximately 40-48

Figure 1

Sketch of the Developmental Cycle of *Chlamydia*.



hours after attachment, EBs are released from the host cells by lysis of the cells or by some form of exocytosis from the host cells (Ward 1988, Todd and Caldwell 1985). Nutritional depletion, pH modification, penicillin treatment or host defense mechanisms affect the growth cycle (Moulder 1991, Mardh 1989).

According to the growth cycle, recovery of infectious CT (EBs) decreases up to 24 hr post-inoculation (pi), then increases after 48 hr pi. Therefore, CT replication can be determined by kinetics of CT recovery.

Immune Responses to Chlamydial Infection

Chlamydial infection in humans elicits both humoral and cellular immune responses (Wang et al. 1982, Brunham et al. 1981). Since details of the humoral and cellular immune responses in human infection are difficult to discern (Qvigstad et al. 1985), the precise roles of the antibody, T cells, macrophages and cytokines remain poorly understood. However, evidence from studies using animal models of chlamydial infection and in vitro cell culture systems, together with epidemiological, serological and clinical observations of human chlamydial infections provide some insight into the human immune response to chlamydial infection.

In culture, CT (biovar trachoma or LGV) and *C. psittaci* have been shown to stimulate purified human or mouse B lymphocytes to proliferate and produce polyclonal antibodies (Bard et al. 1984, Levitt et al. 1986). Stimulated human B cells require T-cell help for antibody production, while mouse cells do not. The B cell-stimulating factor may not be chlamydial LPS, since (a) polymyxin B failed to inhibit

Chlamydia-induced polyclonal B cell activation and (b) C3H/HeJ mice (LPS-nonresponders) responded normally to *Chlamydia* with respect to polyclonal antibody production (Levitt et al. 1986, Moulder 1991). However, studies reporting stimulation of T-cell proliferation by *Chlamydia* were controversial. Qvigstad and colleagues (1983, 1985) observed that T cells from human peripheral blood proliferated in response to UV-inactivated LGV. However, other investigators (Bard et al. 1984) did not observe T cell proliferation in response to CT.

Development of local antibody (secretory IgA) in humans is associated with protection against chlamydial endocervical reinfection (Johnson 1985). Anti-CT antibodies in sera from healthy people neutralized the infectivity of CT (Lin et al. 1992). The neutralization was dependent upon antibody isotype and the cell types tested (Peterson et al. 1993). Neutralization was enhanced in the presence of complement (Lin et al 1992). The neutralization was thought to occur by inhibition of attachment of EBs to host cells (Byrne et al. 1978, Moulder 1991), since monoclonal antibodies, which recognize epitopes on MOMP, inhibited attachment (Zhang et al. 1987, Baehr et al. 1988, Stephens et al. 1988). However, antibodies (polyclonal or monoclonal) to MOMP also inhibited CT inclusion formation without affecting the attachment of EBs to host cells (Caldwell et al. 1982, Peeling et al. 1984). The latter indicated that the developmental cycle was halted at some point beyond attachment. Indeed, it was observed that antibody treatment resulted in fusion of EBs with lysosomes (Friis et al. 1972), thereby inhibiting transport of ATP into RBs (Peeling et al. 1984).

The demonstration of chlamydial antigen on infected cell surfaces satisfies one of the requirements for cytotoxic T lymphocyte (CTL) activity. The presence of appropriate class I major histocompatibility (MHC) antigens is the other requirement for CTL activity. CTL activity was observed in spleen cells from mice immunized with *C. psittaci*, indicating that chlamydial antigen could be presented with MHC class I (Lammert 1982). However, CTL activity was not found in spleens, lymph nodes or peritoneal exudates from mice immunized with LGV (Pavia et al. 1983). The role of CTL activity in CT infection remains to be defined.

In animal models, the cell-mediated immune (CMI) response seems to be essential for resolution of genital CT infections. In female guinea pigs infected by the agent of guinea pig inclusion conjunctivitis (GPIC), CMI and humoral responses cooperated to resolve the infection and provide resistance to reinfection (Rank and Barron. 1983, Rank et al 1989). Female athymic nude (nu/nu) mice infected by mouse pneumonitis agent (MoPn) developed chronic genital infection (Rank et al. 1985). In contrast, B cell-deficient mice were capable of resolving the infection and were immune to challenge infection (Ramsey et al. 1988). Furthermore, nu/nu mice that received MoPn antigen-specific T lymphocyte lines were capable of resolving the infection (Ramsey and Rank 1991, Igietsume et al. 1991). Thus, in the MoPn mouse model, T cells eliciting CMI responses were essential for resolution of the infection and provision of immunity against reinfection. Additionally, either MoPn antigen-specific $CD4^+$ or $CD8^+$ T cells were sufficient to protect animals from infection (Ramsey and Rank 1990). IFN-γ production might be a common mechanism of the

protection by the two T-cell subpopulations since animals treated with monoclonal antibody against murine gamma interferon (IFN-γ) sustained a significantly prolonged infection compared to untreated animals (Ramsey and Rank 1991).

Interaction between Macrophages and CT

Human and animal chlamydial infections are associated with polymorphonuclear and mononuclear (monocyte/macrophage) leukocyte infiltration of affected tissues (Lammont and Nichols. 1981). The roles of these leukocyte populations in protecting and resolving the infections in the host may be important (Brunham et al. 1985, Rank et al. 1985).

In vitro studies indicated that interaction between macrophages and *Chlamydia* is complicated. A survey of 10 human lymphoblastoid-myeloid cell lines, for example, showed that CT biovar LGV multiplied in some lines, entered others but did not multiply and attached to still other lines but did not enter (Bard et al. 1985). Human mononuclear phagocytes cultivated in vitro less than 7 days were almost totally resistant to CT inclusion formation and replication (< 10 IFU/ml recovered). However, LGV survived and replicated (1-9.5 x 10^5 IFU/ml) in phagocytes cultivated for more than 7 days. In contrast, CT biovar trachoma was completely inhibited in such phagocytes (Yong et al. 1987). Similar results were observed by Manor and Sarov (Manor and Sarov. 1986). Replication of LGV was completely inhibited in human peripheral monocytes, but not in monocyte-derived macrophages (MDMϕ). In contrast to LGV, *C. psittaci* replicated in human monocytes and MDMϕ.

Transmission electron microscopy showed two main types of phagosomes in the cytoplasm of CT-infected monocytes at 1 hour pi, one in which the EB was tightly surrounded by the membrane of the phagosome and another in which the EB appeared in an enlarged phagosome. At 24 to 48 hours pi, each phagosome contained a single EB-like particle or an atypical (damaged) RB, indicating that CT was destroyed in phagosomes of human monocytes (Manor and Sarov 1986, Yong et al. 1987). When human monocytes and MDMϕ were added to CT-infected HEp-2 cells, CT recovery and CT DNA production in HEp-2 cells were reduced (Manor and Sarov 1988). Electron microscopy showed that infected HEp-2 cells exposed to MDMϕ contained small vacuoles with abnormal RBs and very few typical EBs. Compared to monocytes, MDMϕ exhibited more pronounced inhibition and were less resistant to chlamydial infection. Direct contact of MDMϕ with the CT-infected cells was required for effective inhibition. However, addition of anti-TNF-α antibody to the system reduced the inhibition of chlamydial DNA production (Manor and Sarov 1988), although the mechanisms involved have not been defined clearly.

The human promyelocyte cell line HL-60 can be induced by phorbol myristate acetate (PMA) or dimethyl sulfoxide (DMSO) to differentiate along the macrophage or the granulocyte pathway, respectively. Compared to uninduced HL-60 cells, PMA- or DMSO-induced cells have a greater ability to bind, ingest and destroy CT (biovar LGV), indicating that the ability of this cell line to interact with and destroy Chlamydiae correlates with distinct stages of differentiation (Bard et al. 1987).

Although CT biovar trachoma cannot replicate in human monocytes or

monocyte-derived macrophages in vitro (Yong et al. 1987), infection of macrophages by CT biovar trachoma may take place in vivo. The evidence for this is that CT EBs and RBs were found in synovial macrophages of patients with Reiter's syndrome or sexually acquired arthritis following urogenital infection by CT serovars D-K (Ishikawa et al. 1986, Keat et al. 1987).

Alveolar macrophages isolated from healthy nonsmoking adults exhibited strong intracellular killing of CT biovar trachoma as well as LGV (Nakajo et al. 1990). But alveolar macrophages obtained from individuals with acquired immune deficiency syndrome (AIDS) exhibited susceptibility to CT infection (Nakajo et al. 1988).

It has been demonstrated that *C. psittaci* and CT biovar LGV can infect and replicate in mouse peritoneal macrophages (Wyrick et al. 1978, Kuo 1978, Zhong and Maza 1988, Huebner and Byrne 1988). No apparent difference between thioglycollate-elicited and resident peritoneal macrophages was observed with regard to chlamydial infection (Wyrick et al. 1978). The intracellular fate of *C. psittaci* in peritoneal macrophages was somehow dependent upon the multiplicity of infection (MOI). Survival and multiplication of Chlamydiae were optimal at an MOI of 1 or less. At a high MOI (100), macrophage damage occurred immediately, which resulted in significant reduction of CT survival (Wyrick et al. 1978). EBs pretreated with heat (56^{0}C for 10-30 minutes) or coated with homologous antibody were rapidly destroyed in phagolysosomes of macrophages (Wyrick et al. 1978).

It was observed that peritoneal macrophages from *C. psittaci*-immunized mice

were resistant to infection by *C. psittaci*, while casein-, protease peptone- or thioglycollate-elicited macrophages from uninfected mice were equally susceptible to infection. Macrophages from *Mycobacterium tuberculosis* BCG- or *Listeria monocytogenes*-immunized mice exhibited intermediate susceptibility to infection (Huebner and Byrne 1988). Resistance of macrophages from the *C. psittaci*-immunized mice was not due to the ingestion of fewer organisms, nor were these cells persistently infected, since *Chlamydia* could not be recovered from the infected macrophages, even after an extended incubation period. Although increased respiratory and oxidative activities were observed in macrophages from the *C. psittaci*-immunized mice, treatment of the macrophages with superoxide dismutase to abrogate O_2^- activity did not affect resistance to chlamydial infection, indicating that resistance involved an oxygen-independent pathway (Huebner and Byrne 1988). However, the mechanism(s) responsible for the resistance have not been defined.

In contrast to *C. psittaci* or CT biovar LGV, growth of CT biovar trachoma in mouse peritoneal macrophages is not well documented. Low levels of CT (10^4 IFU/ml) were recovered at 48 hours pi from peritoneal macrophages inoculated with CT serovar B at an MOI of 5 (Kuo 1978). Since the kinetics of CT recovery were not determined, the observed infectivity recovered may have reflected residual inoculum. Therefore, actual replication of CT biovar trachoma in peritoneal macrophages has not been confirmed.

IFN-γ-activated human monocytes and MDMϕ have been shown to inhibit chlamydial infection. Human monocytes and MDMϕ utilize both respiratory burst-

dependent and -independent antimicrobial mechanisms. IFN-γ can enhance the effect of both pathways (Nathan et al. 1983, Murray et al. 1983, Rothermel et al. 1986). Therefore, oxygen-dependent and -independent pathways have been examined as antichlamydial processes in macrophages. Inhibition of *C. psittaci* growth by IFN-γ-activated human MDMϕ was shown to be an oxygen-independent mechanism (Rothermel et al. 1986). Depletion of intracellular iron was not the mechanism of the inhibition, as iron-saturated transferrin did not reverse the intracellular inhibition (Murray et al 1991). Furthermore, deferoxamine (an iron chelator) did not impair chlamydial replication within unstimulated human monocytes (Murray et al 1991). However, it was reported that IFN-γ treatment of human MDMϕ initially resulted in a microbiostatic inhibition of intracellular chlamydial development (*C. psittaci*) which could be reversed by the addition of exogenous tryptophan (Byrne et al. 1989). Prolonged treatment with IFN-γ before the addition of tryptophan resulted in microbiocidal activity that was irreversible.

Thioglycollate-elicited mouse peritoneal macrophages activated by IFN-γ (with or without LPS) resulted in microbiostatic inhibition of *C. psittaci* (Huebner and Byrne 1988). The inhibitory effect was eliminated shortly after IFN-γ was removed from the culture. Thioglycollate-elicited mouse peritoneal macrophages activated in vivo with recombinant murine IFN-γ significantly restricted CT replication (LGV). Moreover, mice that received IFN-γ-activated macrophages exhibited significant decreases in CT recovery from their spleens and peritoneal fluids (Zhong and de la Maza 1988). The mechanism(s) have not been defined. It was observed that the

restriction of CT replication paralleled expression of Ia^d on the macrophages (Zhong and de la Maza 1988). Increase of Ia expression on macrophages induced by T cell-derived IFN-γ also was observed in mice infected by other organisms such as *Listeria monocytogenes* (Koga et al. 1987). Furthermore, Ia-bearing-macrophage influx was associated with genetic resistance of mice to infection by *Rickettsia tsutsugamushi* (Jerrells 1983). However, the precise role of Ia expression in inhibition of CT remains unknown.

Effect of Cytokines upon Chlamydial Infection

Interferon-γ

Interferon-γ (IFN-γ) is produced by Th_1 lymphocytes (helper T lymphocyte 1) and has multiple biological functions. These include activation of macrophages, participation in T cell differentiation, inhibition of proliferation of Th_2 lymphocytes (helper T lymphocyte 2) and antimicrobial activity (Adams and Hamilton 1992, Auger and Ross 1992, Fitch et al. 1993). The inhibitory effects of IFN-γ upon CT infection have been examined in vitro as well as in vivo. Stimulation of spleen cells from CT (serovar L1)-infected mice by concanavalin A (con A) or heat-killed CT resulted in the release of high levels of IFN-γ at 5-8 days postinfection (Zhong et al. 1989). IFN-γ levels paralleled the clearance of CT from the mice. Treatment of CT-infected mice with monoclonal antibody (MAb) to IFN-γ significantly increased recovery of CT (Zhong et al. 1989). In vitro, IFN-γ activated macrophages (human or mouse) which

inhibited chlamydial growth as described above and restricted chlamydial infection in other cells (Rothermel et al. 1983, Maza et al. 1987, Shemer and Sarov 1985, Byrne et al. 1989). Mouse fibroblasts (L and McCoy cells) and human epithelioid or epidermoid cell lines (T24, HeLa and HEp-2) treated with IFN-γ exhibited inhibition of inclusion formation. Inhibition of chlamydial recovery also was observed in these cells. IFN-γ restricted replication of CT biovar trachoma in primary human conjunctival epithelial cells (Tahija et al. 1990).

Most investigators have focused upon tryptophan degradation as a mechanism of inhibition. IFN-γ can induce a variety of cells to synthesize indoleamine-2, 3-deoxygenase (Shemer and Sarov 1987, Byrne et al. 1986, Tahija et al. 1990, Carlin et al. 1989). This enzyme causes depletion of tryptophan by degrading it to kynurenine and N-formylkynurenine. Exogenous tryptophan partially reversed IFN-γ-induced inhibition of *C. psittaci* in T24 cells or human macrophages (Byrne et al. 1986 and 1989, Carlin et al. 1989). Moreover, tryptophan partially reversed the inhibition of CT biovar LGV in HEp-2 cells (Shemer and Sarov 1987). However, exogenous tryptophan failed to block the action of IFN-γ in McCoy cells infected with CT biovar LGV (de la Maza et al. 1985). This phenomenon also was observed in other murine-derived cell lines (Murray et al. 1989, Mayer et al. 1993). Therefore, it is possible that tryptophan degradation is involved in antichlamydial activity in human cells, but not in murine cells.

Tumor Necrosis Factor α

Tumor necrosis factor α (TNF-α) is produced primarily by macrophages and has been shown to exert an inhibitory activity in chlamydial infections. In vivo, TNF-α was produced in the lungs of CT-infected mice, where it plays an undefined role in host defense against infection (Williams et al. 1990). In culture, the effect of TNF-α upon chlamydial infection (CT biovar LGV) resembled that of IFN-γ (Shemer-Avni et al. 1988, 1989). The TNF-α effect was abolished by cycloheximide, indicating that synthesis of host protein was required (Shemer-Avni et al 1988). Like IFN-γ, TNF-α induced the degradation of tryptophan. This is remarkable, since exogenous tryptophan or anti-IFN-β antibody partially reversed the inhibition of CT replication in HEp-2 cells (Shemer-Avni et al. 1989). Inhibition of CT (biovar LGV) by TNF-α was accompanied by increased production of prostaglandin E_2 (PGE_2) in HEp-2 cells (Holtmann et al. 1990). The role of increased PGE_2 is unclear.

Interleukin-1

Interleukin-1 (IL-1) was induced in human monocytes by live, heat- or UV-inactivated EBs (CT biovar LGV) only at a high MOI (Manor et al. 1990, Rothermel et al. 1989). Recombinant IL-1α inhibited CT growth in HEp-2 cells in the absence of tryptophan degradation. However, PGE2 formation was increased in infected HEp-2 cells treated with IL-1 or TNF-α, but not IFN-γ. Tryptophan inhibited PGE2 production and reversed the inhibition of CT replication induced by exogenous PGE2

(Shemer-Avni et al. 1990). The precise mechanism involved in this inhibition is not clear.

NO Production and Inhibition of Microorganisms in Activated Macrophages

Macrophage activation is known to occur through a series of stages ranging from a level equivalent to resident tissue macrophages and culminating at an activated state whereupon macrophages become competent to kill several pathogens and lyse tumor cells (Adams and Hamilton 1984, 1992). During this activation process, macrophages produce an array of biologically active molecules. Nitric oxide (NO) is one of these products which include superoxide, hydrogen peroxide, TNF-α, IL-1, IL-6, MIP1-α and PGE_2 (Stuehr and Marletta 1987, Adams and Halmilton 1992).

NO, a simple and relatively unstable radical, is a potent and pleiotropic mediator of diverse biological activities (Liew and Cox 1991a, Kolb and Kolb-Bachofen 1992, Moncada et al. 1991). This radical accounts for the activity of an endothelium-derived relaxing factor, acts as a neurotransmitter and serves as a major defense molecule against tumor cells, intracellular parasites, fungal and bacterial pathogens (Liew and Cox 1991, Kolb and Kolb-Bachofen 1992, Moncada et al. 1991, Stuehr and Nathan 1989).

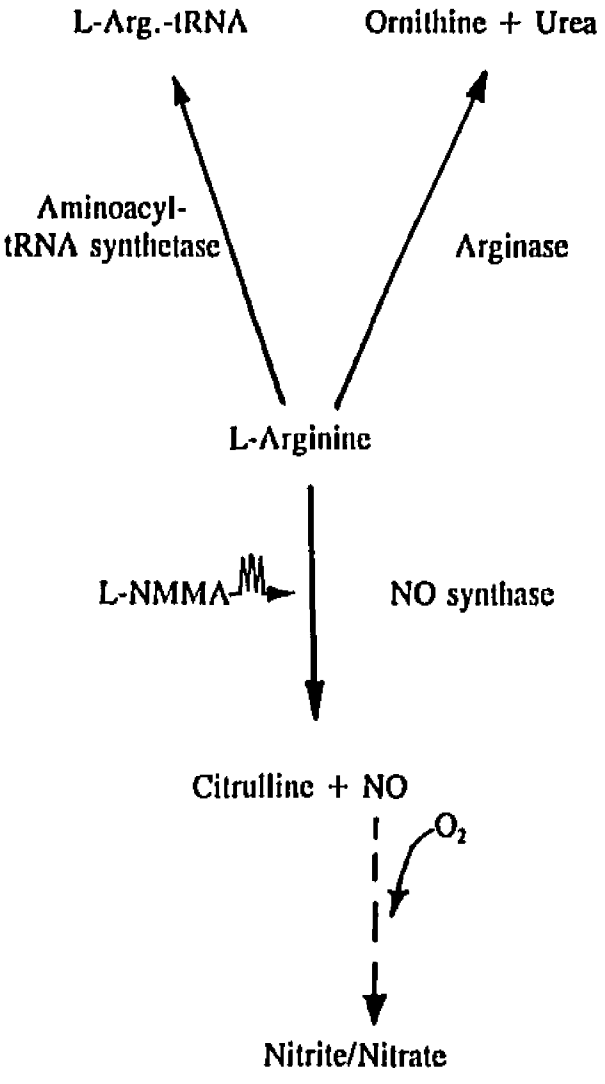
The first evidence to suggest that macrophages produced nitrite (NO_2^-) was that blood levels and urinary excretion of nitrate (NO_3^-) increased after exposure to LPS in LPS-sensitive, but not in LPS-resistant mice (Stuehr and Marletta 1985). These authors also demonstrated that mouse peritoneal macrophages activated by LPS

increased NO_2^- and NO_3^- production in vitro. Subsequent work demonstrated that mouse peritoneal macrophages and macrophage cell lines produced nitrite and nitrate following stimulation by cytokines or LPS (Ding et al. 1991, Stuehr and Marletta 1985, 1987a, 1987b, Moncada et al. 1991). The production of NO was dependent upon the presence of L-arginine which is converted to citrulline (Iyengar et al. 1987). Experiments with ^{15}N-labeled arginine demonstrated that both NO_2^- and NO_3^- were derived from the terminal guanidino nitrogen atom(s) of arginine (Iyengar et al. 1987). It is clear that NO is generated in macrophages from L-arginine catalysis directed by NO synthase (NOS). NO then is converted to nitrite or nitrate. As NO is unstable (half life of seconds), nitrite rather than NO production is measured. Analogues of L-arginine, such as N^g-monomethyl-L-arginine (L-NMMA), specifically inhibited NO synthesis by competitive binding (Figure 2).

There are at least two distinct isoforms of NO synthase. One is calcium- and calmodulin-dependent and is constitutively expressed in some cell types of the body, notably the endothelium and some neurons. The other is not dependent upon calcium or calmodulin, and is not constitutively expressed. When induced, the latter can produce large amounts of NO in macrophages and many other cell types (Moncada et al. 1991). The inducible enzyme is not detectable in macrophage cell lines or freshly isolated macrophages that have not been activated by LPS or cytokines (Stuehr and Marletta 1985, 1987a, 1987b). Cellular protein synthesis is required for its expression (Marletta et al. 1988). There is a lag phase of approximately 8 hours before synthesis of NO_2^- and NO_3^- is detectable (Stuehr and Marletta 1987a). Recently, cDNA of the

Figure 2

The Generation of Nitrite from L-Arginine.



NOS gene has been cloned from the mouse macrophage cell line RAW264.7 (mac-NOS, Lowenstein et al. 1992, Lyons et al. 1992, Xie et al. 1992), from bovine aortic endothelial cells (ecNOS, Sessa et al. 1992) and from rat cerebellum (bNOS, Bredt et al. 1991). Comparison of the deduced amino acid sequence of mac-NOS to that of bNOS and ecNOS revealed only 50-57% identity (Sessa et al 1992, Lowenstein et al. 1992).

The capacities of 12 cytokines to induce NO_2^- production in resident or thioglycollate-elicited mouse peritoneal macrophages were evaluated (Ding et al. 1988). Of these, only IFN-γ induced substantial NO_2^- production. The remainder (IFN-α, IFN-β, TNF-α, TNF-β, CSF-GM, CSF-M, IL-1β, IL-2, IL-3, IL-4 and TGF-β) were incapable of inducing significant nitrite production. Although neither TNF-α nor TNF-β induced nitrite production alone, combination of either with IFN-γ increased nitrite production six-fold over that in macrophages treated with IFN-γ alone. In combination with LPS, IFN-α or IFN-β also induced nitrite production, despite the fact that neither alone was sufficient. IFN-γ and LPS acted synergistically to induce nitrite production. IL-10, a product of Th_2 lymphocytes, inhibited expression of NOS and nitrite production in IFN-γ-activated macrophage cell line J774A.1 cells (Cunha et al. 1992). The response was dose-dependent. Inhibition occurred only when the cells were pretreated with IL-10. Addition of IL-10 after IFN-γ activation was not effective (Cunha et al. 1992). IL-4 and TGF-β also inhibited nitrite production by IFN-γ-activated macrophages (Liew et al. 1991b, Ding et al. 1990).

The effect of NO production upon intracellular killing of microorganisms by mouse peritoneal macrophages has been studied quite extensively. Mouse macrophages activated by LPS and IFN-γ exerted a powerful cytostatic effect in vitro against the fungal pathogen *Cryptococcus neoformans* (Granger et al. 1986a, 1986b). This fungistasis was dependent upon the presence of L-arginine, was inhibited by L-NMMA and correlated with the synthesis of L-citrulline, nitrite and nitrate (Granger et al. 1990). Similarly, the cytostatic action of mouse macrophages against *Toxoplasma gondii* was prevented by L-NMMA (Adams et al. 1990). Intraperitoneal administration of killed *Corynebacterium parvum* in mice led to increased resistance to infection by *Toxoplasma*, an effect which was prevented by L-NMMA (Adams et al. 1990). Intracellular destruction of *Leishmania major* amastigotes (*L. major*) was inhibited by L-NMMA (Green et al. 1990, Liew et al. 1990). Moreover, this parasite was killed directly by exposure to NO (Liew et al. 1990). Mice infected by *L. major* developed larger lesions following in vivo treatment with L-NMMA. The infected footpads of the L-NMMA-treated mice contained 10^4 times more parasites than those of untreated mice (Liew et al. 1990). NO produced by activated mouse macrophages also may be involved in the in vitro killing of *Schistosoma mansoni* (James and Claven 1989) and *Entamoeba histolytica* trophozoites (Lin and Chadee 1992). It is notable that the effect of NO in inhibition of CT by mouse macrophages, to our knowledge, has not yet been reported.

Most studies have indicated that human monocytes and MDMϕ stimulated with IFN-γ in vitro or in vivo did not produce enhanced levels of nitrite (Murray and

Tertelbaum 1992, James et al. 1990, Cameron et al. 1990). Neither L-NMMA nor arginase inhibited intracellular antimicrobial activity (Murray and Tertelbaum 1992). James and his coworkers (James et al. 1990) reported that human MDMϕ activated in vitro by IFN-γ killed *Schistosoma mansoni*, but did not produce nitrite. Cameron and his coworkers (Cameron et al 1990) showed that human alveolar and peritoneal macrophages inhibited replication of intracellular *Cryptococcus neoformans* without producing nitrite or nitrate. The authors observed that L-NMMA did not affect anticryptococcal activity. In addition, the killing of *Mycobacterium avium*, *Toxoplasma gondii*, *Chlamydia psittaci* or *Leishmania donovani* by human monocytes was not diminished by L-NMMA or arginase (Murray and Teitelbaum 1992). At the same time, it remains possible that the generation of NO may play some antimicrobial role in vivo, which for unclear reasons cannot be readily demonstrated in vitro using human cells. Recent studies in cancer patients treated with IL-2, for example, have indicated the presence of cytokine-inducible nitrite production in serum and urine (Billiar et al. 1991, Murray et al. 1989). Furthermore, 14-day old human monocyte-derived macrophages have been reported to generate nitrite after prolonged (7-day) intracellular infection by the slowly growing pathogen *Mycobacterium avium* (Denis 1991). Eight days of concurrent treatment with TNF-α or CSF-GM increased nitrite production and induced killing of *M. avium*. L-NMMA or arginase reversed the effect. It is possible that human macrophages may be stimulated to produce NO only in response to a particular cytokine or pathogen.

Summary

Chlamydial genital infection is prevalent throughout the would. Interaction between macrophages and CT is important in host defense against the infection. *C. psittaci* and CT biovar LGV have been demonstrated to grow in inactivated macrophages, but replication of CT biovar trachoma in macrophages is not well documented. Cytokines such as IFN-γ and TNF-α have been shown to inhibit CT infection. However, the mechanisms involved have not been defined. Although NO has been identified as a major defense molecule against a variety of microorganisms, the effect of NO in inhibition of CT by activated mouse macrophages is unknown. Therefore, this study was initiated to demonstrate replication of CT biovar trachoma (serovar D) in mouse peritoneal macrophages and macrophage cell lines. Subsequently, correlation between NO production and CT inhibition in these cells was analyzed. We observed a strong correlation between nitrite production and inhibition of CT replication in macrophage cell lines (RAW264.7 and J774A.1). L-NMMA partially reversed the inhibition. Our data suggest that NO is one of the mechanisms involved in inhibition of CT replication in IFN-γ-activated macrophages.

CHAPTER 2

Materials and Methods

Materials

Buffers and Solutions

Dulbecco's phosphate buffered saline (DPBS) was purchased from Sigma Chemical Co. DPBS consisted of 0.9 mM calcium chloride, 2.68 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 0.49 mM magnesium chloride, 0.137 M sodium chloride and 8.06 mM sodium phosphate dibasic.

Trypsin-EDTA containing 0.25% trypsin and 1 mM EDTA was purchased from Gibco. Fetal bovine serum (FBS) was purchased from Sigma Chemical Co. FBS was inactivated at 56^{0}C for 30 minutes before use.

IFA (Immunofluorescence Assay) buffer was phosphate buffered saline prepared according to the manufacturer's instructions.

Glucose, Tris-HCl, EDTA (ethylenediaminetetraacetic acid), NaOH, SDS (lauryl sulfate sodium salt), potassium acetate, NaCl, sodium citrate, NaH_2PO_4, Na_2HPO_4, crystal violet, RNase A, ethidium bromide, 37% formaldehyde, MOPS (3-N-morpholinopropanesulfonic acid), DEPC (diethylpyrocarbonate), bromphenol blue, xylene cyanol, sulfanilamide and naphthylethylenediamine dihydrochloride (NEDD) were purchased from Sigma Chemical Co. Phenol, chloroform, isoamylalcohol, isopropanol, ethanol; methanol, HCl, phosphoric acid and ion-exchange resin were

purchased from Fisher Scientific.

Distilled water used to prepare buffers and solutions for RNA isolation and hybridization was treated with DEPC, and then autoclaved for 30 minutes at 121°C.

Formamide was deionized by mixing 50 ml of formamide with 5 g of ion-exchange resin (Bio-Rad AG501-X8), stirring 30 minutes at room temperature and filtering through Whatman filter paper. Deionized formamide was aliquoted and stored at -20°C.

GTE buffer was 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. Alkaline lysis buffer was 0.2 N NaOH with 1% SDS. Potassium acetate solution was 5 M, pH 4.8. TE buffer was 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. TBE was prepared as a 10x stock solution which contained 0.89 M Tris base and 0.89 M boric acid, 20 mM EDTA. 10X MOPS buffer was 0.2 M MOPS, 80 mM sodium acetate (pH 7.0), 10 mM EDTA (pH 8.0). 10x loading buffer (pH 8.0) was 1 mM EDTA, 0.25% bromphenol blue, 0.25% xylene cyanol and 50% glycerol. Ethidium bromide (EB) solution was prepared as a 10,000x stock (5 mg/ml). 20 x SSC was 3 M NaCl and 0.3 M sodium citrate adjusted pH to 7.0 with 1 M HCl. 2 x SSPE was 0.36 M NaCl, 20 mM NaH_2PO_4 (pH 7.4) and 20 mM EDTA.

RNase A was dissolved in TE buffer by boiling for 10 minutes to make a concentration of 10 mg/ml. Aliquots were stored at -20°C.

Denaturing solution 1 for Dot Blot was 4 ml 20x SSC mixed with 1 ml 37% formaldehyde. Denaturing solution 2 for Northern Blot was 50 µl 10x MOPS mixed with 89.4 µl 37% formaldehyde, 250 µl formamide and 110.6 µl DEPC-treated

distilled water. Formaldehyde denaturing agarose gel was 1% agarose in 1 X MOPS buffer with 2.2 M formaldehyde and 0.5 μg/ml EB.

Prehybridization solution was 0.5 M phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 7.5, with 7% SDS and 100 μg/ml denatured herring sperm DNA. Washing buffer 1 was 0.1 M phosphate buffer with 1% SDS. Washing buffer 2 was 0.01 M phosphate buffer with 0.1% SDS.

A commercially prepared 10x buffer coming with restriction endonuclease Not I (Promega) was 60 mM Tris-HCl, pH 7.9, 1.5 M NaCl, 60 mM $MgCl_2$ and 10 mM DTT.

Griess reagent for nitrite assay was 1% sulfanilamide, 0.1% NEDD and 2.5% phosphoric acid.

Crystal violet solution (10x) for TNF-α assay was 2% crystal violet in 21% ethanol.

Thioglycollate broth was purchased from Difco. 3% (w/v) of thioglycollate was prepared, autoclaved to sterilize and stored in the dark at room temperature.

Media

RPMI-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM) powder, 10x MEM (Eagle's minimum essential medium), glutamine, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), sodium bicarbonate, sodium pyruvate, cycloheximide (CH), gentamicin sulfate and ampicillin were purchased from Sigma Chemical Co. Ham's Nutrient Mixture F12 (Ham's F12) powder was

purchased from Gibco. Tryptone, yeast extract, Bacto-agar were purchased from Difco. IPTG (Isopropyl-1-thio-β-D-galactoside) and Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) were purchased from Promega.

McCoy cell growth medium was 1X MEM. For 500 ml growth medium, 50 ml 10X MEM were diluted with 450 ml sterile distilled water, then supplemented with 10 ml 27% glucose, 10 ml 1 M HEPES (pH 7.3), 50 ml FBS (unless otherwise indicated in separate experiments), 5 ml 7.5% sodium bicarbonate, 0.2 ml 50 mg/ml gentamicin sulfate and 5 ml 200 mM glutamine. Fresh glutamine was added after four days of storage. CH medium was McCoy cell growth medium complemented with CH (1 μg/ml). Macrophage growth medium was RPMI-1640 with 5% FBS (unless otherwise indicated in separate experiments), 10 mM HEPES (pH 7.3), 2 mM glutamine, 1 mM sodium pyruvate and 50 μg/ml gentamicin sulfate.

OVCAR3 growth medium was RPMI-1640 medium with 10% FBS, 20 μg/ml gentamicin sulfate, 20 mM HEPES, 2 mM glutamine and 10 μg/ml insulin.

KLE growth medium was a 1:1 mixture of 1x DMEM and 1x Ham's F12 with 10% FBS, 20 μg/ml gentamicin sulfate and 20 mM HEPES adjusted to pH 7.4 with sodium bicarbonate.

Medium for *E. coli* growth was LB medium containing 10 g tryptone, 5 g yeast extract, 5 g sodium chloride and 1 ml of 1 N NaOH in 1000 ml of medium. Selective antibiotic plates were prepared by melting 1.5 g Bacto-agar (Difco) in 100 ml LB medium supplemented with ampicillin (50 μg/ml), IPTG (0.1 mM), Xgal (20 μg/ml). The selective antibiotic plates were prepared one day before use.

Reagents

Recombinant mouse tumor necrosis factor (rTNF-α, 10 μg/ml) was purchased from Genzyme. The specific activity of rTNF-α was $4X10^7$ units/mg. Polyclonal rabbit anti-mouse TNF-α antibody (anti-TNF-α Ab, primarily IgG and IgM) was purchased from Genzyme. One μl of anti-TNF-α Ab neutralized approximately 1000 units of mouse TNF-α bioactivity in the standard L929 cell cytotoxicity assay. Recombinant mouse interferon-γ (IFN-γ, 1×10^5 units/ml) was purchased from Gibco BRL Life Technologies. The specific activity of IFN-γ was approximately 1×10^7 units/mg. Lipopolysaccharide (LPS, Escherichia coli, 026:B6), N^G-monomethyl-L-arginine acetate (L-NMMA) and L-tryptophan were purchased from Sigma Chemical Co.

Ultraspec™ RNA (a total RNA isolation reagent) was purchased from Biotecx Laboratories. RNA markers, DNA markers (lambda DNA/Hind III), herring sperm DNA, restriction endonuclease Not I and Prime-a-Gene labeling system were purchased from Promega. Deoxycytidine 5'-[α-^{32}P] triphosphate (^{32}P-dCTP) was purchased from Amersham.

Plasmid and cDNA

The plasmid CL-BS-mac-NOS was generously provided by C.J. Lowenstein, Johns Hopkins University, Baltimore, MD. The recombinant plasmid was made from the Bluescript vector sold by Stratagene, with the 4100 bp (base pairs) fragment from the macrophage nitric oxide synthase cDNA (mac-NOS) inserted into the unique Not I

site. Chicken β-actin cDNA was originally isolated by Cleveland et al. (1980) and generously provided by R. D. Stout, East Tennessee State University (ETSU), Johnson City, TN.

Stains

An anti-MOMP (major outer membrane protein) monoclonal antibody conjugated with FITC was purchased from Syva (Microtrak CT culture Confirmation System). Jones' iodine was prepared by mixing 50 ml 95% ethanol, 5 g iodine, 5 g potassium iodide and 50 ml distilled water in order and filtering through two Whatman #1 filters.

Cells

McCoy cells were a generous gift from Dr. J. Schachter, San Francisco, CA. L929 cells were generously given by Dr. R.D. Stout, ETSU, Johnson City, TN. Mouse macrophage cell lines: J774A.1 and RAW264.7 cells, human epithelial cell lines: OVCAR-3 and KLE, and JM109 (a strain of *E. Coli*) cells were purchased from American Type Culture Collection (ATCC).

Chlamydia trachomatis

The strain of CT used in this study was a serovar D. The organism was isolated in our clinical laboratory from a conjunctional swab obtained from the left eye of a two-

week old girl suffering from a perinatal infection. The organism has been passed an indeterminate number of times in McCoy cells since isolated.

Animals

Inbred female BALB/c mice were purchased from Charles River Laboratories. The mice were housed in isolation in the Division of Laboratory Animal Resources (DLAR), James H. Quillen College of Medicine.

Miscellaneous

XAR-5 X-ray film was purchased from Sigma Chemical Co. SeaKem LE agarose was purchased from FMC Bioproducts. Nytran plus nylon membranes were purchased from Schleicher & Schuell. Whatman papers (3M) were purchased from Fisher Scientific. DEAE-cellulose membrane was purchased from Schleicher and Schuell, and treated as follows before use: soaking the membrane for 5 minutes in 10 mM EDTA, pH 8.0, then soaking the membrane in 10 mM EDTA with 0.5 N NaOH for 5 minutes, finally washing the membrane six times in sterile water.

Methods

Cell Cultures

McCoy, OVCAR-3 and KLE cells were grown and maintained in McCoy growth medium, OVCAR-3 growth medium and KLE growth medium, respectively. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. Confluent monolayers were trypsinized, washed and counted. Viability of cells were determined by trypan blue exclusion (Mishell and Shiigi 1980).

RAW264.7, J774A.1 and L929 cells were grown and maintained in macrophage growth medium at 37°C in a humidified atmosphere with 5% CO_2. Confluent monolayers were scraped gently using a sterile rubber policeman. Number and viability of cells were determined as described as above.

Preparation of CT Seed Pool

Confluent monolayers of McCoy cell were trypsinized. 5 x 10^5 cells in 1 ml of McCoy growth medium were added to each shell vial containing a 12 mm coverslip. Each vial was closed with a sterile #0 neoprene stopper and incubated at 37°C overnight. After aspiration of supernatant, the cells were inoculated with CT (0.1 ml inoculum/vial) at a multiplicity of infection (MOI) of 0.5-1 and centrifuged at 900 X g for 45 minutes at room temperature. 1 ml of CH medium was added to each vial and the vials incubated at 37°C for 48 hours. After incubation, the supernatant was aspirated and 0.5 ml of fresh macrophage growth medium with sterile 4 mm glass

beads were added to each vial. The vials were vortexed 10 seconds for three times. The crude lysates from 70 vials were pooled, dispensed into 0.5 or 1.0 ml aliquots and stored at -80^{0}C. The lysate served as CT seed pool. Titration of the CT Seed Pool was performed on McCoy cells (see CT recovery).

Preparation of Peritoneal Macrophages

Thioglycollate-elicited peritoneal macrophages (thio-macrophages) were generated as described Mishell and Shiigi, 1980. Briefly, mice were injected intraperitoneally with 3% thioglycollate. At day five post-injection, mice were sacrificed by cervical dislocation. Peritoneal exudative cells were harvested by washing peritoneal cavity with DPBS containing 2% FBS, and resuspended to 5 X 10^5 cells/ml in macrophage growth medium. The cell suspension (1 ml) was added to each shell vial containing a 12 mm coverslip. The vials incubated at 37^{0}C overnight and nonadherent cells were removed by washing with DPBS.

CT Recovery on McCoy Cells

The procedure used for CT recovery was described by Smith, 1979. Briefly, 5 x 10^5 freshly trypsinized McCoy cells in 1 ml of McCoy growth medium were dispensed into each shell vial containing a 12 mm coverslip. The vials incubated at 37^{0}C overnight, at which time medium was aspirated from the monolayers and 100 μl of CT inoculum were added to each vial. After centrifugation at 900 X g for 45 minutes at room temperature, the inoculum was removed and 1 ml CH medium was

added to each vial. After incubation at 37^{0}C for 48 hours, the monolayers on coverslips were fixed with methanol for 30 minutes and stained with Jones' iodine for 10 minutes. The coverslips were inverted and mounted on microscope slides with glycerol:Jones' iodine (1:1). The number of inclusions on each coverslip was counted under a light microscope. The number of IFU/ml (inclusion forming units per ml) was determined by a formula: No. IFU/ml = No. Inclusions/coverslip x reciprocal dilution x 10. Quantitation of CT recovery reflected extension of CT replication.

FITC-conjugated Anti-MOMP Stain

For determination of initial inclusions, infected monolayers were stained with FITC-conjugated anti-MOMP according to the manufacturer's instructions. Briefly, cells on coverslips were fixed and incubated with FITC-conjugated anti-MOMP at 37^{0}C for 30 minutes in a moist chamber, rinsed with IFA buffer and mounted on slides. Inclusions were counted under a Reichert fluorescent microscope.

To restain coverslips previously stained with iodine, the coverslips were placed in 2 ml IFA buffer for at least one hour to remove all traces of the iodine. Then the coverslips were stained with FITC-conjugated anti-MOMP as described above. Yield of CT was determined as follows: No. IFU ml^{-1} / total initial inclusions (i.e., the number of IFU recovered per inclusion).

Initial Inclusions and Kinetics of CT Recovery

In order to demonstrate CT infection and replication in cells, initial inclusions

were measured and kinetics of CT recovery performed. Peritoneal macrophages, macrophage cell lines (J774A.1 and RAW264.7) or McCoy cells (control) were planted in shell vials (5 X 10^5 cells/ml/vial) and incubated at 37^{0}C overnight. Following aspiration of the medium, 0.1 ml of CT Seed Pool (1:3 dilution) were added to each vial (MOI: 1-2). The vials incubated at 37^{0}C for 90 minutes. After adsorption, the inoculum was aspirated and the monolayers were washed gently three times with DPBS. Macrophage growth medium (1 ml) was added to each vial. The vials incubated at 37^{0}C for 3 or 6 days. Addition of inoculum to shell vials without cells served to define background. At different time points pi, recovery of CT from supernatant or lysate of infected cultures was measured on McCoy cells as described previously. Replicate monolayers were fixed and stained with iodine or FITC-conjugated anti-MOMP to quantitate inclusion formation.

Nitrite Assay

The method used to measure nitrite production was based upon that described by Stuehr and Marletta (1987). Briefly, 50 μl of supernatant from each culture was mixed with an equal volume of Griess reagent in a 96-well plate. The plate was incubated at room temperature for 10 minutes. The OD_{570} was determined by an automated plate reader (VMAX, Molecular Devices Corporation, Menlo Park, CA). Sodium nitrite served as a standard. Nitrite concentration was quantitated by using the standard curve generated in each assay.

TNF-α Assay

The TNF-α assay was described by Ruff et al. (1981) and Hansen et al. (1989). Briefly, 100 μl of supernatant from each culture were mixed with 100 μl of the L929 cell suspension containing 4 μg/ml of actinomycin D in a 96-well plate. The plate was incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. Medium was discarded and the monolayers were stained with a crystal violet solution (0.2%) for 10 minutes. After the plate was washed 6-7 times with tap water, 100 μl of 1% SDS was added to each well and the plate was shaken for 1 minute. The OD_{558} was measured as described above. Mouse rTNF-α served as a standard. Concentration of TNF-α was determined by using the standard curve generated at the same time.

Treatment of Uninfected Cells with LPS and/or IFN-γ

RAW264.7, J774A.1 and McCoy cells were planted as described previously. After incubation at 37°C overnight, the medium was aspirated and 1 μg LPS, 100 U IFN-γ or both was added to each vial in 1 ml of macrophage growth medium. The vials were incubated at 37°C for 6 days. During incubation, half of medium (0.5 ml) was replaced by fresh growth medium at day 3 post-treatment (pt). In a second set of vials, medium was completely replaced by fresh growth medium every 24 hours. TNF-α and nitrite production were measured as described previously in supernatants at different time points.

LPS Dose Curve

RAW264.7, J774A.1 and McCoy cells were planted as described as previously. At the time of treatment, 1 ml of macrophage growth medium containing different concentrations of LPS (from 0.001 to 10 μg/ml) was added to the cells. At 48 hours pt, nitrite production in supernatant was measured as described previously. LPS at a concentration 100 ng/ml was optimal for stimulation of nitrite production. Therefore, 100 ng/ml of LPS was used in subsequent experiments.

Treatment of CT-Infected Cells with LPS and/or IFN-γ

RAW264.7, J774A.1, McCoy, KLE and OVCAR-3 cells were planted and inoculated with CT as described in Kinetics of CT Recovery. At 24 hours pi, the infected cells were treated with LPS (100 ng) and/or IFN-γ (100 U) as described. At different time points pt, nitrite and TNF-α production was measured in the supernatants. CT recovery from supernatants or lysates was quantitated on McCoy cell cultures as described previously.

Blocking of Nitrite Production by L-NMMA

Dose curve of L-NMMA. RAW264.7 cells were planted and treated with rIFN-γ and LPS as described previously. At the time of treatment, L-NMMA (from 0.001 mM to 10 mM) was added to each vial. At 24, 48 and 72 hours pt, nitrite production was measured as described.

Specific inhibition of nitrite production by L-NMMA. RAW264.7, J774A.1

and McCoy cells were planted and treated with IFN-γ, LPS or both as described. At the time of treatment, 1 mM of L-NMMA was added to each vial. At 24, 48, 72 hours pt, nitrite and TNF-α production were measured in the supernatants as described.

Abrogation of CT inhibition by L-NMMA. RAW264.7, J774A.1 and McCoy cells were planted and inoculated with CT as described previously. At 24 hours pi, the CT-infected cells were treated with LPS and/or IFN-γ in presence of different concentrations of L-NMMA (from 0.1 to 5 or 10 mM) as described above. At 48 hours pt, nitrite and TNF-α production were measured as described. Concomitantly, CT recovery from lysate was quantitated in McCoy cell cultures as described.

Treatment of CT-infected Cells with Sodium Nitrite

RAW264.7 cells were planted and inoculated with CT as described previously. At 24 hours pi, medium was aspirated and fresh macrophage growth medium containing different concentrations of sodium nitrite (from 50 to 200 nmol/ml) was added to the infected cells. At 48 hours pt, TNF-α production and nitrite concentration in supernatant were measured. CT recovery from lysate of the infected cultures was quatitated as described previously.

Role of Tryptophan Degradation in Inhibition of CT Replication

RAW264.7 cells were planted, inoculated with CT and treated with IFN-γ and LPS as described previously. At the time of treatment, different amounts of

tryptophan (from 40 to 1000 μg/ml) were added to each vial. In a separate experiment, the amount of tryptophan and 1 mM of L-NMMA were added to each vial. At 48 hours pt, nitrite and TNF-α production were measured. Concomitantly, CT recovery was quantitated in McCoy cell cultures as described.

Isolation of Total RNA

Total RNA was isolated from cell cultures using a commercial total RNA isolation reagent according to the manufacturer's instructions. Briefly, monolayers were lysed directly in shell vials by adding Ultraspec™RNA and pipeting the cell lysate several times. The cell lysate was transferred immediately to a microcentrifuge tube which was immersed in ice. Five minutes were allowed to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml per 1 ml of Ultraspec™RNA) was added and the tubes were shaken vigorously for 15 seconds. The homogenate was centrifuged at 12,000 X g for 15 minutes at 4°C . The top aqueous phase was carefully transferred to a fresh tube. An equal volume of isopropanol was added and the samples were cooled at 4°C for 10 minites. Total RNA was pelleted by centrifugation at 12,000 X g for 10 minutes at 4°C. The pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation at 7,500 X g for 5 minutes at 4°C. The pellet was briefly dried, then dissolved in 50 μl of DEPC-treated distilled water. This method generally yielded 10 μg of total RNA from 1 x 10^6 cells. The OD_{260}/OD_{280} ratio was approximately 1.5.

Preparation of NOS cDNA Probe

Transformation of JM109 cells with Plasmid DNA

JM109 cells were transformed with CL-BS-mac-NOS plasmid DNA using a calcium chloride procedure (Ausubel et al. 1992). Briefly, JM109 cells were grown in 50 ml of LB medium to OD_{600}=0.437. The bacterial cells were centrifuged at 800 X g for 10 minutes. The cell pellet was resuspended gently in 25 ml of an ice-cold, sterile solution of 50 mM $CaCl_2$ and placed in an ice bath for 15 minutes. The cells were centrifuged again, resuspended in 2 ml of an ice-cold 50 mM $CaCl_2$ and incubated at 4°C overnight. Two hundred μl of the cell suspension were mixed with 50 ng of the plasmid DNA (in 5 μl TE buffer, pH 8.0) in a microcentrifuge tube. The cells were mixed with TE buffer alone as a control. The mixtures were incubated on ice for 30 minutes and then placed in a heated water bath (42°C) for 2 minutes. Following the heat shock, LB medium (0.5 ml) was added to each tube and the tubes were incubated at 37°C for 1 hour. Then selective antibiotic plates were inoculated with the transformed cell suspension (100 μl). After incubation of the plates at 37°C for 16 hours, colorless colonies appeared only in the plates inoculated with the plasmid-transformed cells. No colonies were observed in the plates inoculated with the control cells.

Isolation of Plasmid DNA

One colony isolated as described above was picked to inoculate 30 ml LB

medium containing 50 μg/ml ampicillin. After overnight shaking at 37°C , the plasmid DNA was isolated by a method for minipreps described by Davis et al. (1986). Briefly, the cells were resuspended in 700 μl of GTE buffer containing lysozyme (2 mg/ml), incubated at room temperature for 5 minutes and mixed with alkaline lysis buffer (1.7 ml) by inversion of the tube. After cooling on ice for 5 minutes, the cell lysate was mixed with 1.26 ml of ice cold potassium acetate solution (5 M, pH 4.8). After centrifugation at 6,000 X g at 4°C for 10 minutes, the supernatant was collected and treated with RNase A (50 μg/ml) at 37°C for 1 hour. The treated supernatant was subsequently extracted twice with an equal volume of a phenol:chloroform: isoamylalcohol (25:24:1) mixture. The plasmid DNA was precipitated in ice cold isopropanol and centrifuged at 7,500 X g for 15 minutes. The plasmid DNA pellet was washed with 75% ethanol, dehydrated with 100% ethanol, briefly dried and dissolved in 300 μl of TE buffer. The OD_{260}/OD_{280} ratio of the sample was 1.93.

Isolation of Insert (NOS cDNA) from the Plasmid DNA

Isolation of the insert was performed by modification of a procedure described by Sambrook et al. (1989a). Briefly, 10 μg of the plasmid DNA was digested by Not I (1 U/μl) in 100 μl of the reaction solution at 37°C overnight. The digisted plasmid DNA was run on a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide at 18 V (1.3 V/cm) overnight. Then a piece of the treated DEAE-cellulose membrane (see materials) was inserted in front of the leading edge of the band with 4100 bp. Electrophoresis was resumed at 80 V for 25 minutes to allow migration of all DNA of

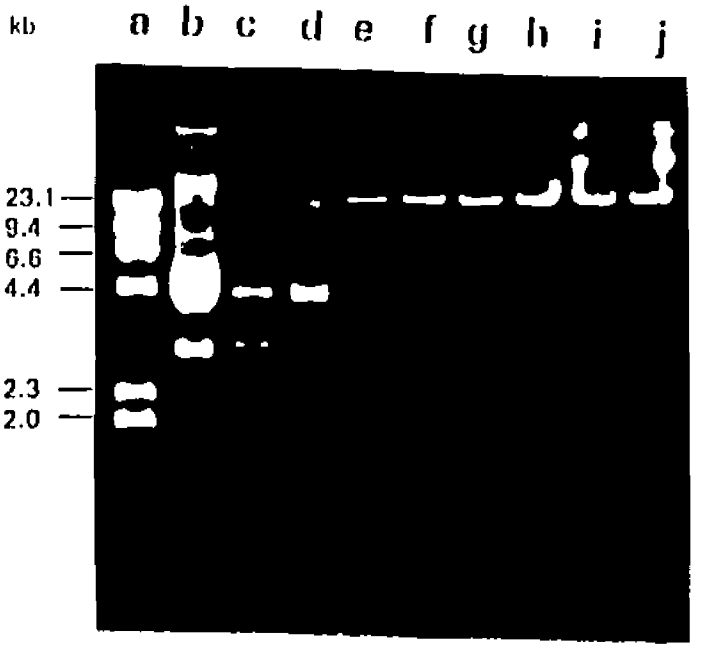
the band onto the membrane. The membrane was rinsed with a low salt washing buffer (50 mM Tris·Cl, pH 8.0, 0.15 M NaCl, 10 mM EDTA, pH 8.0). The fragment was eluted from the membrane by washing with a high salt elution buffer (50 mM Tris·Cl, pH 8.0, 1 M NaCl, 10 mM EDTA pH 8.0) at 65^{0}C for 60 minutes. The eluate was subsequently extracted once with a mixture of phenol:chloroform:isoamylalcohol as described previously. Then, the fragment was precipitated with isopropanol and dissolved in TE buffer. Figure 3 shows the band of isolated NOS-cDNA and the recombinant plasmid DNA before and after the digestion.

Radiolabelling of NOS cDNA

The NOS cDNA isolated as above was labeled with ^{32}P-dCTP by random primer labeling using a Prime-a-Gene Labeling System Kit according to manufacturer's instructions. Briefly, 40 μl sterile water, 20 μl 5 X labeling buffer, 4 μl mixture of the dATP, dTTP and dGTP, 20 μl denatured NOS cDNA (50-100 μg), 4 μl nuclease-free BSA, 100 μCi [α-^{32}P] dCTP, 10 units of Klenow enzyme were mixed in a microcentrifuge tube on ice. The total reaction volume was 100 μl. The mixture was incubated at room temperature for 60 minutes. The reaction was terminated by heating at 100^{0}C for 2 minutes and subsequently chilling in an ice bath. The labeled probe was stored at -20^{0}C. Probes with specific activities of more than 10^9 cpm/μg were routinely generated by this method.

Figure 3

NOS cDNA isolated from CL-BS-mac-NOS plasmid. Lane a, lambda DNA/Hind III markers; Lane b, CL-BS-mac-NOS plasmid DNA; Lane c, Not I-digested CL-BS-mac-NOS plasmid DNA; Lane d, isolated NOS cDNA; Lanes e-j, lambda DNA 15.6, 31.2, 62.5, 125, 250 and 500ng, respectively. Ethidium bromide-stained agarose gel.



Dot Blot Hybridization

The dot blot method was a modification of that described by Sambrook et al. (1989b). Briefly, RNA samples (1 μg of sample RNA in 100 μl of denaturing solution 1) were denatured by heating at 65^{0}C for 15 minutes and subsequently chilling on ice. For titration of NOS mRNA, serial dilution of the RNA sample (4 μg - 8 ng) from LPS- and IFN-γ-treated RAW264.7 cells was performed. To confirm the hybridization with RNA, 4 μg of the RNA sample from LPS- and IFN-γ treated RAW264.7 cells were treated with RNase A (10 μg/μg RNA) before denaturation. The denatured RNA samples were blotted onto Nytran plus nylon membranes using a "dot blot" apparatus (Gibco BRL Life Technologies). The membranes were UV-crosslinked and baked at 80^{0}C for 1 hour. Then, the membranes were prehybridized in prehybridization solution at 68^{0}C for 3 hours and subsequently hybridized with denatured ^{32}P-labeled NOS-cDNA probe in the same solution at 68^{0}C for 16 hours. After hybridization, the membranes were washed twice in washing buffer 1 for 15 minutes at room temperature and twice in washing buffer 2 for 15 minutes at 65^{0}C. The membranes were then exposed to X-ray films in cassettes with intensifying screens (Fisher Scientific) at -80^{0}C for 1-2 days. The films were developed and fixed using Kodak GBX developer and fixer solutions. The density of the dots was measured by Bio-Image Analysis System (Millipore Corporation Imaging Systems, Ann Arbor, MI). Hybridization with a β-actin cDNA probe served as a control. Blots were stripped of probe by washing with 2 x SSPE containing 50% formamide at 68^{0}C for 2 hours, then with 0.7 x SSPE containing 50% formamide at 80^{0}C for 2 hours.

Northern Blot Hybridization

The Northern blot method was based upon that described by Sambrook et al. (1989b). Briefly, RNA samples (10 μg of RNA in 20 μl denaturing solution 2) were denatured by heating at 65°C for 15 minutes. The denatured RNA samples were chilled on ice. Two μl of 10x loading buffer were added to each of the denatured RNA samples. Five μg of a RNA markers were denatured as above. Also, two μg of the loading buffer were added to the denatured markers. Then, the RNA samples and the markers were run on a 1% formaldehyde denatured agarose gel (20 x 20 cm^2) in 1 x MOPS buffer at 90 V for 4 hours. The gels were photographed over a UV-transilluminator (FisherBiotech™, Fisher Scientific). RNA was transferred from the agarose gel onto a Nytran plus nylon membrane using a capillary transfer method (Sambrook et al. 1989b). The membranes were UV-crosslinked and baked at 80°C for 1 hour. The membranes were prehybridized and hybridized as described above.

Kinetics of NOS mRNA Expression

RAW264.7 cells were planted, inoculated with CT and treated with LPS and/or IFN-γ as described previously. At 6, 24 and 48 hours pt, total RNA was isolated as described previously. NOS mRNA expression was determined by Northern blot hybridization as described.

Effect of Anti-TNF-α Ab upon NO Production and

Inhibition of CT Replication in RAW264.7 Cells

RAW264.7 cells were planted and inoculated with CT as described previously. At 24 hours pi, the cells were divided into 16 groups as shown in Table 1.

Table 1

EXPERIMENTAL PROTOCOL FOR INVESTIGATING INHIBITION OF CT REPLICATION IN RAW264.7 CELLS

Treatments	None	Anti-TNF-α Ab	L-NMMA	Anti-TNF-α Ab & L-NMMA
None	1	2	3	4
IFN-γ	5	6	7	8
LPS	9	10	11	12
IFN-γ & LPS	13	14	15	16

Group 1 served as an untreated control. Group 2 was treated with anti-TNF-α Ab. Group 3 was treated with L-NMMA. Group 4 was treated with the Ab and L-NMMA. Group 5 was treated with IFN-γ. Group 6 was treated with IFN-γ and the Ab. Group 7 was treated with IFN-γ and L-NMMA. Group 8 was treated with IFN-γ, the Ab and L-NMMA. Group 9 was treated with LPS. Group 10 was treated with LPS and the Ab. Group 11 was treated with LPS and L-NMMA. Group 12 was treated with LPS, the Ab and L-NMMA. Group 13 was treated with IFN-γ and LPS.

Group 14 was treated with IFN-γ, LPS and the Ab. Group 15 was treated with IFN-γ, LPS and L-NMMA. Group 16 was treated with IFN-γ, LPS, the Ab and L-NMMA.

At 6 hours pt, total RNA was isolated and NOS-mRNA was measured by dot blot and Northern blot hybridization as described previously. At 48 hours pt, nitrite and TNF-α production in supernatant were measured as described previously. Concomitantly, recovery of CT from lysates of the infected cell cultures was performed as described. For these experiments, 100 ng/ml LPS, 100 units/ml IFN-γ, 1 mM L-NMMA and 8 μl/ml anti-TNF-α Ab were used.

Effect of Nitrite Production Upon CT Replication in LPS- and/or IFN-γ-treated Peritoneal Macrophages

Peritoneal macrophages were generated and inoculated with CT as described previously. At 24 hours pi, the infected macrophages were divided into 16 groups and each group was treated as described above. At 48 hours pt, nitrite and TNF-α production in supernatant were measured. At the same time, CT recovery from lysates of the infected cultures also was performed as described.

Treatment of Cells with rTNF-α

Post-inoculation treatment. McCoy cells were plated and incubated as described. At the time of inoculation, the medium was aspirated, then 0.1 ml of CT inoculum (MOI=0.006) was added to each vial. The vials were centrifuged at 900 X

g at room temperature for 45 minutes. After centrifugation, the inoculum was aspirated and 250 ng of rTNF-α in 1 ml of McCoy growth medium (5% FBS) was added to each vial. The vials were incubated at 37^{0}C for 2 days.

Pretreatment. McCoy cells was plated as described. After incubation at 37^{0}C overnight, 250 ng of rTNF-α (1 x 10^4 units) in 1 ml of McCoy growth medium (5%) was added to each vial. The vials were incubated at 37^{0}C for 24 hours. The medium containing rTNF-α was removed. Then, the pretreated cells were inoculated with CT as described above. After inoculation, McCoy growth medium without rTNF-α (5% FBS) was added to each vial. The vials were incubated at 37^{0}C for 2 days.

After incubation at 37^{0}C for two days, the cells were fixed and stained with Johns' iodine, then destained and restained with FITC-conjugated anti-MOMP. Meanwhile, CT recovery from lysates of the infected cultures also was performed as described.

Statistical Analysis

Student's t test was used for comparison of means in two groups. Analysis of Variance was used for comparison of means in more than two groups. The Least Significant Difference (LSD) was used to determine where the differences occurred if a significant F is found. Linear Regression was used for analysis of correlation between nitrite production or NOS mRNA expression and CT inhibition.

CHAPTER 3

Results

Initial Inclusion Formation in CT-Infected Macrophages and McCoy Cells

Initial inclusions were formed in peritoneal macrophages infected by CT in vitro, which was demonstrated by FITC-conjugated anti-MOMP antibody. However, the pattern of initial inclusion formation in peritoneal macrophages was quite different from that in McCoy cells. The number of inclusions in CT-infected peritoneal macrophages reached a peak at 24 hr pi (4313 $\pm$ 273 inclusions /coverslip), then continuously decreased during the period of observation (Figure 4). At day 6 pi, the number of initial inclusions decreased to 340 $\pm$ 46 per coverslip. In contrast, in McCoy cells 1917 $\pm$ 130 inclusions /coverslip were observed at 24 hr pi and increased after 48 hr pi. The number of inclusions reached a plateau (5481 $\pm$ 98 inclusions/coverslip) at day 4 pi which was sustained through day 6 (Figure 4). The percentage of infected peritoneal macrophages was 0.9% at 24 hr pi and decreased to 0.1% at day 6 pi (Table 2). However, the percentage of infected McCoy cells was 0.2% at 24 hr pi and increased to 0.9% at day 6 pi.

CT Replication in Peritoneal Macrophages

In order to determine if CT replicated in peritoneal macrophages, recovery of infectious CT (EBs) from infected cells was analyzed. CT recovery from lysates of

Figure 4

Different patterns of initial inclusion formation in CT-inoculated macrophages and McCoy cells. Peritoneal macrophages and McCoy cells were inoculated with CT (MOI=1). Initial inclusions were determined by FITC-conjugated anti-MOMP. Data presented are mean of triplicate ± S.D..

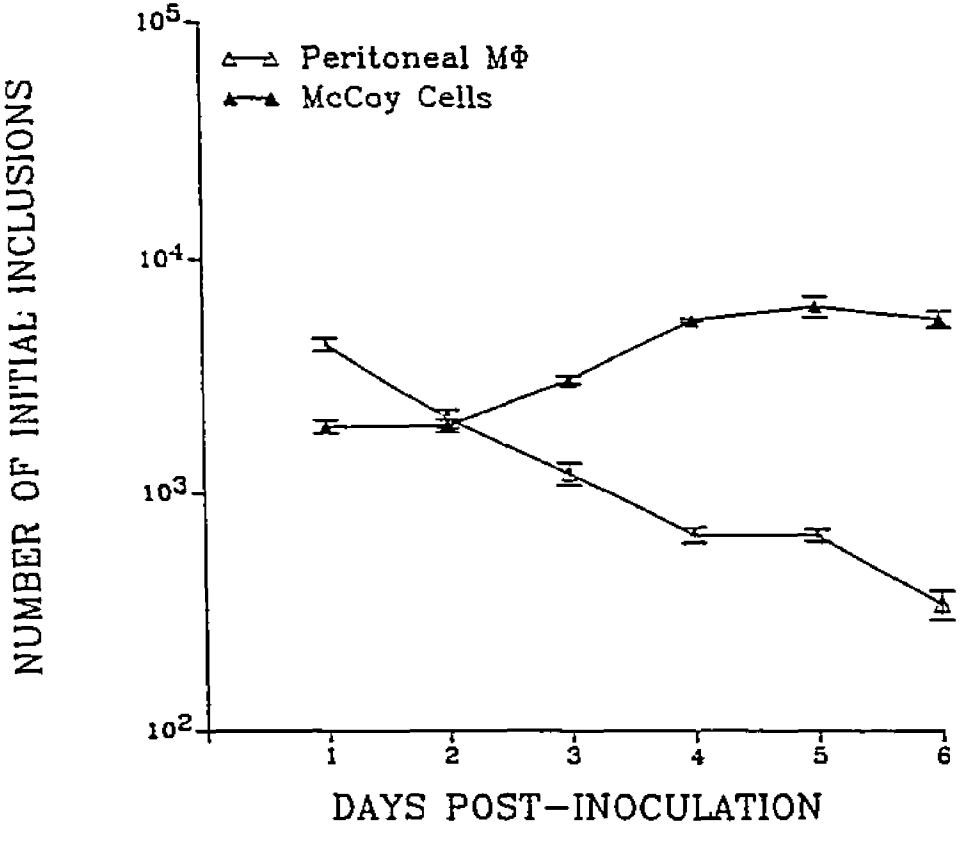


Table 2

PERCENTAGE OF CT-INFECTED CELLS
DURING IN VITRO INFECTION

Cell Type	% Cells Infected					
	Days Post-infection					
	1	2	3	4	5	6
RAW264.7	0.1	<0.1	0.3	0.4	0.5	0.6
J774A.1	1.6	0.6	9.8	12.1	27.4	48.2
Mϕ	0.9	0.4	0.3	0.2	0.2	0.1
McCoy	0.2	0.2	0.3	0.6	1.0	0.9

Peritoneal macrophages, RAW264.7, J774A.1 and McCoy cells were inoculated with CT (MOI=1). Infected cells were detected and enumerated by FITC-conjugated anti-MOMP, at 48 hours pi.

CT-infected peritoneal macrophages decreased from 10^6 IFU at the time of inoculation to 10^3 IFU at 24 hours pi (Figure 5). At 48 hours pi, CT recovery dramatically increased to 2.8×10^4 IFU, which demonstrated replication. The shape of curve of CT recovery from infected macrophages was similar to that of infected McCoy cells. However, the level of CT recovery was two magnitudes lower than that from the infected McCoy cells (Figure 5). CT recovery from coverslips without cells (background control) decreased from 10^6 IFU at inoculation to 0 after 48 hours pi (Figure 5).

The yield of CT production (IFU/inclusion) in peritoneal macrophages reached a peak between 48 and 72 hr pi and decreased thereafter. The pattern in McCoy cells was similar to that in peritoneal macrophages (Figure 6), since the yield reached a peak at 48 hr pi and decreased thereafter. However, the highest levels of yield from peritoneal macrophages were 21.2 (day 2) and 22.8 (day 3) IFU/inclusion, which was significantly less than from McCoy cells (355 and 262 IFU/inclusion at days 2 and 3, respectively). Thus the number of EBs produced by each inclusion was significantly less in peritoneal macrophages than in McCoy cells.

Initial Inclusion Formation in Macrophage Cell Lines

Initial inclusion formation was demonstrated by FITC-conjugated anti-MOMP stain in CT-infected macrophage cell lines (RAW264.7 and J774A.1). The number of initial inclusions in CT-infected RAW264.7 was 908 $\pm$ 142 per coverslip at day 1 pi and decreased at day 2 pi (419 $\pm$ 64 Inclusions/coverslip). At day 3 pi, the number

Figure 5

Kinetics of CT recovery from peritoneal macrophages. Peritoneal macrophages and McCoy cells were inoculated with CT (MOI=1). Addition of inoculum to shell vials without cells served to define background. CT recovery was quantitated at 0, 1.5, 10. 24, 48 and 72 hours pi, respectively in susceptible McCoy cells. Data presented are mean of triplicate ± S.D..

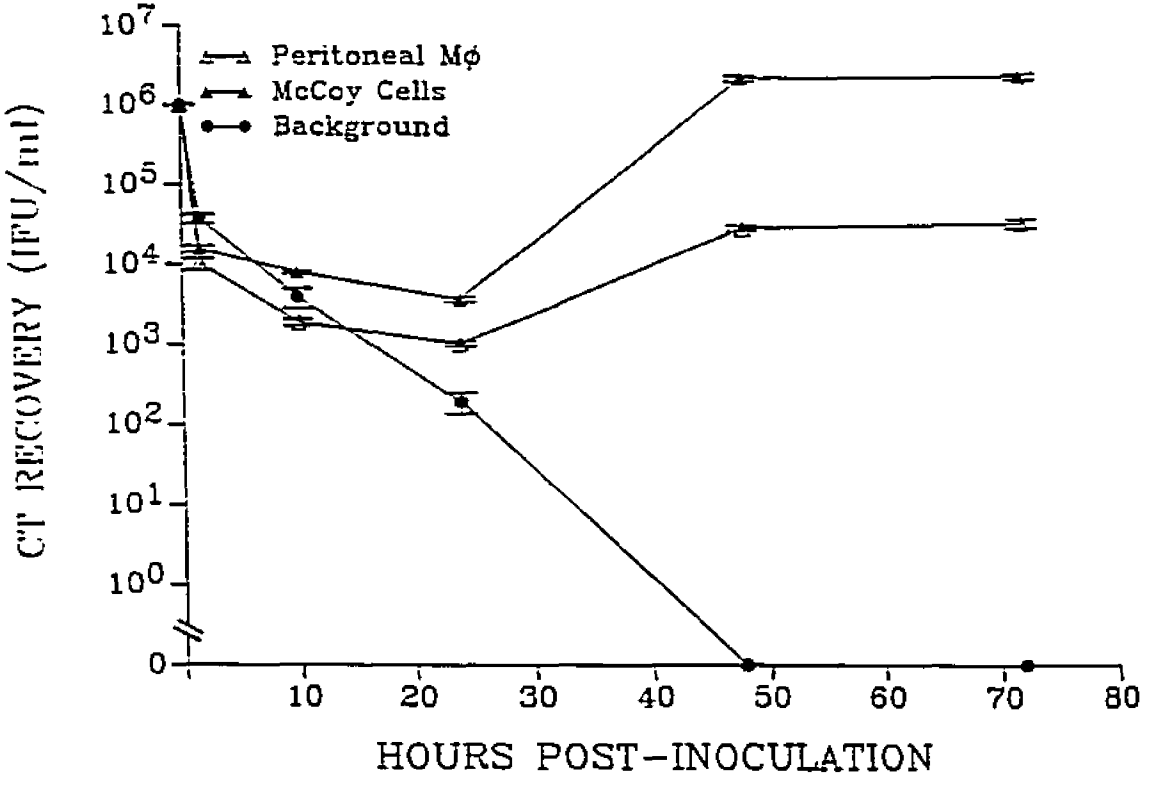
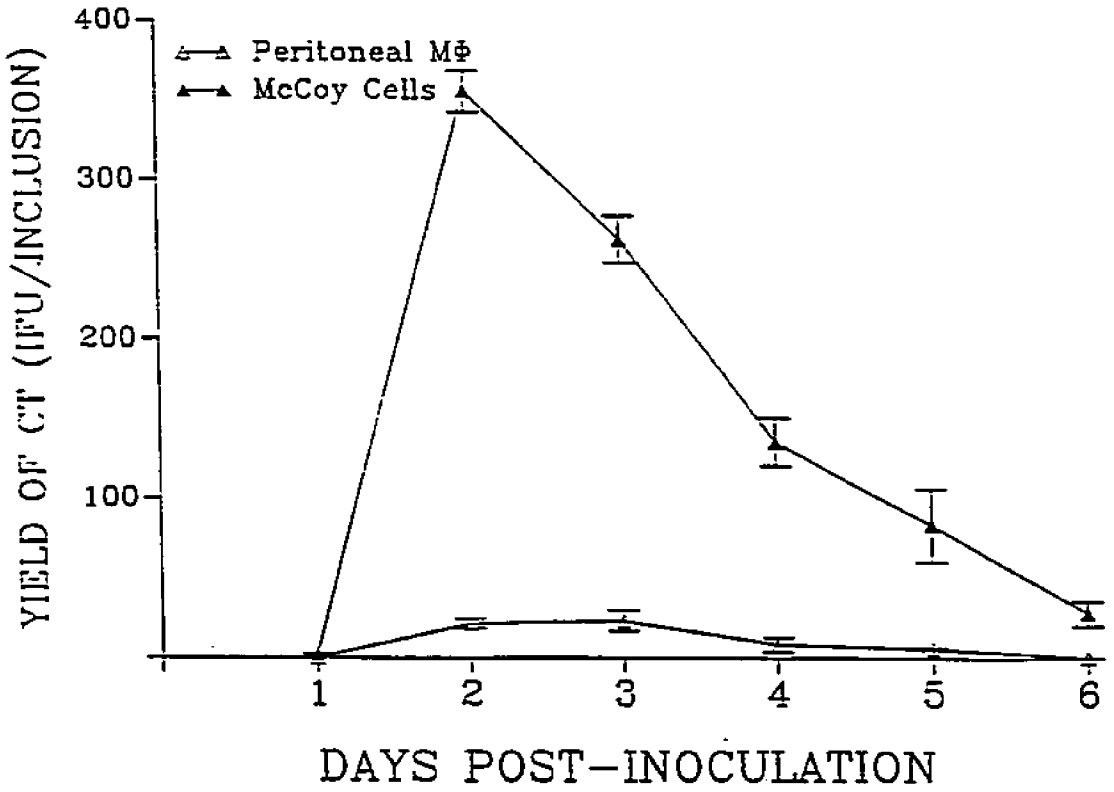


Figure 6

Comparative yield of CT production in macrophages and McCoy cells. Peritoneal macrophages and McCoy cells were inoculated with CT (MOI=2). Initial inclusions and CT recovery were quantitated. Yield of CT was determined by a formula: No. IFU/inclusion = No. IFU ml^{-1} / total initial inclusions per coverslip. Data presented are mean of triplicate ± S.D..



of inclusions in RAW264.7 cells was increased (3000 ± 410 Inclusions/coversilp) and maintained the same level thereafter (Figure 7). The number of initial inclusions in CT-infected J774A.1 cells was 9,521 ± 629 per coverslip at 24 hr pi. As in RAW264.7 cells, the number of initial inclusions in J774A.1 cells decreased at day 2 pi (3,771 ± 130 Inclusions/coverslip) but increased at day 3 pi (29,250 ± 1442 Inclusions/coverslip). However, the number of initial inclusions in J774A.1 cells gradually decreased after day 3 pi. The kinetics of initial inclusions in peritoneal macrophages was different from that in macrophage cell lines. The number of initial inclusions reached a peak at 24 hr pi and continuously decreased during the period of observation (Figure 7). The pattern of initial inclusion kinetics in McCoy cells was similar to that in RAW264.7 cells with the exception that a decrease in the number of initial inclusions was not observed at day 2 pi in the former (Figure 7).

The number of J774A.1 cells decreased by more than 80% after day 3 pi in the infected cultures, but not in the infected RAW264.7 or McCoy cells (Figure 8). As a result, the percentage of infected cells at day 3 pi was 9.8% in J774A.1 cells (Table 2). At day 6 pi, the percentage of infected J774A.1 cells was much higher (48.2%) than that of infected RAW264.7 (0.6%) or McCoy cells (0.9%). As in J774A.1 cells, the number of peritoneal macrophages decreased by about 30% at day 6 pi. However, this decrease in cell number did not result in an increase in the proportion of infected cells. In fact, the percentage of infected cells decreased from 0.9% at day 1 to 0.1% at day 6 pi (Table 2).

Figure 7

Comparison of initial inclusion formation in macrophage cell lines and peritoneal macrophages. Peritoneal macrophages, macrophage cell lines (RAW264.7 and J774A.1) and McCoy cells were inoculated with CT (MOI=1). Initial inclusions were determined by FITC-conjugated anti-MOMP. Data presented are mean of triplicate ± S.D..

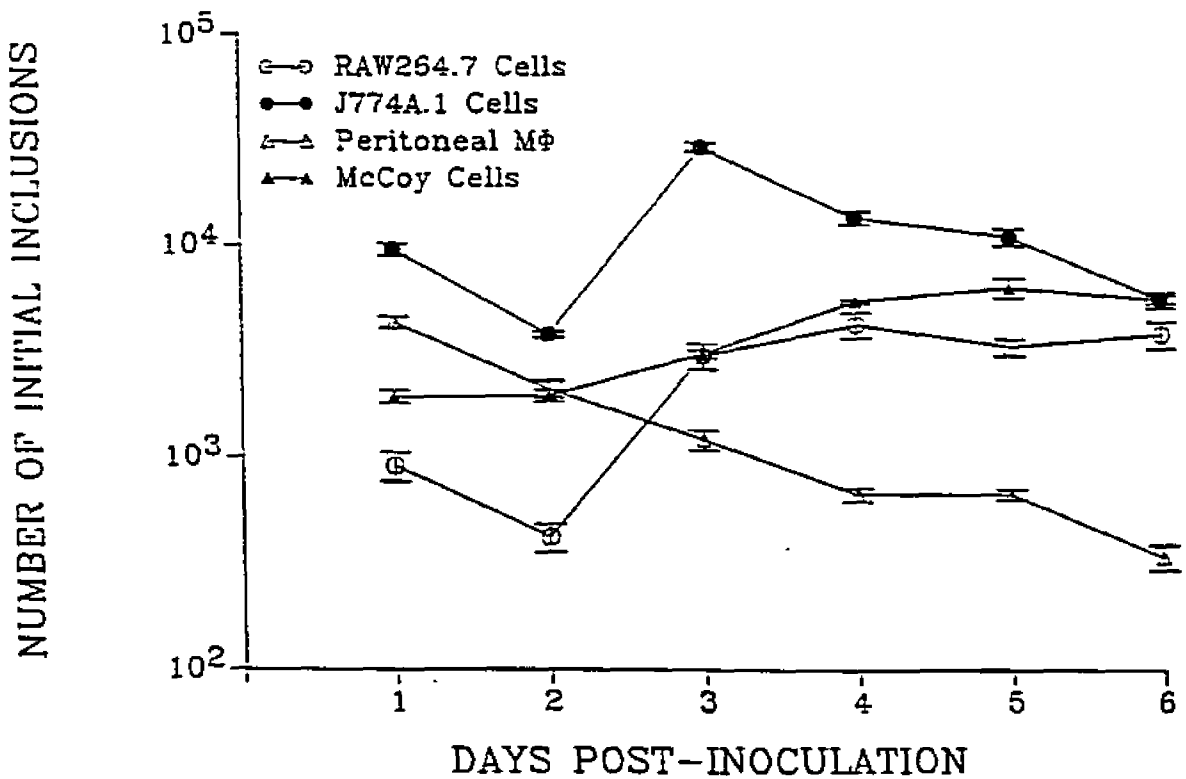
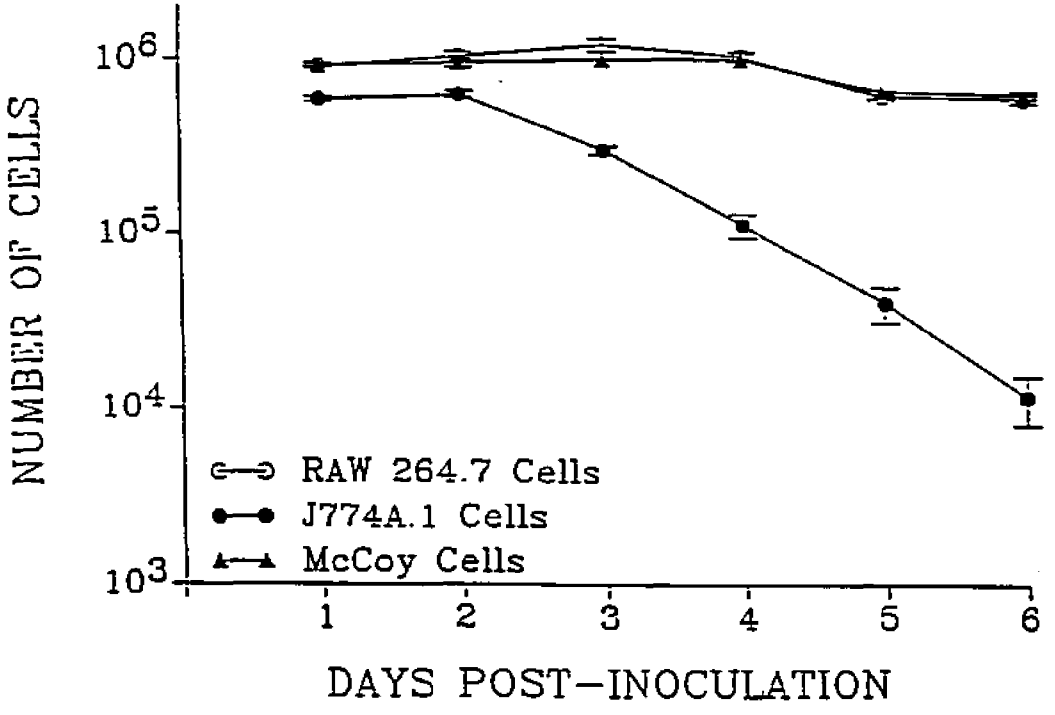


Figure 8

Comparative cell numbers in CT-infected cell lines. RAW264.7, J774A.1 and McCoy cells were inoculated with CT (MOI=2). Number of cells were estimated by using a light microscope at 400 magnification. Data presented are mean of triplicate ± S.D..



Effect of Medium Change Upon CT Replication

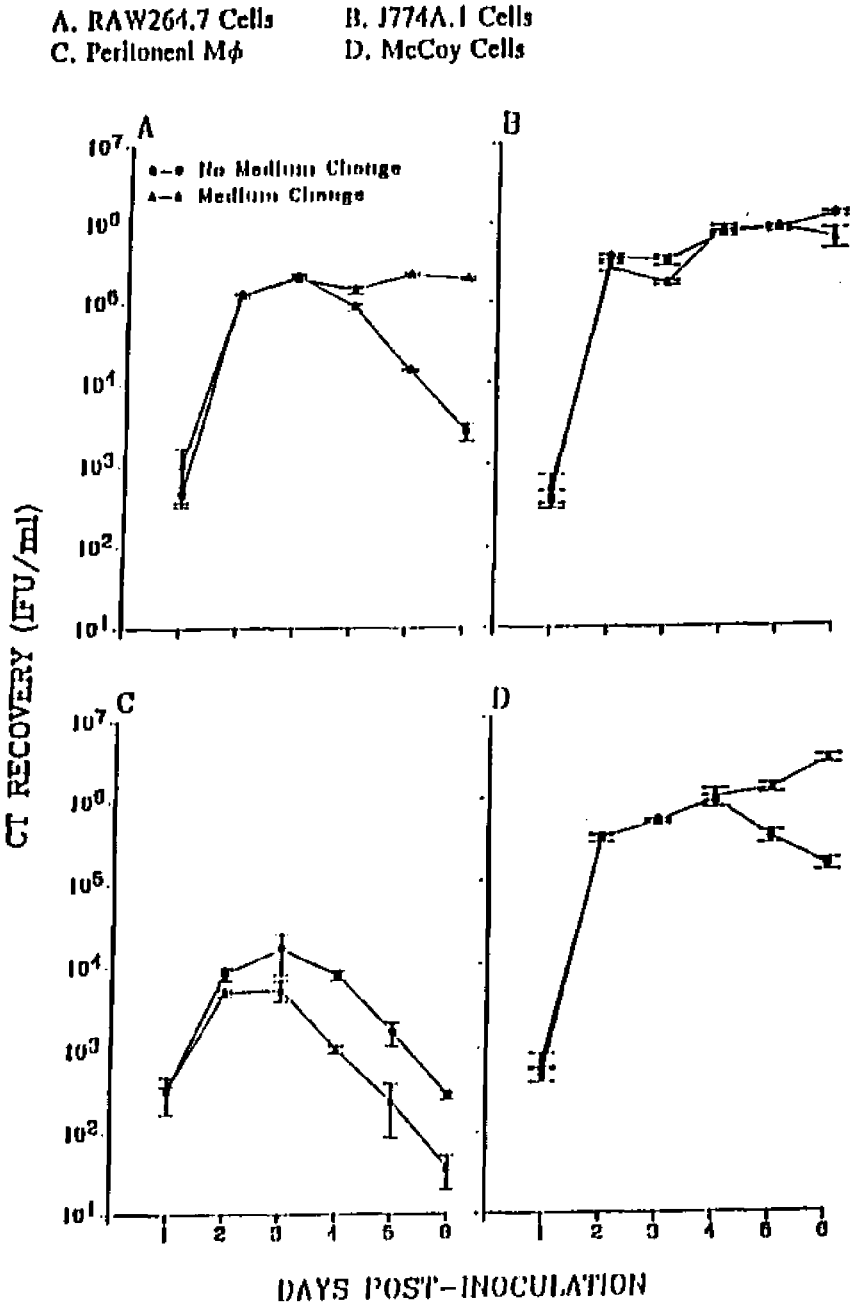
Since subsequent experiments would require addition of inhibitors or cytokines after infecton, it was important to determine the effect of medium change upon CT replication. The recovery of CT from supernatants of CT-infected J774A.1 cells was 355,278 ± 29,832 IFU/ml at day 2 pi. The highest recovery was at day 6 pi (1,177,083 ± 81,570 IFU/ml). The level of CT replication in McCoy cells was as high as in J774A.1 cells (Figrue 9). CT recovery from McCoy cells was 322,361 ± 38,424 IFU/ml at day 2 pi. The highest recovery was at day 4 pi (885,444 ± 128,467 IFU/ml). In RAW264.7 cells, CT replication (214,028 ± 18,490 IFU/ml) was lower than in McCoy cells (885,444 ± 128,467 IFU/ml), but higher than in peritoneal macrophages (16,319 ± 8308 IFU/ml). Medium change significantly increased CT replication in RAW264.7 (Figure 9A) and McCoy cells (Figure 9D). Medium change did not affect CT replication in J774A.1 cells (Figure 9B). However, changing medium daily actually decreased CT recovery in peritoneal macrophages (Figure 9C).

Inhibition of CT Replication by LPS or IFN-γ

The levels of CT recovery from supernatants of infected RAW264.7 cells were 1.2×10^5 and 6.6×10^4 IFU/ml on days two and three, respectively. Figure 10A shows that CT recovery from the supernatants was greatly reduced in IFN-γ-treated RAW264.7 cells (0.6 and 1.2 logs inhibition, $p<0.01$ determined by LSD). Greater inhibition was observed in RAW264.7 cells treated with LPS (2.0 and 2.9 logs inhibition, $p<0.002$ determined by LSD) or LPS plus IFN-γ (2.4 and 4.8 logs

Figure 9

Effect of medium change upon CT replication. Peritoneal macrophages, RAW264.7, J774A.1 and McCoy cells were inoculated with CT (MOI=1). In some vials, maintenance medium was changed every 24 hours after inoculation. In others, the medium was not changed. CT recovery from the supernatants of infected cultures was quantitated. Data presented are mean of triplicate ± S.D..



inhibition, $p<0.002$ determined by LSD). Significant inhibition by LPS or LPS plus IFN-γ appeared one day earlier than that by IFN-γ alone (Figure 10A). Similar decreases in intracellular CT recovery also were observed in lysates of RAW264.7 cells treated with IFN-γ and/or LPS (data not shown).

In J774A.1 cells, CT recovery was dramatically inhibited ($p<0.002$ determined by LSD) by IFN-γ- or LPS-treatment (Figure 10B). However, IFN-γ treatment induced greater inhibition of CT recovery (1.7 logs) than LPS (0.9 logs). IFN-γ and LPS induced inhibition synergistically (2.7 logs).

Inhibition of CT recovery was far less pronounced in IFN-γ- and/or LPS-treated McCoy cells than in the macrophage cell lines (Figure 10C). At day 3, LPS slightly reduced CT recovery. IFN-γ or IFN-γ plus LPS exhibited significant inhibition (0.3 and 0.4 logs of inhibition, $p<0.01$ determined by LSD).

Nitrite Production Induced by LPS or IFN-γ

LPS or IFN-γ induced significant nitrite production ($p<0.001$ determined by Student's t test) in uninfected RAW264.7 cell cultures without medium change (Figure 11), but nitrite production induced by LPS was higher. Peak nitrite production occurred at 48 hr pt. Combination of LPS and IFN-γ exhibited a synergistic effect upon nitrite production. No detectable nitrite production was observed in untreated cells (Figure 11A). Medium changes every 24 hr pt dramatically reduced nitrite production in the LPS- and/or IFN-γ-treated RAW264.7 cells (Figure 11B), indicating that persistent stimulation was necessary for continued production of nitrite.

Figure 10

Inhibition of CT replication in cells treated with IFN-γ and LPS. CT-inoculated RAW264.7, J774A.1 and McCoy cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. CT recovery was quantitated at days 1, 2 and 3 pt. Data are mean of triplicate ± S.D..

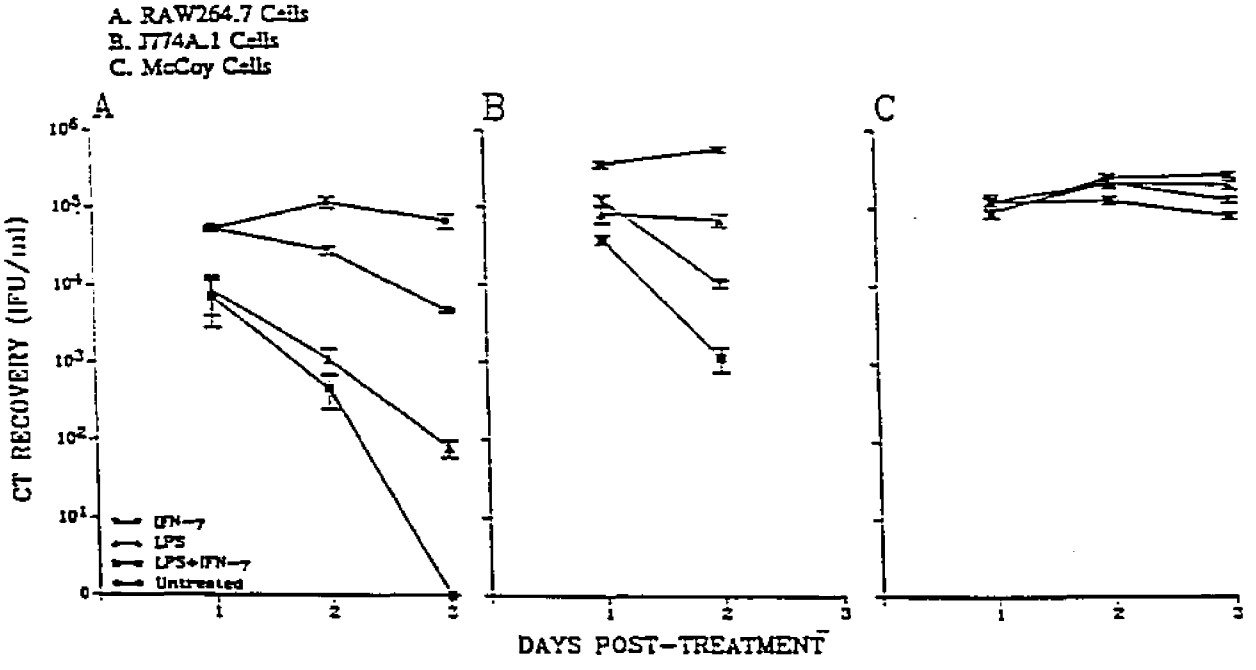
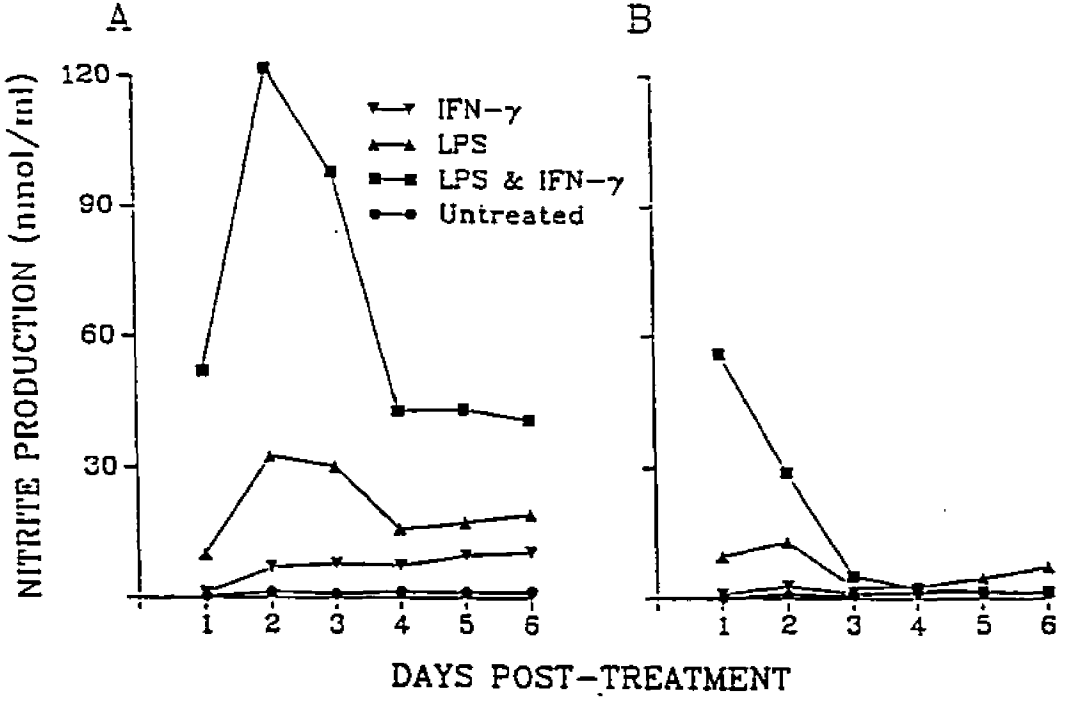


Figure 11

Effect of medium change upon nitrite production in RAW264.7 cells. Uninfected RAW264.7 cells were treated with IFN-γ (100 U/ml), LPS (1 μg/ml) or IFN-γ plus LPS. In some vials, the maintenance medium was changed every 24 hours after treatment. In others, the medium was not changed. Nitrite production was determined by the Griess reagent.

A. No Medium Change
B. Medium Change



Compared to RAW264.7 cells, J774A.1 (Figure 12) and McCoy cells (Figure 13) produced low levels of nitrite in response to LPS and IFN-γ. The pattern of nitrite production in J774A.1 cells treated with IFN-γ and LPS was similar to that observed in McCoy cells. The levels of nitrite production in LPS-treated J774A.1 cells were much higher than in McCoy cells.

LPS Dose Curve

In order to determine an optimal concentration of LPS for stimulation of nitrite production, increasing concentrations (from 0.001 to 10 μg/ml) were tested in uninfected RAW264.7, J774A.1 and McCoy cells (Figure 14). As the concentration of LPS was increased from 0.001 μg/ml to 0.1 μg/ml, nitrite production proportionally increased in RAW264.7 and J774A.1 cells. Nitrite production reached a plateau at 0.1 μg/ml of LPS. Nitrite production was not remarkable in LPS-treated McCoy cells, even at a concentration of 10 μg/ml. Therefore, the optimal concentration of LPS for nitrite production in macrophage cell lines was 0.1 μg/ml, which was used in subsequent experiments.

Nitrite Production in CT-infected and Uninfected Cells

CT infection alone was not sufficient to induce nitrite production in RAW264.7 (Figure 15B) or McCoy cells (Figure 16B). However, CT-infected RAW264.7 cells treated with IFN-γ produced more nitrite (17.6 ± 0.5 nmols/ml) than uninfected RAW264.7 cells (6.9 ± 3.0 nmols/ml, Figure 15). CT-infected

Figure 12

Effect of medium change upon nitrite production in J774A.1 cells. Uninfected J774A.1 cells were treated with IFN-γ (100 U/ml), LPS (1 μg/ml) or IFN-γ plus LPS. In some vials, the maintenance medium was changed every 24 hours after treatment. In others, the medium was not changed. Nitrite production was determined by the Griess reagent.

A. No Medium Change
B. Medium Change

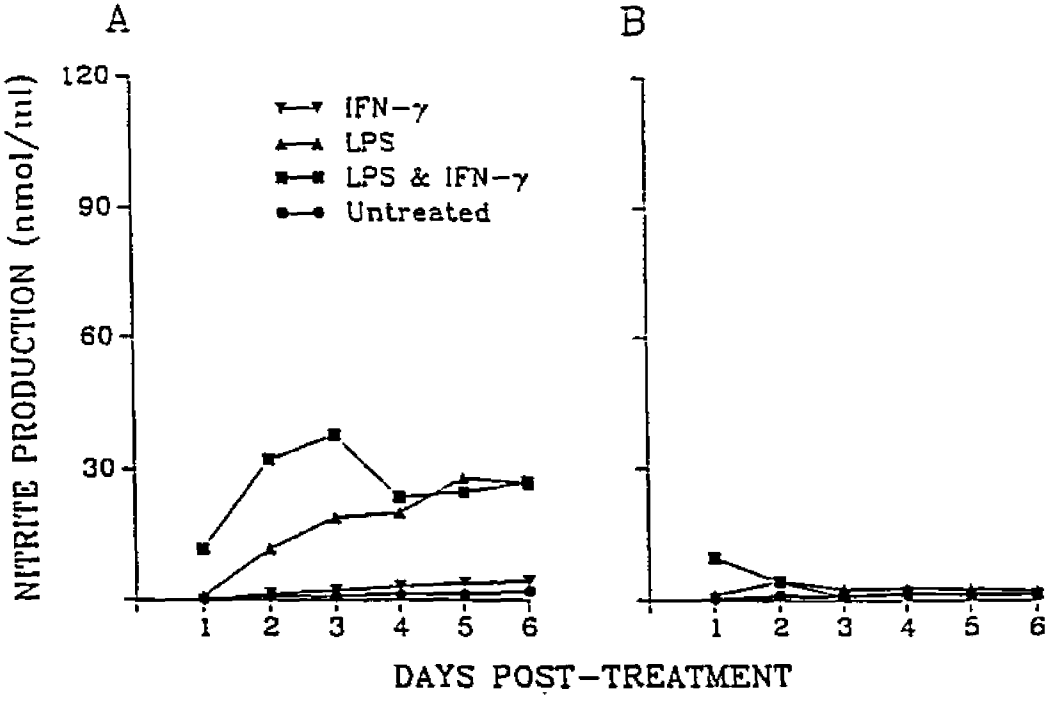


Figure 13

Effect of medium change upon nitrite production in McCoy cells. Uninfected McCoy cells were treated with IFN-γ (100 U/ml), LPS (1 μg/ml) or IFN-γ plus LPS. In some vials, the maintenance medium was changed every 24 hours after treatment. In others, the medium was not changed. Nitrite production was determined by the Griess reagent.

A. No Medium Change
B. Medium Change

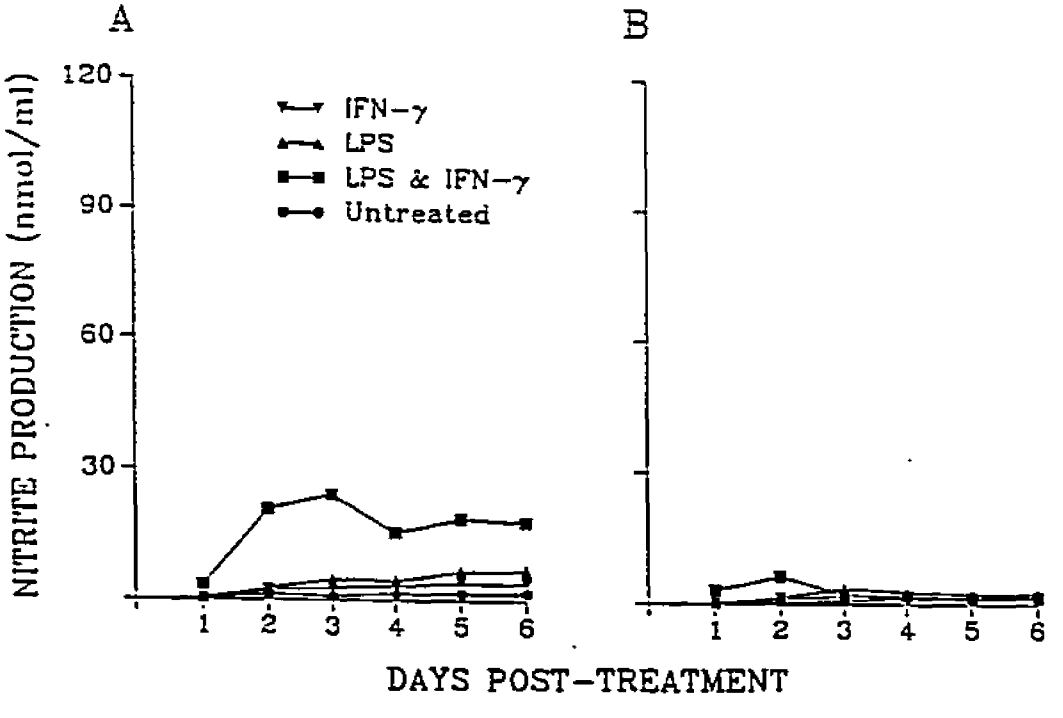


Figure 14

Uninfected RAW264.7, J774A.1 and McCoy cells were treated with LPS (0.001, 0.01, 0.1, 0.5, 1 or 10 μg/ml). At 48 hours pt, nitrite production was determined by the Griess reagent. Data presented are mean of triplicate $\pm$ S.D..

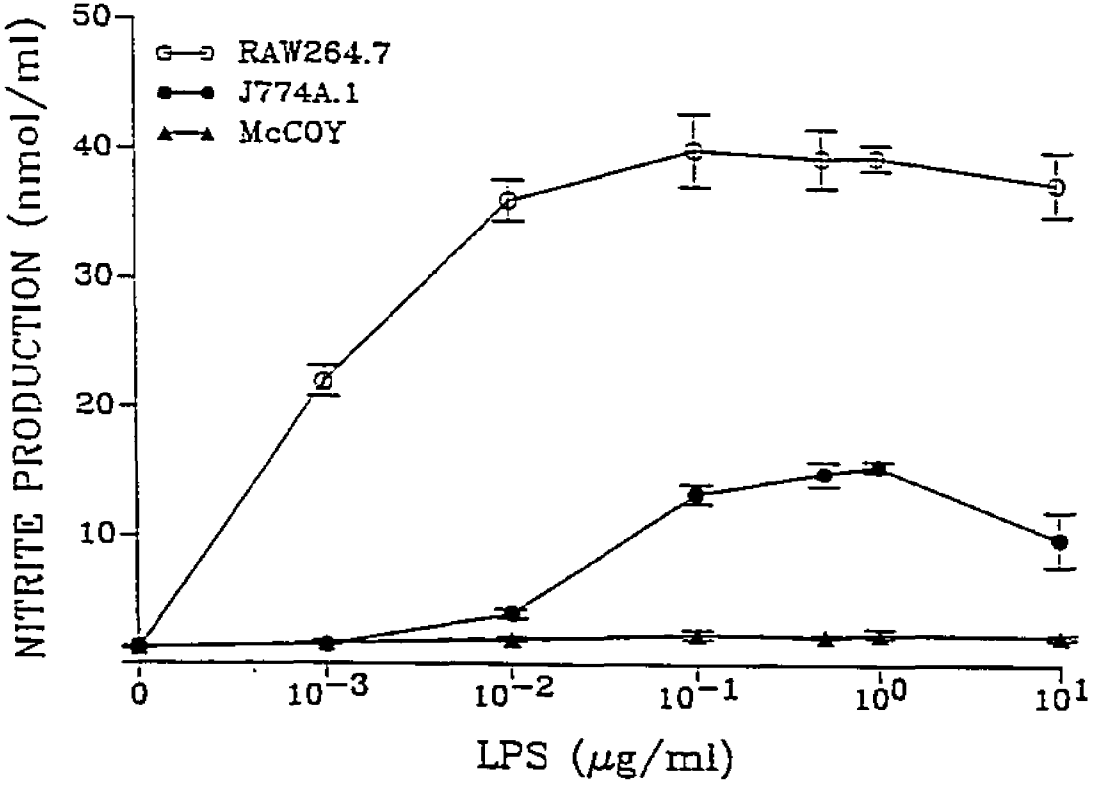
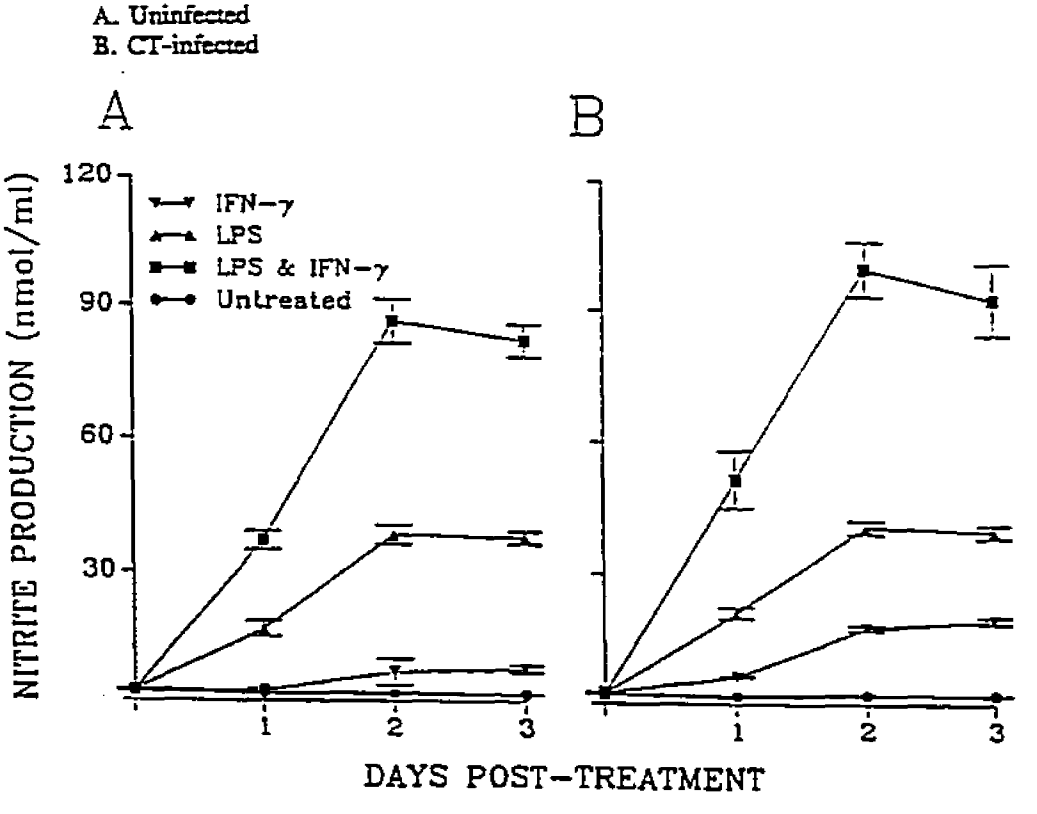


Figure 15

Comparison of nitrite production in CT-infected and uninfected RAW264.7 cells. Uninfected or CT-infected (MOI=1) RAW264.7 cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. Nitrite production was determined by the Griess reagent. Data presented are mean of triplicate ± S.D..



McCoy cells also produced more nitrite (4.1 nmol/ml) than uninfected cells (2.0 nmol/ml) following treated with IFN-γ (Figure 16). Nitrite levels were the same in CT-infected and uninfected cells treated with LPS or LPS plus IFN-γ.

Correlation of Nitrite Production and CT Inhibition

RAW264.7 cells. A high level of CT replication in the absence of nitrite production was observed in untreated RAW264.7 cells (Figure 17A). IFN-γ treatment induced a high level of nitrite production (17.64 ± 0.511 nm/ml) and inhibition of CT replication (0.6 log reduction). LPS treatment induced a higher level of nitrite production (40.77 ± 1.598) and a 2 log (99%) inhibition of CT replication. The highest levels of nitrite production (99.96 ± 6.376) and inhibition of CT replication (2.4 logs) were observed in LPS- plus IFN-γ-treated cells. Linear regression analysis demonstrated a strong correlation between nitrite production and CT inhibition (correlation coefficient: -0.93, $P<0.001$, Figure 17A).

J774A.1 cells. A strong correlation (correlation coefficient: -0.94, $P<0.001$) also was observed between nitrite production and CT inhibition in J774A.1 cells (Figure 17B). The slope of the regression curve was slightly greater in J774A.1 cells (2.453) than in RAW264.7 cells (2.061).

Figure 16

Comparison of nitrite production in CT-infected and uninfected McCoy cells. Uninfected or CT-infected (MOI=1) McCoy cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. Nitrite production was determined by the Griess reagent. Data presented are mean of triplicate ± S.D..

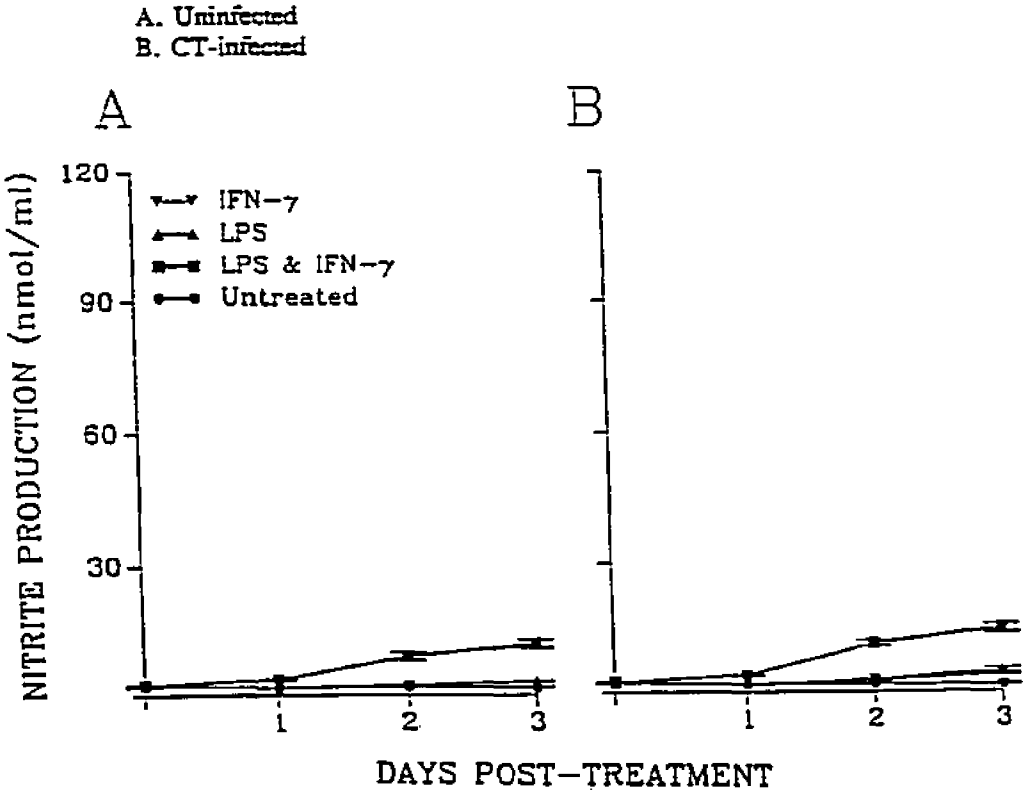
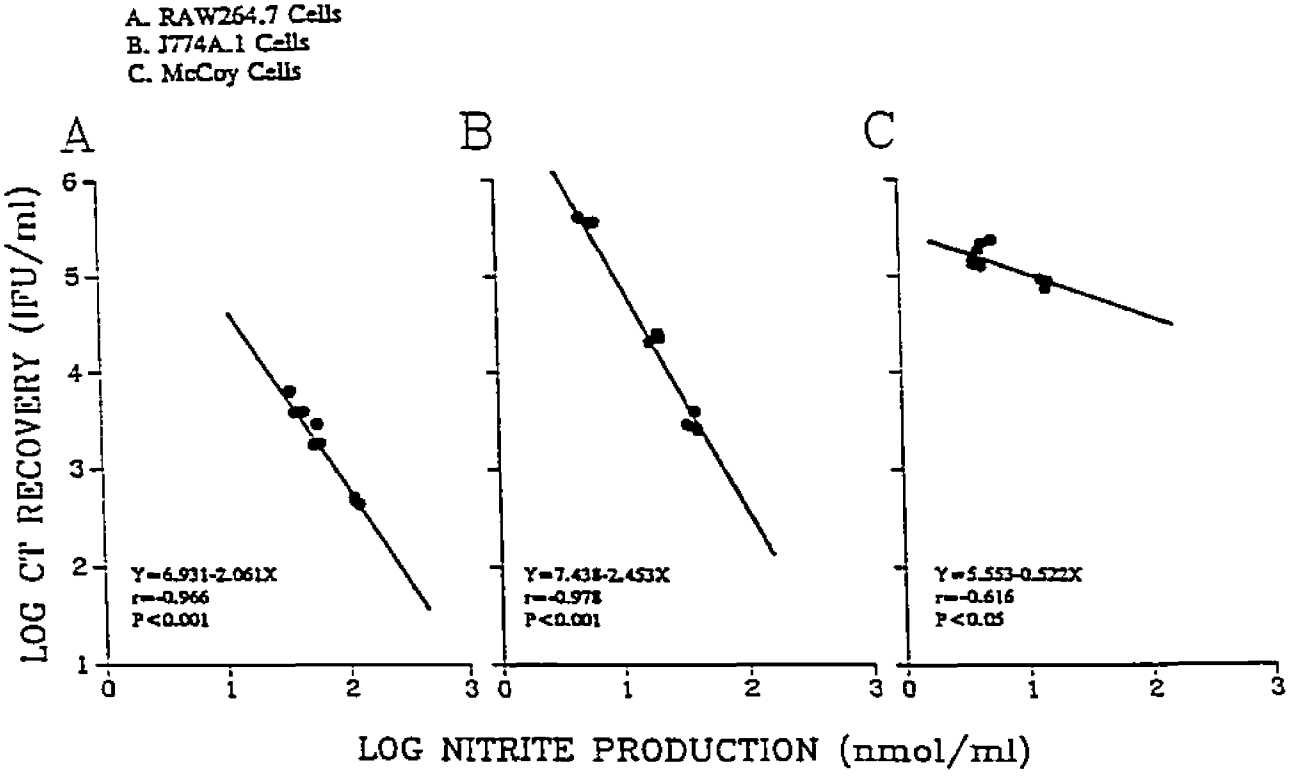


Figure 17

Correlation of nitrite production and CT replication. RAW264.7, J774A.1 and McCoy cells were inoculated with CT (MOI=2). At 24 hours pi, infected cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. Nitrite production and CT recovery were determined at 48 hours pt. Regression analysis of nitrite production and CT recovery was performed.



McCoy cells. Figure 17C shows the correlation of nitrite production and CT inhibition in McCoy Cells (correlation coefficient: -0.67, $P<0.05$). The slope of the regression curve was small (0.522).

Dose-dependent Effect of L-NMMA upon Inhibition of Nitrite Production

Since a combination of LPS and IFN-γ induced the highest level of nitrite production in RAW264.7 cells, this combination was chosen to titrate the effect of L-NMMA upon nitrite production. At a concentration as low as 0.01 mM, L-NMMA exhibited an inhibitory effect upon nitrite production (Figure 18). As the concentration was increased from 0.01 to 10 mM, inhibition was increased proportionally. For example, 1 mM L-NMMA inhibited 63-75% of nitrite production and 10 mM L-NMMA inhibited 93-97% of nitrite production. The L-NMMA dose curves at 24, 48 and 72 hours paralleled one another.

Inhibition of Nitrite Production by L-NMMA in Uninfected Cells

Since 1 mM L-NMMA was shown to inhibit nitrite production in LPS- and IFN-γ-treated RAW264.7 cells (Figure 18), this concentration was used to test inhibition of nitrite production in uninfected RAW264.7, J774A.1 and McCoy cells treated with LPS or IFN-γ.

L-NMMA (1 mM) inhibited nitrite production by 74 or 85% at 24 hr pt in RAW264.7 cells treated with LPS or IFN-γ (Figure 19). Inhibition of nitrite

Figure 18

Dose-dependent effect of L-NMMA upon inhibition of nitrite production in RAW264.7 cells treated with IFN-γ and LPS. Uninfected RAW264.7 cells were treated with IFN-γ (100 U/ml) and LPS (100 ng/ml). At the time of treatment, L-NMMA (0.001-10 mM) was added. Nitrite production was measured at 24, 48 and 72 hours pt. Data presented are mean of triplicate ± S.D..

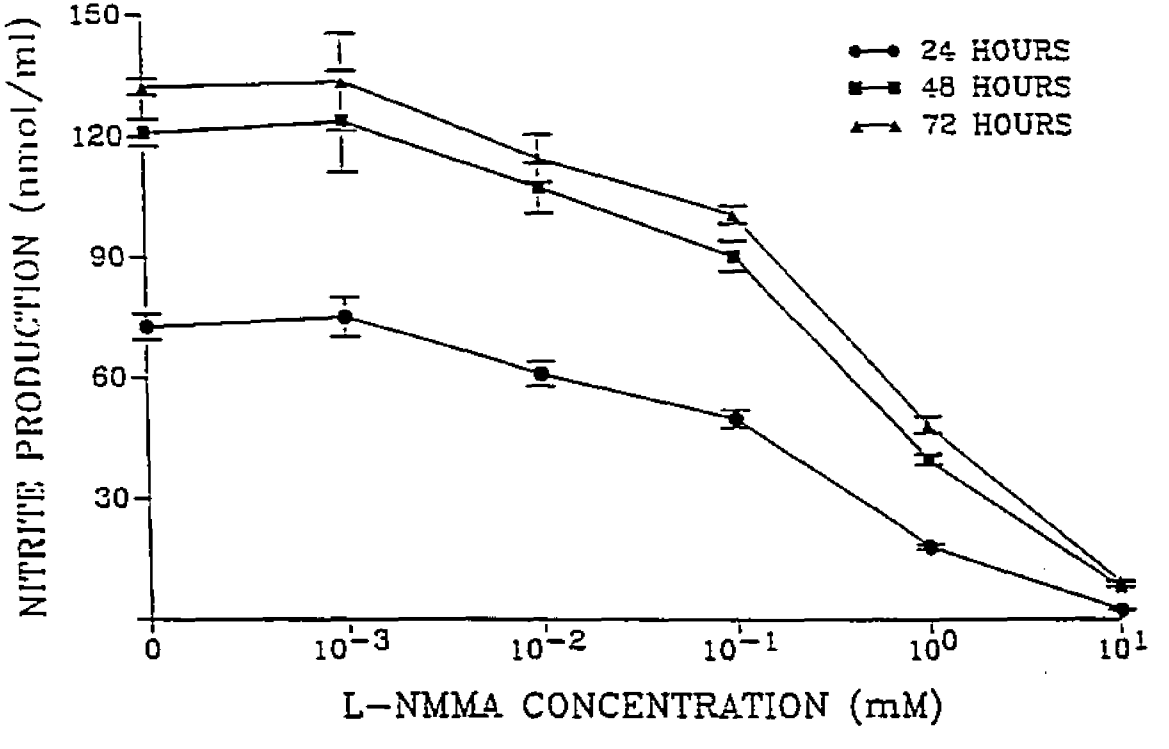
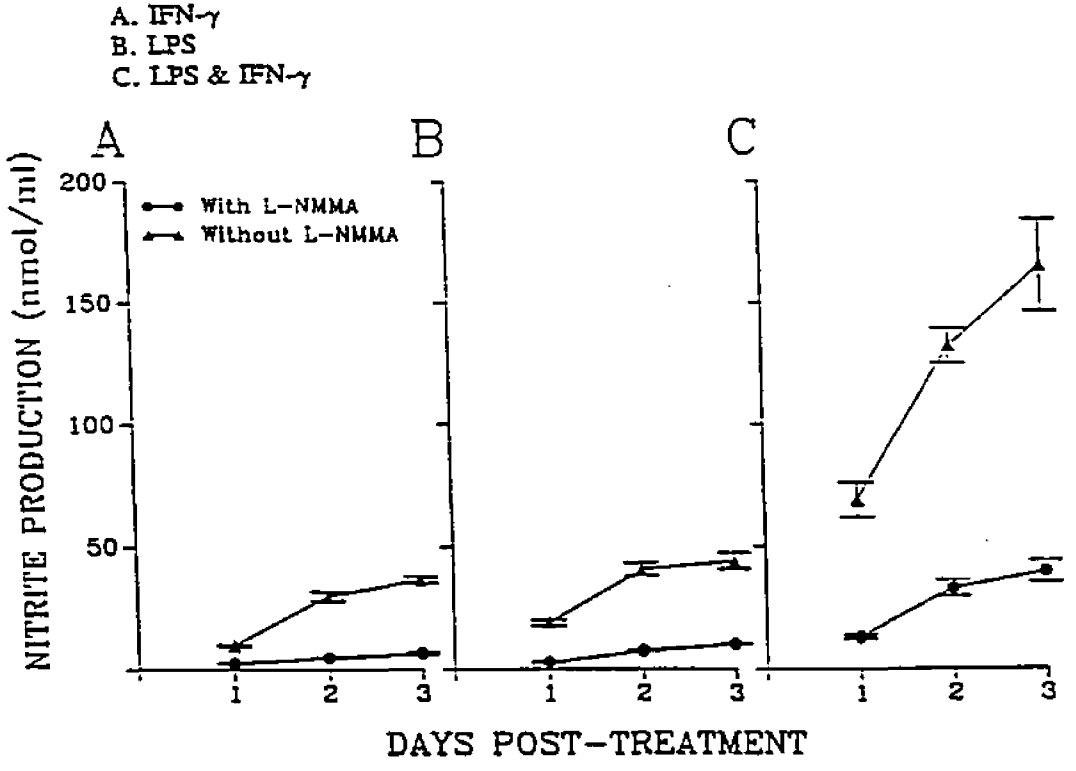


Figure 19

Inhibition of nitrite production by L-NMMA in uninfected RAW264.7 cells. Uninfected RAW264.7 cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At the time of treatment, L-NMMA (1 mM) was added. Nitrite production was measured at days 1, 2 and 3. Data presented are mean of triplicate ± S.D..



production at 48 or 72 hr pt (77 or 84%) was similar to that at 24 hr pt. Inhibition of nitrite production induced by L-NMMA in RAW264.7 cells treated with LPS and IFN-γ was 81%, 75% and 76% at 24, 48 and 72 hr pt, respectively (Figure 19).

In J774A.1 cells, IFN-γ induced a minimal level of nitrite production. L-NMMA did not significantly affect such low levels of nitrite production (Figure 20). LPS induced 8 and 13 nmol/ml of nitrite at 2 and 3 days pt, respectively. L-NMMA reduced this production to 3 and 4 nmol/ml (Figure 20). Inhibition of nitrite production induced by L-NMMA (1 mM) in LPS- and IFN-γ-treated J774A.1 cells was 56 and 73% at days 2 and 3, respectively (Figure 20).

In McCoy cells, LPS or IFN-γ induced negligible levels of nitrite production at days 1 and 2 pt. L-NMMA (1 mM) did not significantly affect this production (Figure 21). At day 3 pt, LPS or IFN-γ induced 6.3 or 4.4 nmol/ml of nitrite, respectively. L-NMMA reduced this production to 2.3 nmol/ml. Inhibition of nitrite production induced by L-NMMA in LPS- and IFN-γ-treated McCoy cells was 85 and 86% at 48 and 72 hours, respectively (Figure 21).

Inhibition of CT Infection by rTNF-α in McCoy Cells

To investigate the direct effect of TNF-α upon CT infection, rTNF-α was used to treat McCoy cells before or after inoculation. TNF-α treatment after CT inoculation significantly inhibited initial inclusion formation and CT replication (Table 3). TNF-α treatment caused development of atypical inclusions which contained unusually low levels of glycogen. The latter were not detected when stained by

Figure 20

Inhibition of nitrite production by L-NMMA in uninfected J774A.1 cells. Uninfected J774A.1 cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At the time of treatment, L-NMMA (1 mM) was added. Nitrite production was measured at days 1, 2 and 3. Data presented are mean of triplicate ± S.D..

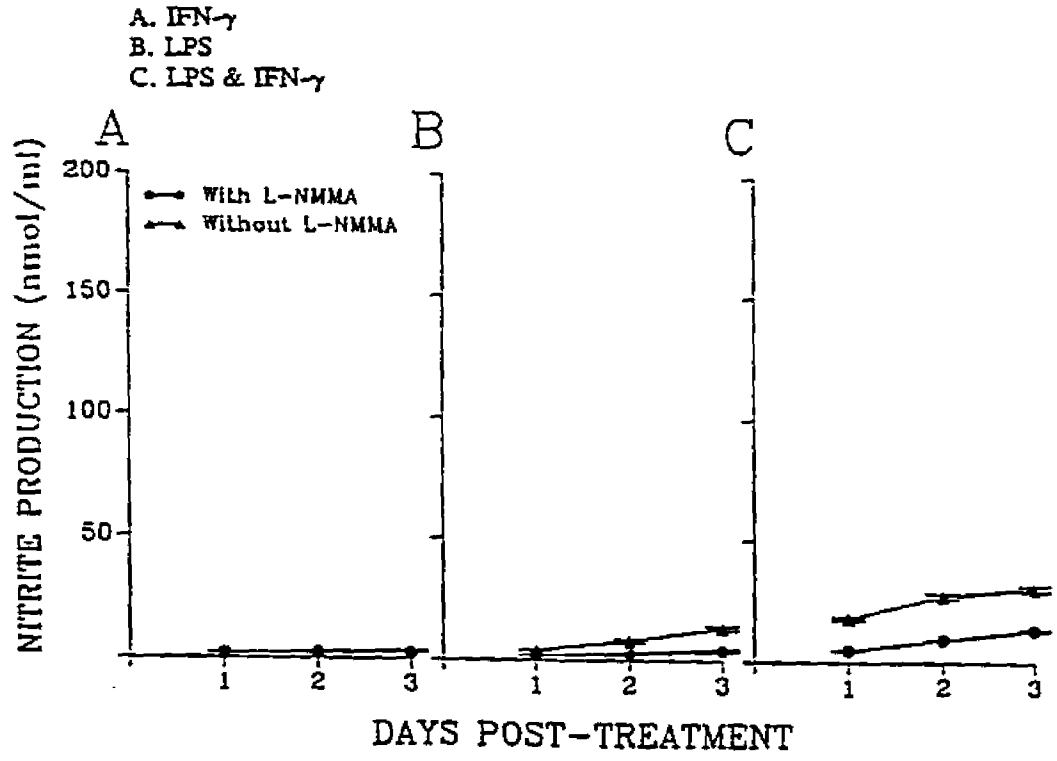


Figure 21

Inhibition of nitrite production by L-NMMA in uninfected McCoy cells. Uninfected McCoy cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At the time of treatment, L-NMMA (1 mM) was added. Nitrite production was measured at days 1, 2 and 3. Data presented are mean of triplicate ± S.D..

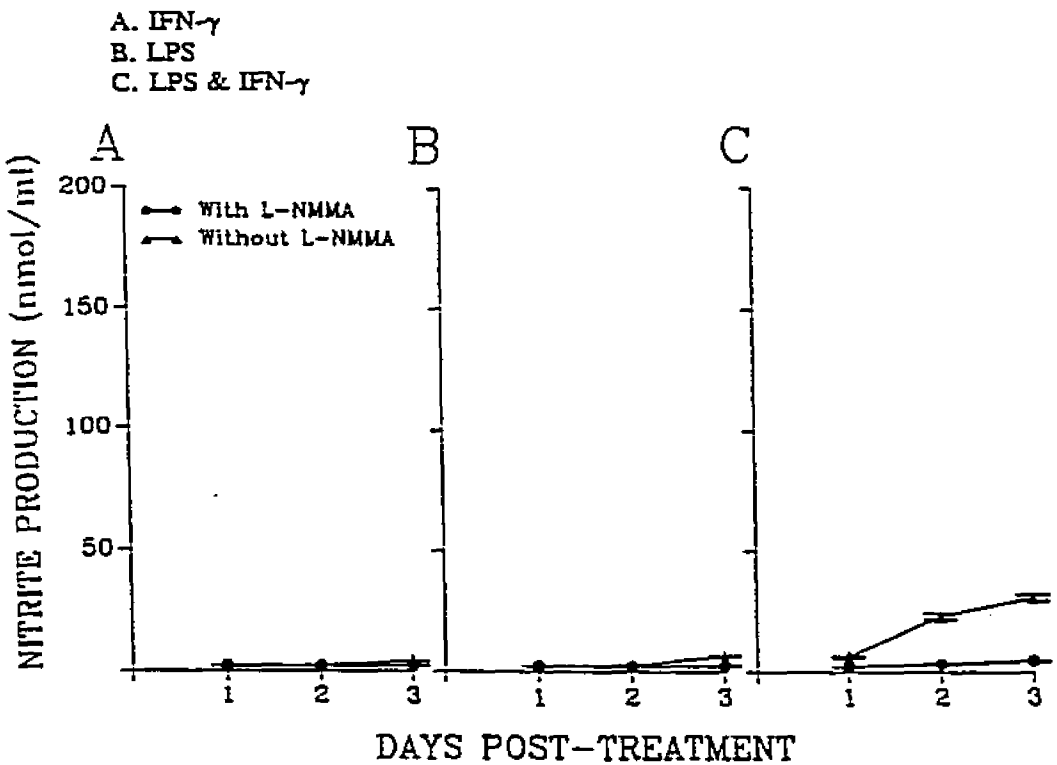


Table 3

EFFECT OF TNFα UPON CT REPLICATION IN MCCOY CELLS

TNF-α	Post-inoc.		Initial Inclusions		Recovery	Yield*
Pretreat	TNFα	Cyclohx.	Iodine	MAb-MOMP	(IFU/ml)	IFU/Inclusion
+	–	–	585±12	2,092±221	54,933±5,119***	26.3
–	+	–	100±9.9*	1,249±265**	13,500±3,984***	10.8
–	–	–	659±120	2,310±88	139,000±23,388	60.2
–	–	+	4,558±530	3,076±736	815,667±178,240	265.2

CT-infected McCoy cells (MOI=0.006) were treated with TNF-α (250 ng/ml) 24 hours before inoculation or immediately after inoculation. Initial inclusions were determined by iodine or anti-MOMP stain at 48 hours pi. Concomitantly, CT recovery was quantitated in susceptible McCoy cells. Untreated McCoy cells with or without cycloheximide (2 μg/ml) served as controls. Data (duplicate) presented are mean ± S.D.. * Yield computed from number of inclusions enumerated by FITC-anti-MOMP.
** $p<0.05$, *** $p<0.01$ determined by Student's t test.

iodine, but were confirmed by staining with FITC-conjugated anti-MOMP antibody. The inclusions in cells treated with TNF-α produced abnormally low numbers of infectious EBs (10.8 IFU/inclusion) that is, the yield was lower than that in untreated cells (60.2 IFU/inclusion). Pretreatment of McCoy cells with TNF-α for 24 hr before inoculation inhibited CT replication, but did not inhibit initial inclusion formation (Table 3).

L-NMMA Did not Reduce TNF-α Production

Since TNF-α inhibited CT replication (Table 3) and LPS also is a strong stimulator of TNF-α production (Jue et al. 1990), the effect of L-NMMA upon TNF-α production was determined. No detectable TNF-α production was observed in untreated cells (data not shown). IFN-γ induced a very low level of TNF-α production in uninfected RAW264.7 cells (Figure 22A), but no detectable TNF-α in J774A.1 cells (Figure 23A). LPS or LPS plus IFN-γ induced a high level of TNF-α production in J774A.1 cells (Figure 23B-C) and even higher levels in RAW264.7 cells (Figure 22B-C). Peak production occurred at approximately 6 hours pt (data not shown) and remained in culture fluids for 72 hours. L-NMMA did not inhibit TNF-α production. Rather, it somehow slightly increased TNF-α production in macrophage cell lines treated with LPS or LPS plus IFN-γ (Figure 22B-C, 23B-C). In McCoy cells, LPS or LPS plus IFN-γ did not induce TNF-α production (data not shown). Therefore, it was not necessary to determine the inhibitory effect of L-NMMA upon TNF-α production in McCoy cells.

Figure 22

Effect of L-NMMA upon TNF-α production in uninfected RAW264.7 cells. Uninfected RAW264.7 cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At the time of treatment, L-NMMA (1 mM) was added. TNF-α production was measured at 6, 24, 48 and 72 hours pt. Data presented are mean of triplicate ± S.D..

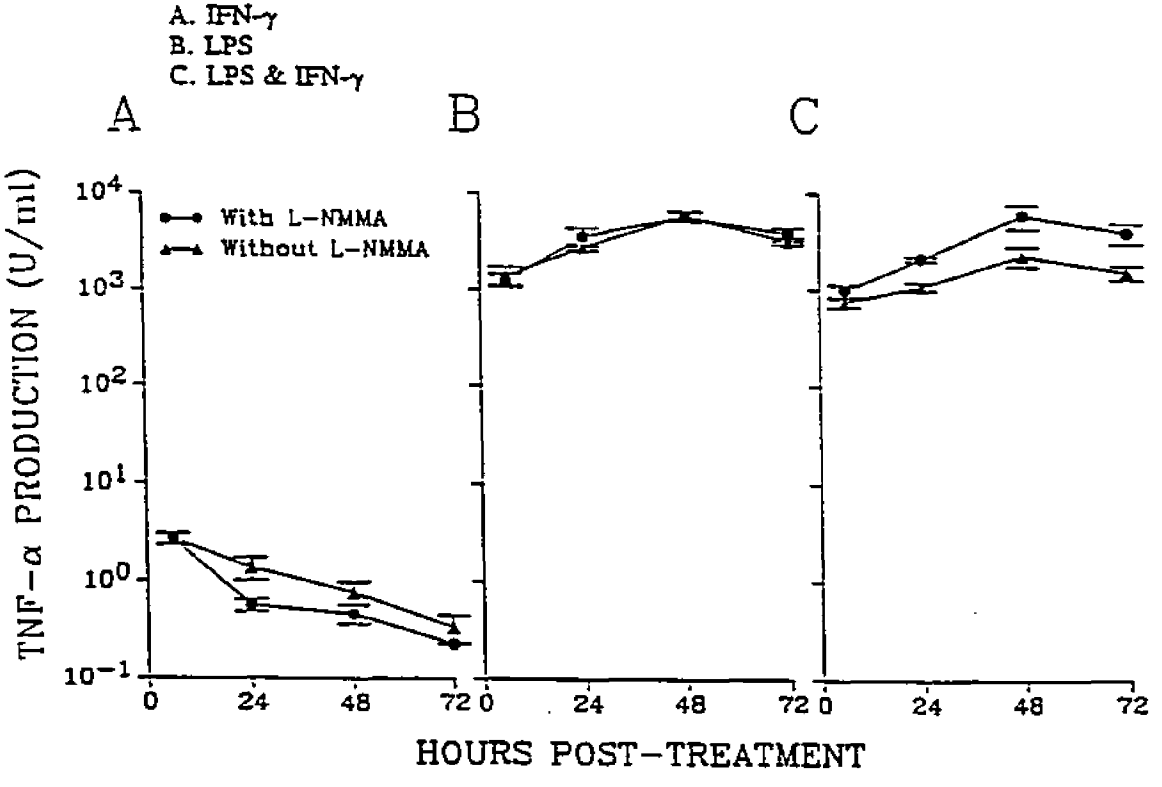
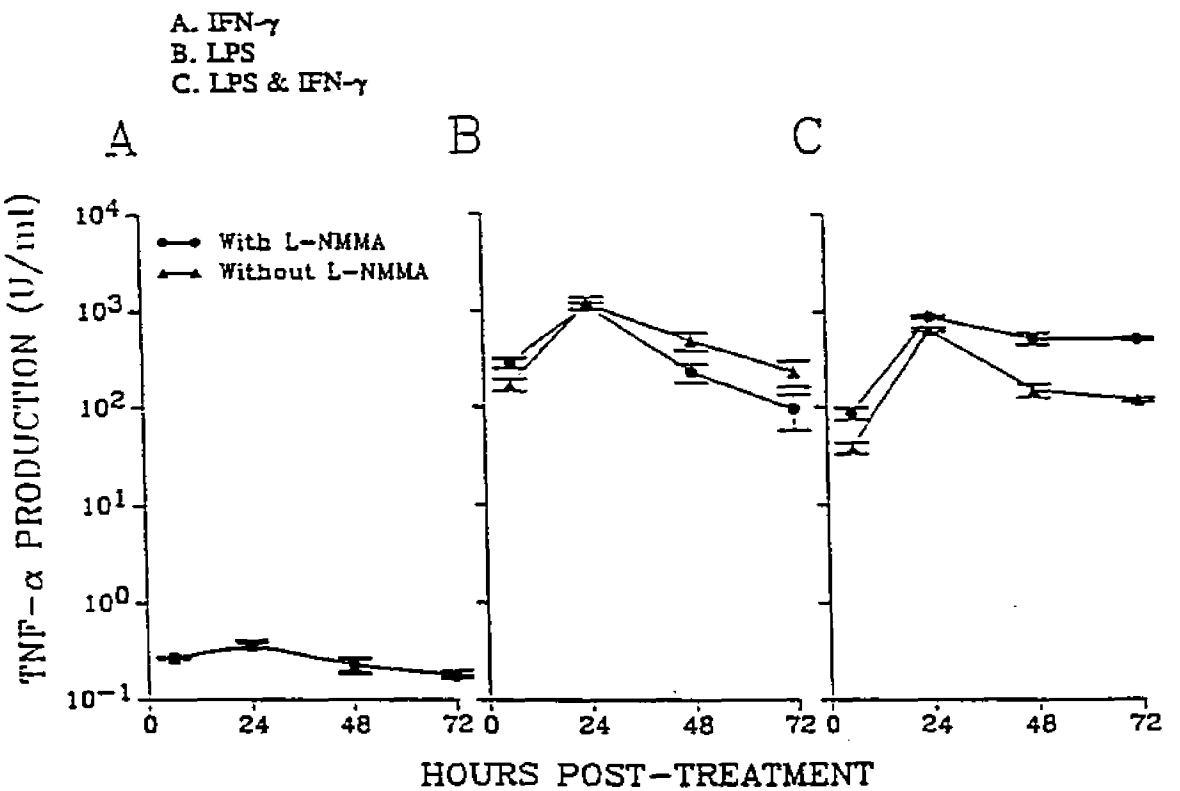


Figure 23

Effect of L-NMMA upon TNF-α production in uninfected J774A.1 cells. Uninfected J774A.1 cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At the time of treatment, L-NMMA (1 mM) was added. TNF-α production was measured at 6, 24, 48 and 72 hours pt. Data presented are mean of triplicate ± S.D..



Correlation between Inhibition of Nitrite Production and Abrogation of CT Inhibition

The strong correlation between nitrite production and CT inhibition led us to invoke the hypothesis that nitrite production was a mechanism of CT inhibition. In order to test this hypothesis, a series of concentrations of L-NMMA (from 0.1 to 10 mM) was used to determine the correlation between inhibition of nitrite production and abrogation of CT inhibition. As expected, in the absence of L-NMMA, IFN-γ and/or LPS induced high levels of nitrite production (Figure 24) and inhibition of CT replication in RAW264.7 cells (Figure 25). L-NMMA dramatically inhibited nitrite production (Figure 24) and remarkably abrogated the inhibition of CT replication in a dose-dependent pattern (Figure 25). However, as the concentration of L-NMMA was increased from 5 mM to 10 mM, restoration of CT replication was not increased. Although 10 mM L-NMMA almost completely inhibited nitrite production, restoration of CT replication was only 71% in IFN-γ-treated RAW264.7 cells and 55% in LPS-treated cells (Figures 24, 25). Furthermore, restoration of CT replication induced by L-NMMA in LPS-treated RAW264.7 cells was less than that in IFN-γ-treated cells.

Similar results were observed with CT-infected J774A.1 cells. As expected, IFN-γ or LPS induced nitrite production (Figure 26) and partial inhibition of CT replication by 0.7 and 1.9 logs, respectively (Figure 27). L-NMMA dramatically inhibited nitrite production (Figure 26) and significantly abrogated inhibition of CT replication (Figure 27) in a dose-dependent pattern. L-NMMA (5 mM) increased CT recovery by 0.9 logs and 1.4 logs in IFN-γ or IFN-γ plus LPS-treated J774A.1 cells,

Figure 24

Inhibition of nitrite production by L-NMMA in CT-infected RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, L-NMMA (0.1, 1, 5 or 10 mM) was added. Nitrite production was measured at 48 hours pt. Data presented are mean of triplicate ± S.D..

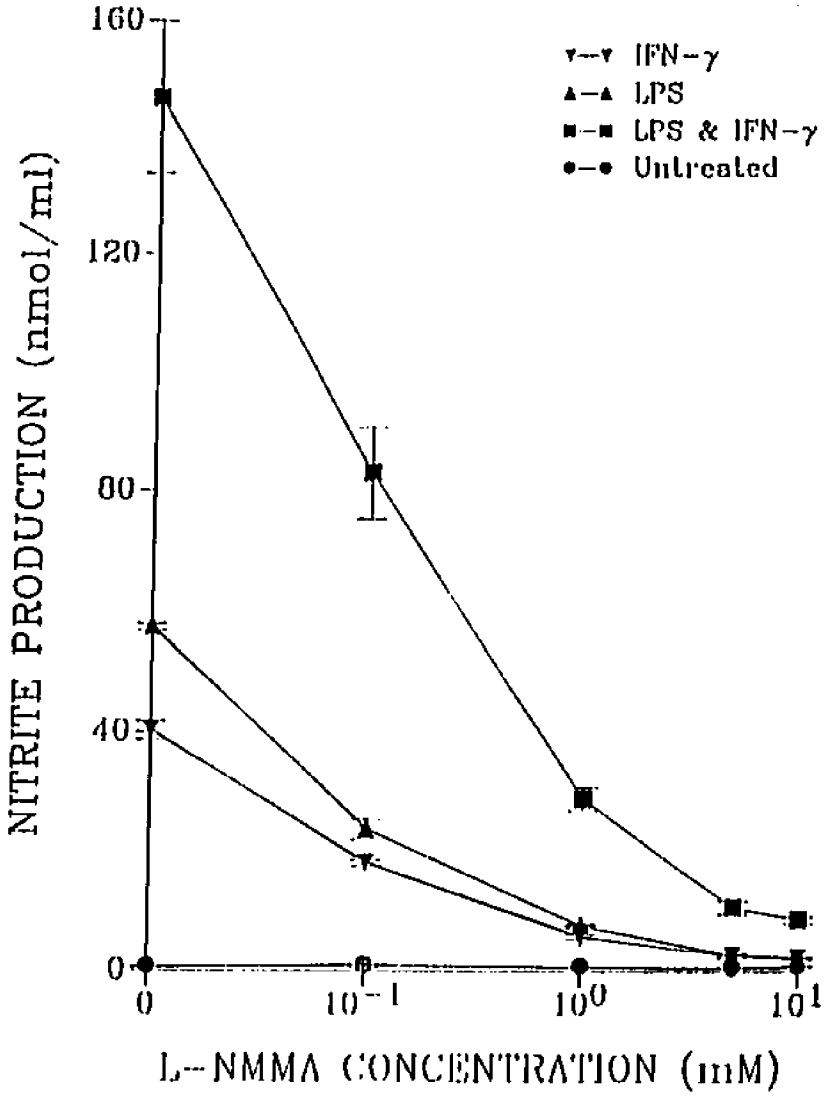


Figure 25

Abrogation of CT inhibition by L-NMMA in RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated as described in Figure 24. At 48 hours pt, CT recovery was quantitated. Data presented are mean of triplicate ± S.D..

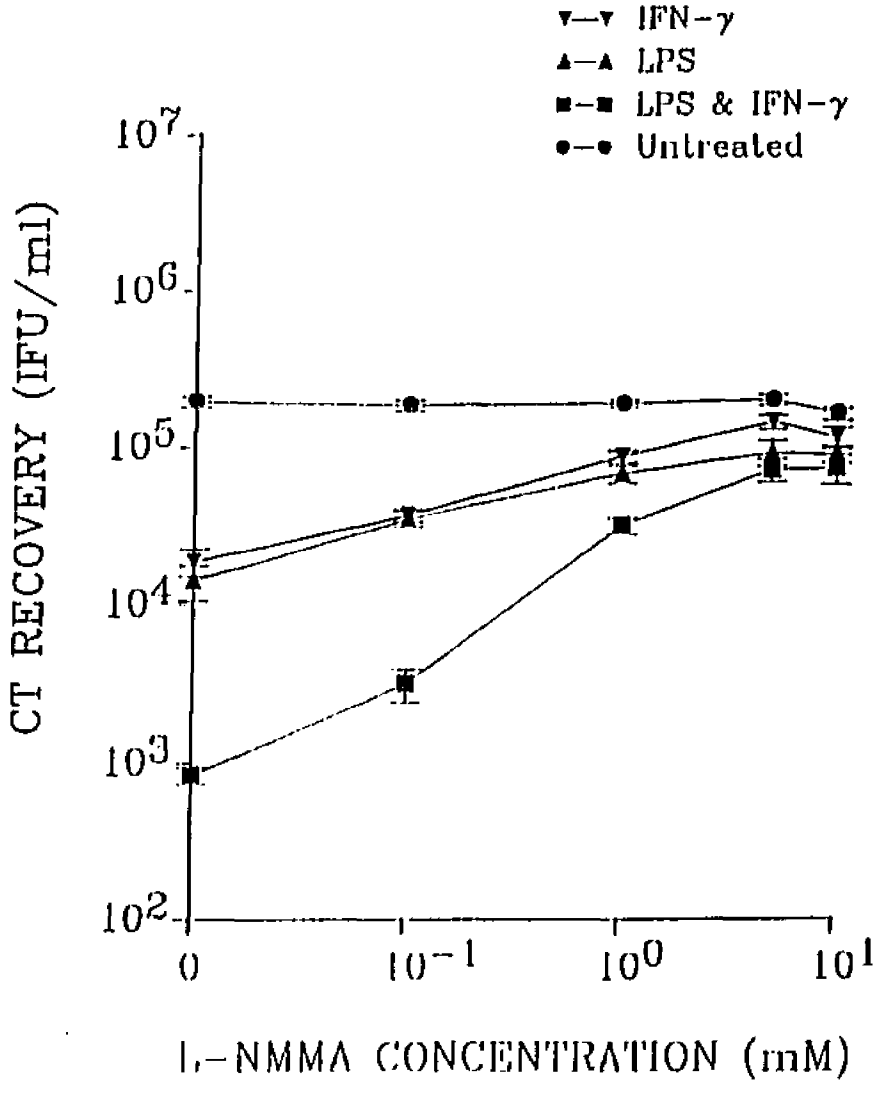


Figure 26

Inhibition of nitrite production by L-NMMA in CT-infected J774A.1 cells. CT-inoculated J774A.1 cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, L-NMMA (0.1, 1 or 5 mM) was added. Nitrite production was measured at 48 hours pt. Data presented are mean of triplicate ± S.D..

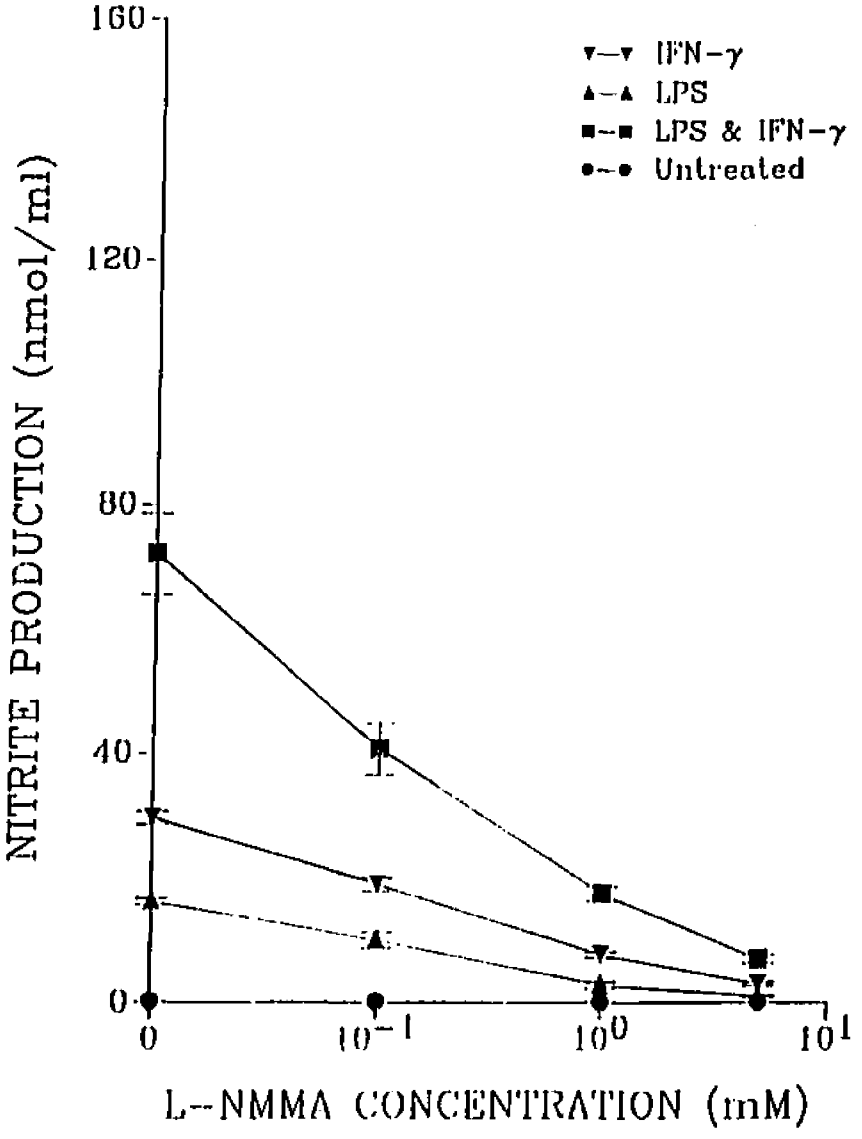
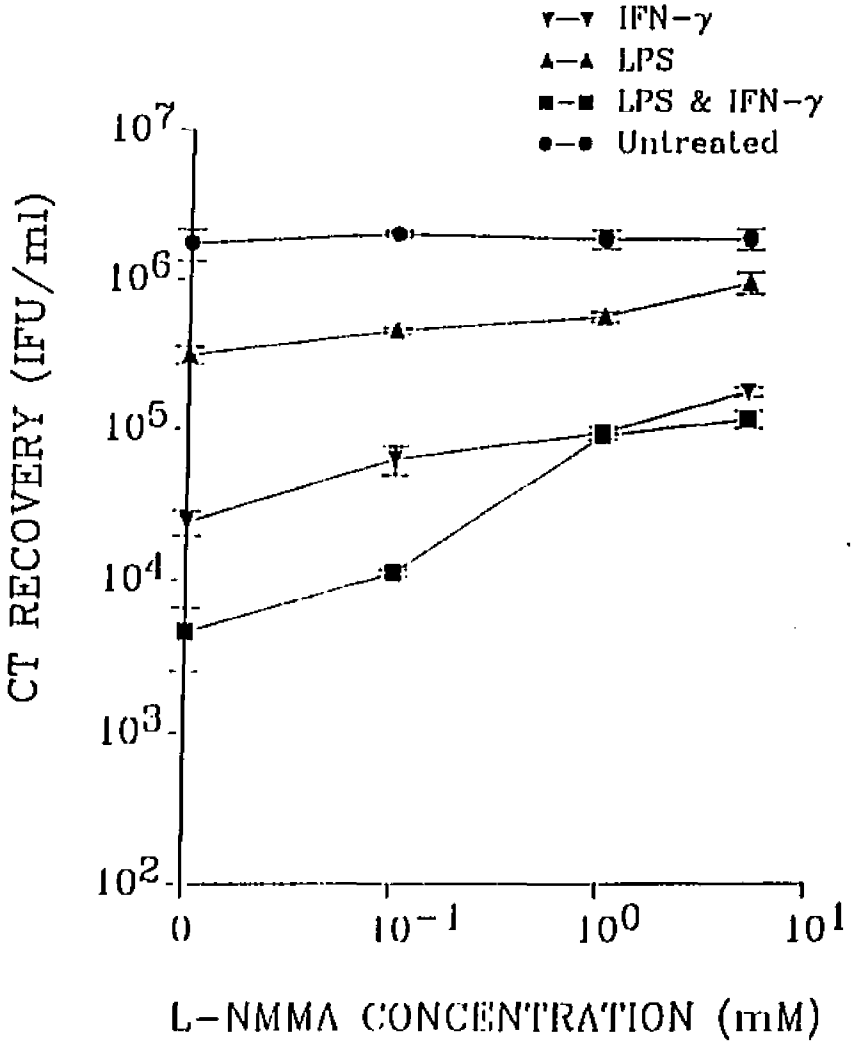


Figure 27

Abrogation of CT inhibition by L-NMMA in J774A.1 cells. CT-inoculated J774A.1 cells (MOI=1) were treated as described in Figure 26. At 48 hours pt, CT recovery was quantitated. Data presented are mean of triplicate ± S.D..



respectively. However, this increased recovery approached the untreated control by only 9.5% and 6.3%. In contrast, restoration induced by L-NMMA in LPS-treated cells (5 mM) was 52% (Figures 26, 27).

In CT-infected McCoy cells, neither significant nitrite production nor CT inhibition was observed by treatment with LPS (Figures 28-29). IFN-γ or IFN-γ plus LPS induced low levels of nitrite production (Figure 28) and concomitant inhibition of CT replication by 1.3 and 1.4 logs IFU/ml, respectively (Figure 29). L-NMMA significantly increased CT replication by 1.5- or twofold ($p < 0.05$ determined by Student's t test) in IFN-γ- or IFN-γ plus LPS-treated cells. However, restoration of CT replication was only 7-9%. Furthermore, as the concentration of L-NMMA was increased from 0.1 to 5 mM, no further increase in CT replication was observed in these cells (Figure 29), although inhibition of nitrite production continued to increase. In a separate but similar experiment, IFN-γ or IFN-γ plus LPS induced lower nitrite production and less inhibition of CT replication in McCoy cells (data not shown). L-NMMA did not affect CT replication in the cells treated with IFN-γ or IFN-γ plus LPS (data not shown).

Nitrite Did Not Inhibit CT Replication in RAW264.7 Cells

Since our previous results suggested that nitrite production may be a mechanism for inhibition of CT replication in RAW264.7 cells, it was necessary to determine if nitrite served as the effector molecule. CT-infected RAW264.7 cells were treated with nitrite (concentrations from 50 to 200 nmol/ml). This treatment

Figure 28

Inhibition of nitrite production by L-NMMA in CT-infected McCoy cells. CT-inoculated McCoy cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, L-NMMA (0.1, 1 or 5 mM) was added. Nitrite production was measured at 48 hours pt. Data presented are mean of triplicate ± S.D..

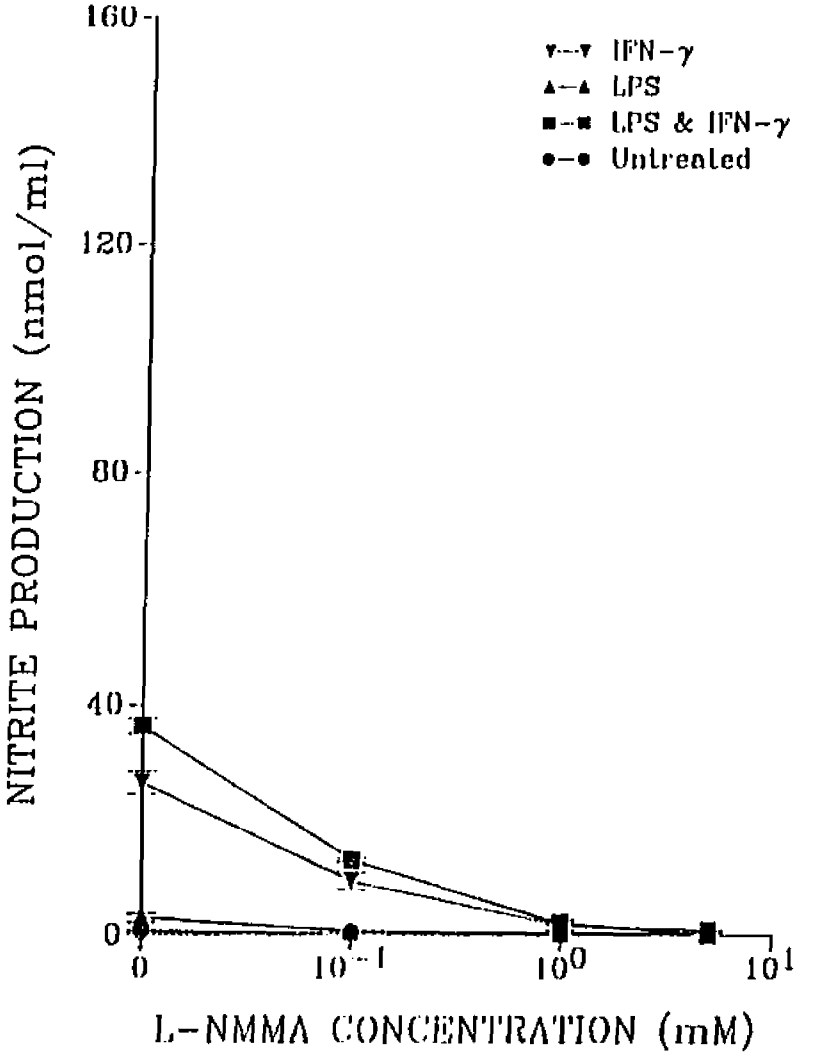


Figure 29

Abrogation of CT inhibition by L-NMMA in McCoy cells. CT-inoculated McCoy cells (MOI=1) were treated as described in Figure 28. At 48 hours pt, CT recovery was quantitated. Data presented are mean of triplicate ± S.D..

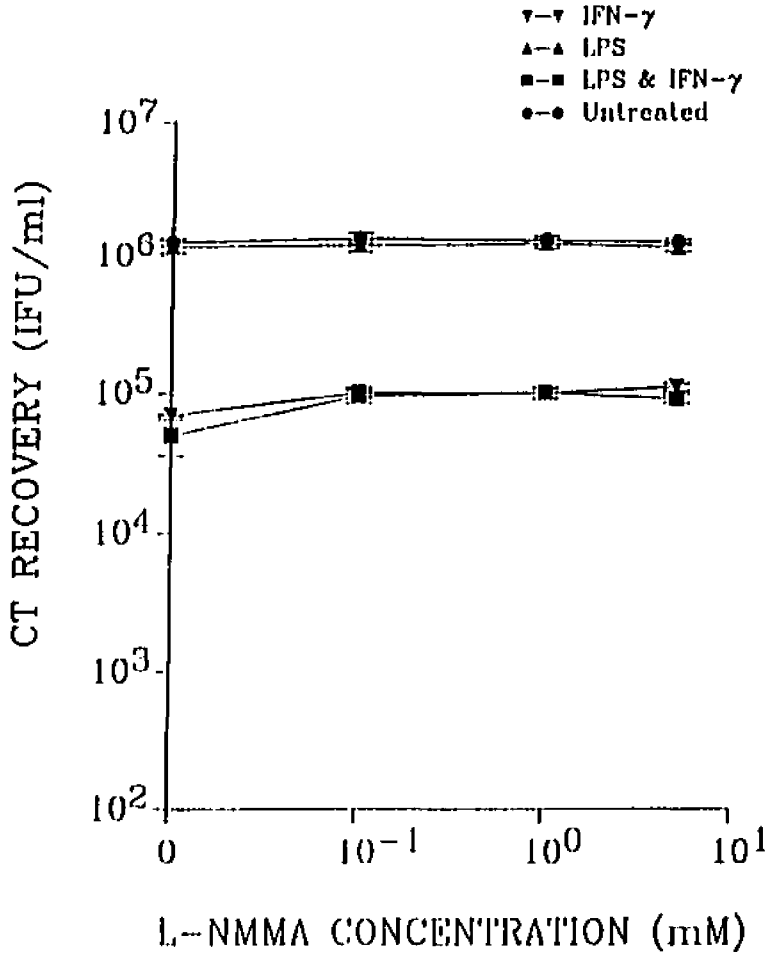
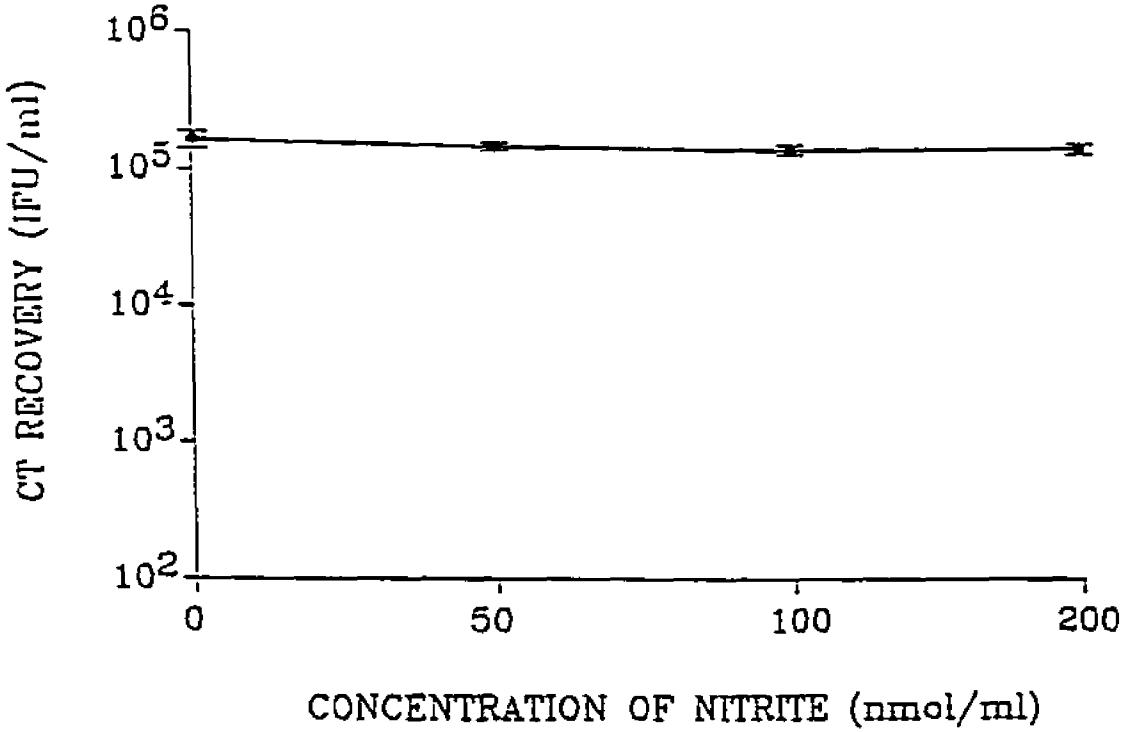


Figure 30

Effect of nitrite upon CT replication in RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated with sodium nitrite (50-200 nmol/ml) at 24 hours pi. CT recovery was quantitated at 48 hours pt. Data presented are mean of triplicate ± S.D..



neither affected TNF-α production (data not shown) nor inhibited CT replication (Figure 30), indicating that nitrite probably is not an effector molecule involved in inhibition of CT replication.

Kinetics of NOS mRNA Expression in RAW264.7 Cells

NO is a mediator of nitrite production but is difficult to measure because of its short half life (several seconds). Therefore, NOS mRNA was measured by Northern blot hybridization with a NOS-cDNA probe. Total RNA was isolated from uninfected or CT-infected RAW264.7 cells which had been treated with LPS and/or IFN-γ. Figure 31 shows total RNA following electrophoresis on a formaldehyde-denaturing agarose gel (1%) stained with ethidium bromide. Similar bands of 18S and 28S rRNA were exhibited in all samples isolated from uninfected, infected and treated cells. Figure 32 shows the results of a Northern blot hybridization. No detectable NOS mRNA expression was observed in uninfected or CT-infected cells which had not been treated (Lanes a, b). In CT-infected RAW264.7 cells, IFN-γ (Lanes c, g & k), LPS (Lanes d, h & l) or IFN-γ plus LPS (Lanes e, i & m) induced low, intermediate and high levels of NOS mRNA expression, respectively. Anti-TNF-α antibody did not reduce NOS mRNA expression induced by LPS at any of the time points tested (Lanes f, j & n). Peak expression occurred at 6-24 hr pt (Lanes c-j). Expression of mRNA was diminished at 48 hours pt (Lanes k-n) in IFN-γ- and/or LPS-treated cells. Therefore, 6 hr was chosen as the time point to determine NOS mRNA expression in subsequent experiments.

Figure 31

Total RNA isolated from RAW264.7 cells. Lane a, uninfected; Lane b-n, CT-infected (MOI=1); Lane b, CT-infected alone (24 hours pi); Lanes c, g and k, IFN-γ-treated (100 U/ml); Lanes d, h and l, LPS-treated (100 ng/ml); Lanes e, i and m, IFN-γ- and LPS-treated; Lanes f, j and n, LPS- and anti-TNF-α Ab-treated; Lanes c, d, e and f, 6 hours pt; Lanes g, h, i and j, 24 hours pt; Lanes k, l, m and n, 48 hours pt. 10 μg of total RNA loaded onto each lane. Ethidium bromide-stained formaldehyde denaturing 1% agarose gel.

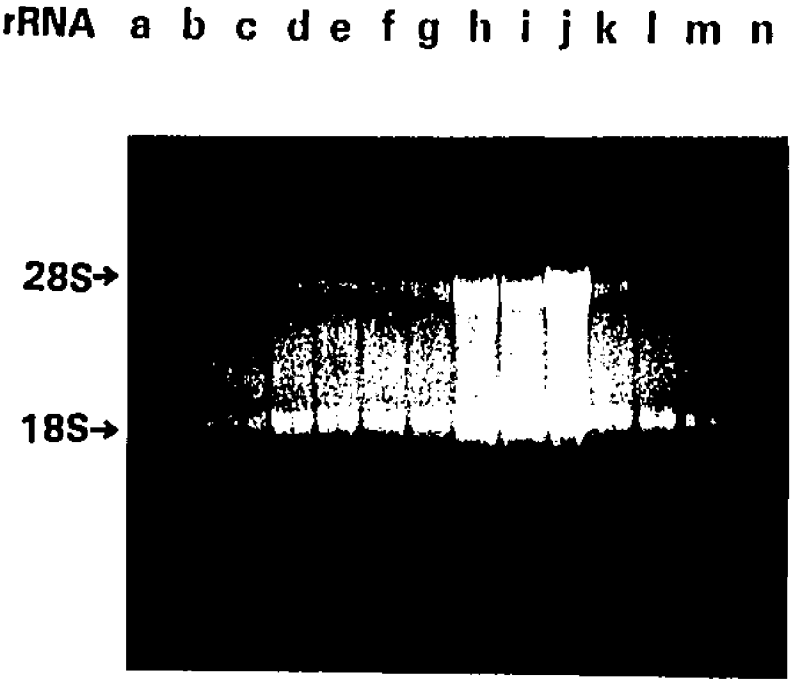


Figure 32

Kinetics of NOS mRNA expression in RAW264.7 cells. Lane a, uninfected; Lanes b-n, CT-infected (MOI=1); Lane b, CT-infected alone (24 hours pi); Lanes c, g and k, IFN-γ-treated (100 U/ml); Lanes d, h and l, LPS-treated (100 ng/ml); Lanes e, i and m, IFN-γ- and LPS-treated; Lanes f, j and n, LPS- (100 ng/ml) and anti-TNF-α Ab-treated; Lanes c, d, e and f, 6 hours pt; Lanes g, h, i and j, 24 hours pt; Lanes k, l, m and n, 48 hours pt. Total RNA (10 μg) was electrophoresed on formaldehyde denaturing 1% agarose gel, transferred onto Nytran plus nylon membrane, and hybridized with [^{32}P]-labeled NOS cDNA. XAR-5 X-ray film was exposured to this membrane for 24 hours. Size markers (in kilobases) are given at left.

Correlation between NOS mRNA Expression and CT Inhibition in RAW264.7 Cells

Total RNA was isolated from CT-infected RAW264.7 cells treated for 6 hours with LPS and/or IFN-γ. NOS mRNA expression was determined by Northern and dot blot hybridization. Both the Northern (Figure 33A) and the dot blot (Figure 34A) hybridizations showed that low, intermediate and high levels of NOS mRNA expression were induced by IFN-γ, LPS or IFN-γ plus LPS, respectively. CT infection alone (without IFN-γ or LPS) did not induce detectable NOS mRNA expression, which was consistent with the observed lack of nitrite production in these cells. Control hybridization with a β-actin cDNA probe showed that similar amounts of RNA from each sample were used (Figures 33B, 34B). Concomitantly, CT recovery at 48 hours pt was analyzed. As expected, CT replication was dramatically inhibited by LPS and/or IFN-γ treatment. Densities of NOS mRNA dots from the dot Blot hybridization was used for correlation analyses. Figure 35 shows a strong correlation between density of NOS mRNA dots and inhibition of CT replication (correlation coefficient: -0.97, $P<0.05$ determined by Linear Regression).

Effect of Anti-TNF-α Ab upon NOS mRNA Expression, Nitrite Production and CT Replication in RAW264.7 Cells

In order to explore the effects of TNF-α upon nitrite production and CT inhibition, polyclonal anti-TNF-α Ab (neutralizing Ab) was employed. As expected, IFN-γ, LPS or both induced nitrite levels in RAW264.7 cells of 47, 60 and 130

Figure 33A

Effect of anti-TNF-α antibody upon NOS mRNA expression in CT-infected RAW264.7 cells (MOI=1). Lanes a-d, untreated; Lanes e-h, IFN-γ-treated (100 U/ml); Lanes i-l, LPS-treated (100 ng/ml); Lanes m-p, IFN-γ- and LPS-treated; Lanes b, f, j & n, anti-TNF-α Ab-treated; Lanes c, g, k & o, L-NMMA-treated (1mM); Lanes d, h, l & p, L-NMMA- and anti-TNF-α Ab-treated. Total RNA (10 μg) was isolated at 6 hours pt, electrophoresed on formaldehyde denaturing 1% agarose gel, transferred onto Nytran plus nylon membrane, and hybridized with [^{32}P]-labeled NOS cDNA probe. XAR-5 X-ray film was exposured to the membrane for 24 hours. Size markers (in kilobases) are given at left.

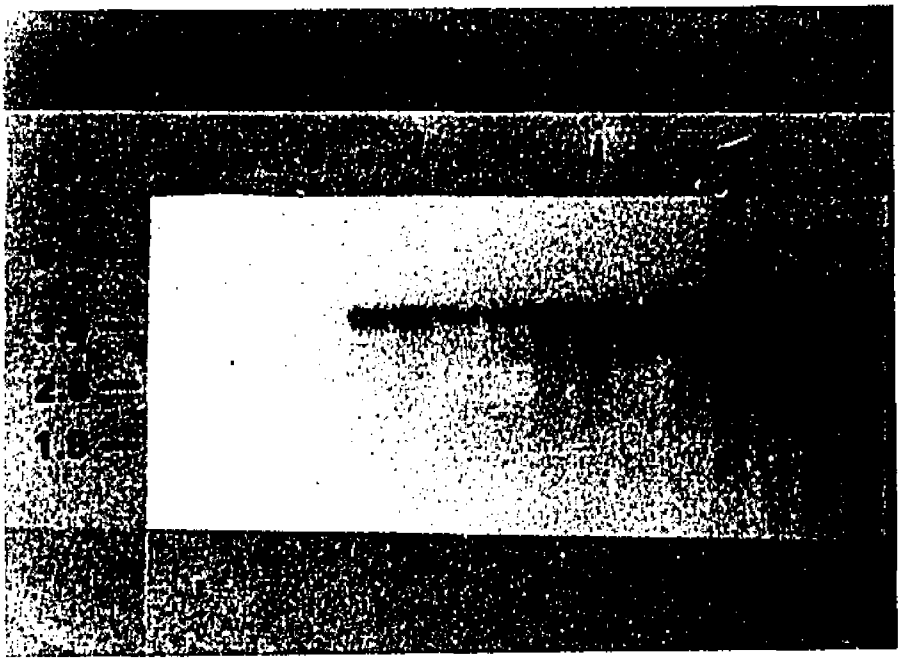


Figure 33B

Control hybridization by β-actin cDNA probe in CT-infected RAW264.7 cells (MOI=1). Lanes a-d, untreated; Lanes e-h, IFN-γ-treated (100 U/ml); Lanes i-l, LPS-treated (100 ng/ml); Lanes m-p, IFN-γ- and LPS-treated; Lanes b, f, j & n, anti-TNF-α Ab-treated; Lanes c, g, k & o, L-NMMA-treated (1mM); Lanes d, h, l & p, L-NMMA- and anti-TNF-α Ab-treated. The membrane used for hybridization with NOS cDNA probe in Figure 33A was washed and rehybridized with [^{32}P]-labeled β-actin cDNA probe. XAR-5 X-ray film was exposured to this membrane for 48 hours. Arrows at left indicate NOS mRNA and β-actin mRNA, respectively.

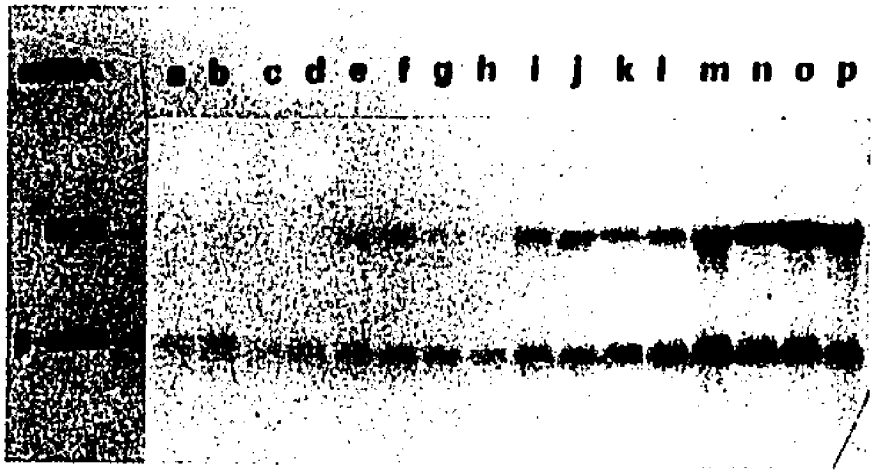


Figure 34A

Effect of anti-TNF-α antibody upon NOS mRNA expression in CT-infected RAW264.7 cells (MOI=1). A1-10, IFN-γ- (100 U/ml) and LPS-treated (100 ng/ml), total RNA 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, 0.008 μg, respectively; A11, 4 μg RNA (from IFN-γ- and LPS-treated cells) treated with RNase (40 μg); B1-4, untreated; B5-8, IFN-γ-treated (100 U/ml); C1-4, LPS-treated (100 ng/ml); C5-8, IFN-γ- and LPS-treated; B2, B6, C2 & C6, anti-TNF-α Ab-treated; B3, B7, C3 & C7, L-NMMA-treated (1mM); B4, B8, C4 & C8, L-NMMA- and anti-TNF-α Ab-treated. Total RNA (1 μg) was isolated at 6 hours pt, blotted onto Nytran plus nylon membrane and hybridized with [^{32}P]-labeled NOS cDNA probe. XAR-5 X-ray film was exposured to this membrane for 48 hours.

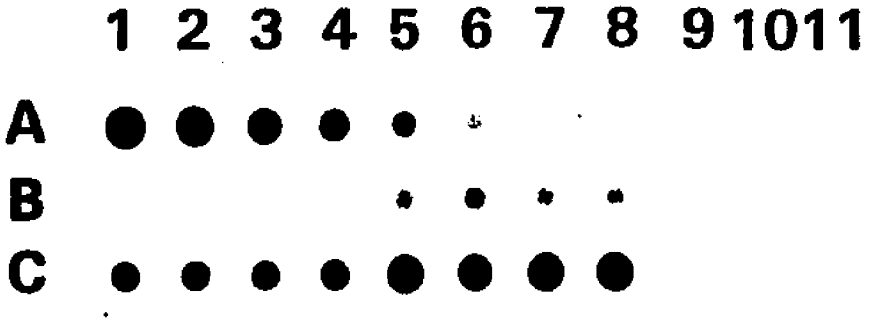


Figure 34B

Control Hybridization by β-Actin cDNA Probe in CT-infected RAW264.7 cells (MOI=1). A1-10, IFN-γ- (100 U/ml) and LPS-treated (100 ng/ml), total RNA 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, 0.008 μg, respectively; A11, 4 μg RNA (from IFN-γ- and LPS-treated cells) treated with RNase (40 μg); B1-4, untreated; B5-8, IFN-γ-treated (100 U/ml); C1-4, LPS-treated (100 ng/ml); C5-8, IFN-γ- and LPS-treated; B2, B6, C2 & C6, anti-TNF-α Ab-treated; B3, B7, C3 & C7, L-NMMA-treated (1mM); B4, B8, C4 & C8, L-NMMA- and anti-TNF-α Ab-treated. Total RNA (1 μg) was isolated at 6 hours pt, blotted onto Nytran plus nylon membrane and hybridized with [^{32}P]-labeled β-actin cDNA probe. XAR-5 X-ray film was exposured to this membrane for 48 hours.

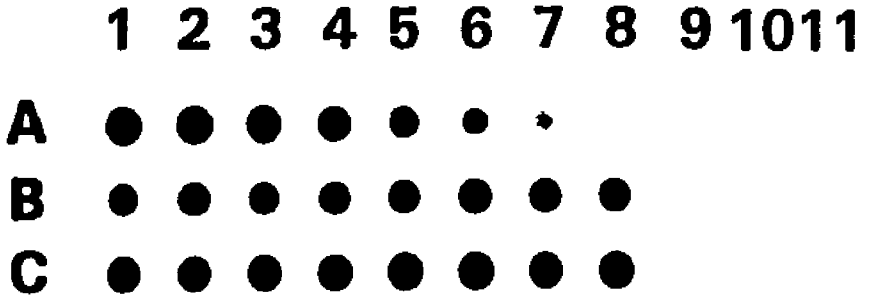
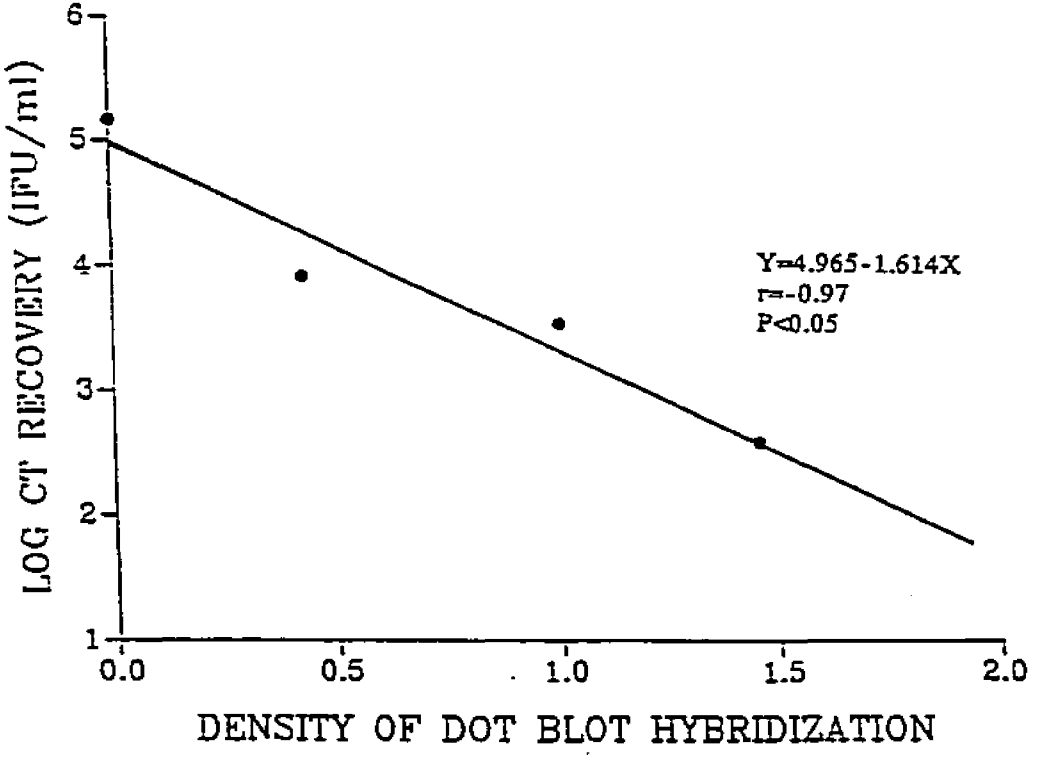


Figure 35

Inverse correlation between NOS mRNA expression and CT replication in RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. Total RNA was isolated at 6 hours pt and hybridized with [^{32}P]-labeled NOS cDNA probe as shown in Figure 34A. The density of the dots was measured by Bio-Image Analysis System. CT recovery was quantitated at 48 hours pt. Regression analysis of CT recovery and density of dot blot hybridization was performed.



nmol/ml, respectively (Figure 37). L-NMMA inhibited nitrite production by 85%, 86% and 76%. TNF-α production in LPS- or LPS- plus IFN-γ-treated cells was 3123 and 1753 units/ml, respectively. Although anti-TNF-α antibody neutralized TNF-α biological activity in LPS- and/or IFN-γ-treated cells (Figure 36), the antibody did not inhibit NOS mRNA expression (Figure 33A, 34A) or nitrite production (Figure 37). The antibody slightly reduced nitrite production (from 130.3+4.3 to 117.4+4.5 nmol/ml) in LPS- and IFN-γ-treated cells (Figure 37). As expected, L-NMMA (1 mM) did not affect NOS mRNA expression (Figure 33A, 34A). Combination of the antibody and L-NMMA did not greatly affect inhibition of nitrite production by L-NMMA (Figure 37).

As expected, treatment with LPS, IFN-γ or both inhibited CT replication by 1.6 logs, 1.3 logs or 2.6 logs, respectively. L-NMMA (1 mM) partially restored CT replication (21% with LPS, 39% with IFN-γ or 8% with IFN-γ plus LPS). The antibody alone did not abrogate this inhibition. However, combination of the antibody with L-NMMA increased the restoration (Figure 38), particularly in cells treated with LPS (42%) or LPS plus IFN-γ (17%, $p<0.01$ determined by Student's t test).

Relationship between Nitrite Production and CT Replication in Peritoneal Macrophages

As in previous experiments with macrophage cell lines, peritoneal macrophages were induced by LPS to produce TNF-α (Figure 39). However, the levels of TNF-α were lower (39 units/ml) than in the macrophage cell lines. No

Figure 36

Specific antibody neutralizes TNF-α produced by CT-infected RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, anti-TNF-α antibody was added. TNF-α biological activity was measured at 48 hours pt by biological assay in L929 cells (see Materials and Methods for detail). Data presented are mean of triplicate ± S.D..

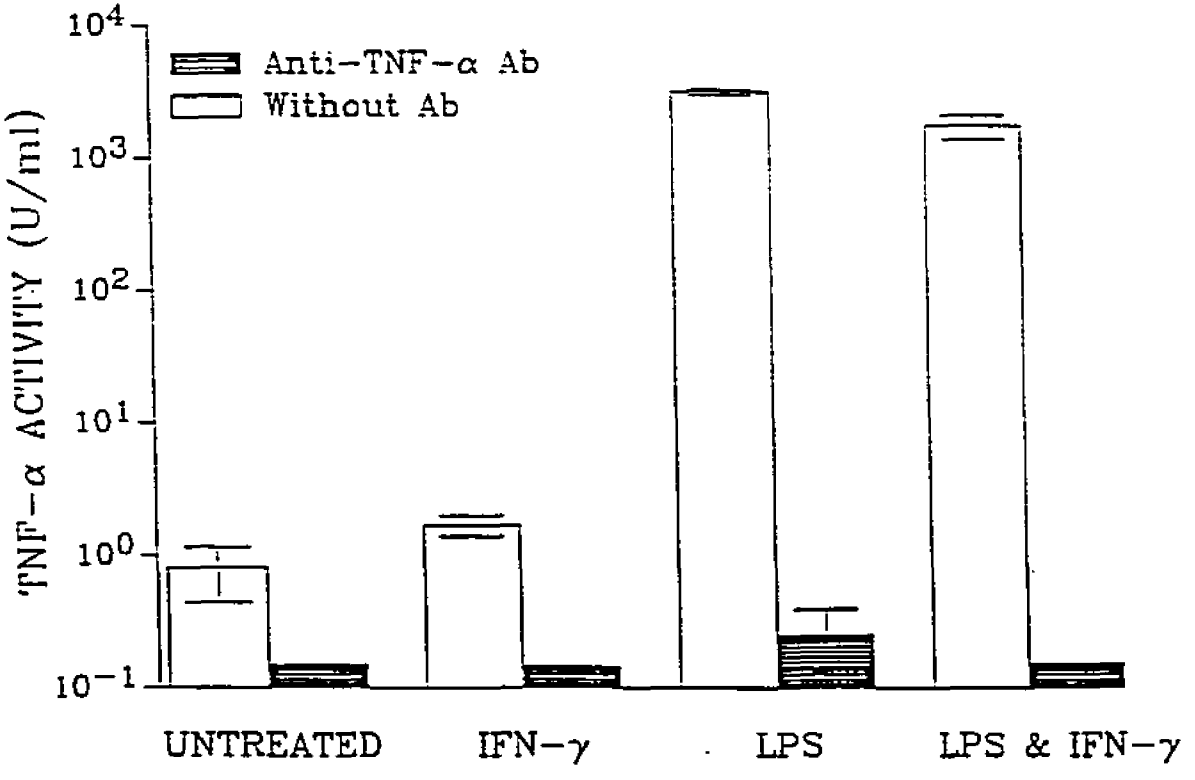


Figure 37

Effect of anti-TNF-α antibody and L-NMMA upon nitrite production in CT-infected RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, anti-TNF-α antibody, L-NMMA (1 mM) or the antibody plus L-NMMA was added. Nitrite production was determined at 48 hours pt by the Griess reagent. Data presented are mean of triplicate ± S.D..

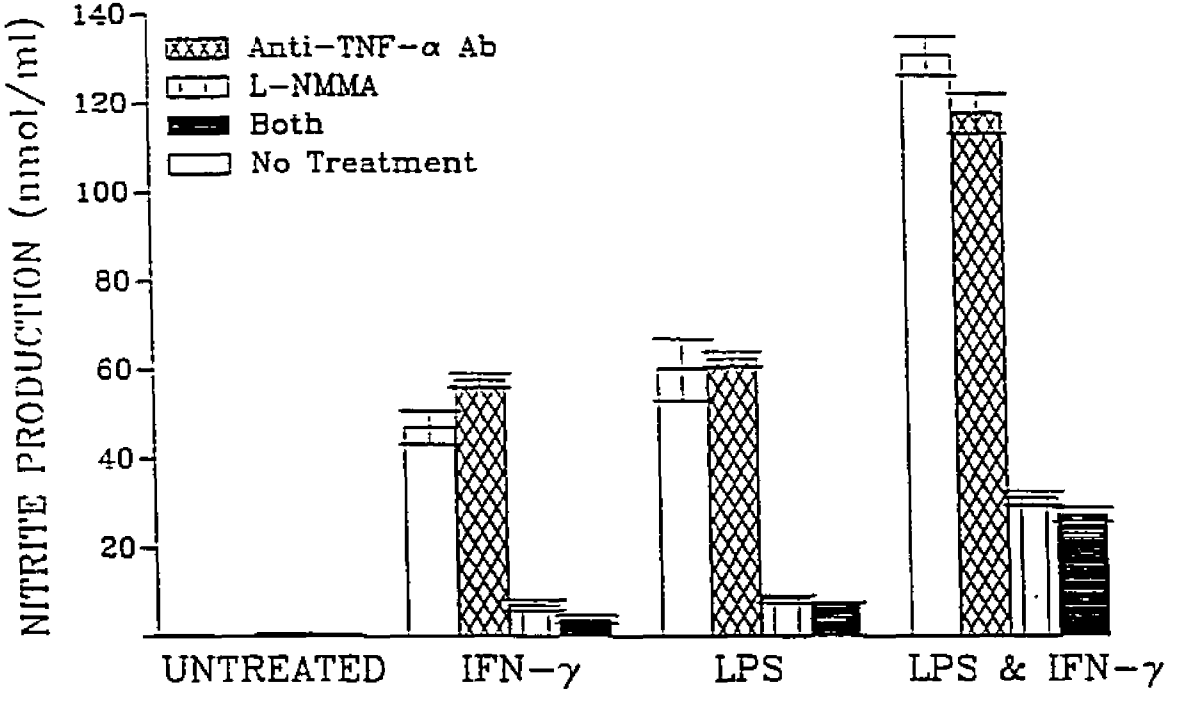
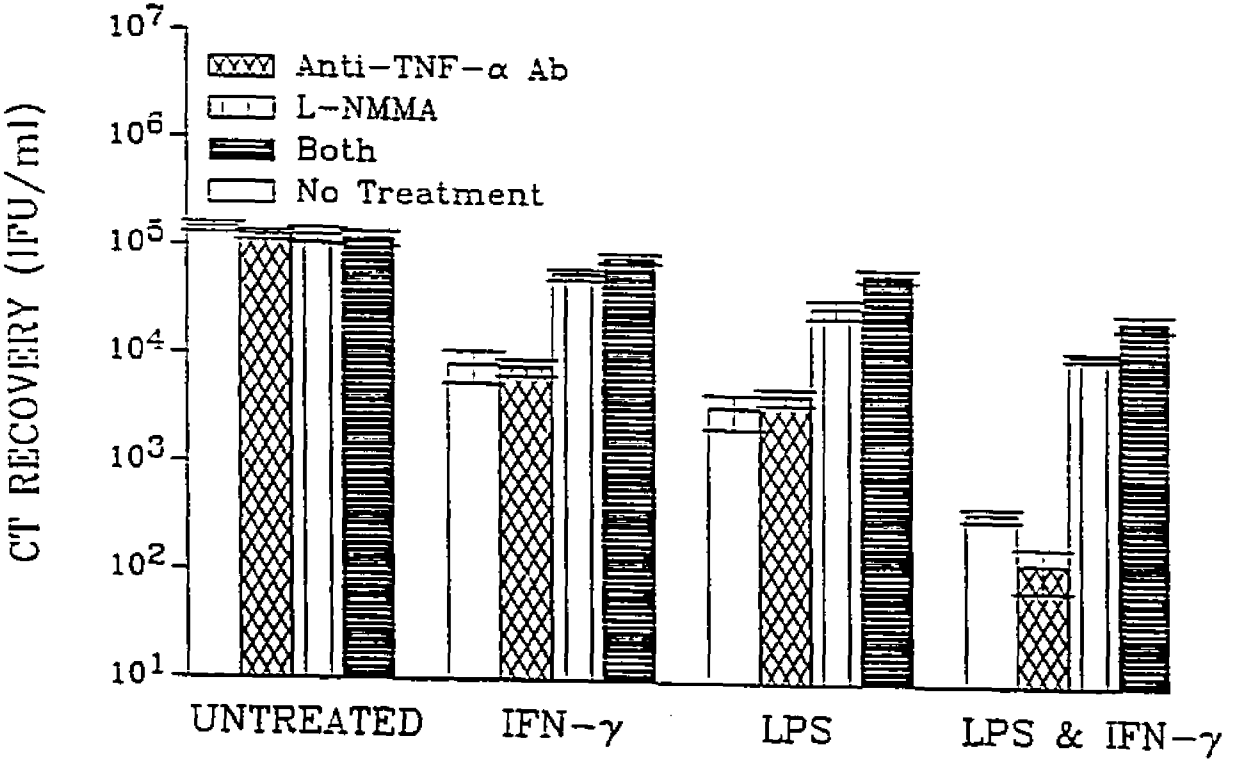


Figure 38

Effect of anti-TNF-α antibody and L-NMMA upon CT recovery in RAW264.7 cells. CT-inoculated RAW264.7 cells (MOI=1) were treated as described in Figure 37. CT recovery was quantitated at 48 hours pt in susceptible McCoy cells. Data presented are mean of triplicate ± S.D..



detectable TNF-α production was observed at 48 hr in CT-infected peritoneal macrophages treated with IFN-γ (Figure 39). Anti-TNF-α antibody completely neutralized the biological activity of TNF-α secreted by peritoneal macrophages treated with LPS or LPS plus IFN-γ (Figure 39).

As shown in Figure 40, levels of nitrite production in peritoneal macrophages treated with IFN-γ, LPS or both (33, 36 and 72 nmol/ml, respectively) were comparable to those in macrophage cell lines (Figure 37). No detectable nitrite production was observed in untreated peritoneal macrophages (Figure 40) or RAW264.7 cells (Figure 37). As expected, L-NMMA dramatically inhibited nitrite production (Figure 40). The inhibition of nitrite production induced by L-NMMA (1 mM) was more pronounced in peritoneal macrophages treated with IFN-γ or LPS (93% with IFN-γ and 100% with LPS) than in RAW264.7 cells (86% with IFN-γ and 87% with LPS). In contrast to RAW264.7 cells, nitrite production in peritoneal macrophages was inhibited by anti-TNF-α antibody (Figure 40). Levels of inhibition induced by the antibody in the macrophages treated with LPS, IFN-γ or both were 64%, 73% and 23% , respectively. Combination of antibody with L-NMMA slightly enhanced inhibition of nitrite production (Figure 40).

Treatment of peritoneal macrophages with IFN-γ, LPS or LPS plus IFN-γ inhibited CT replication by 1.1, 1.2 and 1.9 logs, respectively (Figure 41). Anti-TNF-α antibody significantly increased CT replication from 478 to 1822 IFU (34% restoration) in peritoneal macrophages treated with IFN-γ. L-NMMA also restored CT replication to 2755 IFU (35%) in these cells. Combination of the antibody and L-

Figure 39

Specific antibody neutralizes TNF-α produced by CT-infected peritoneal macrophages. CT-inoculated peritoneal macrophages (MOI=2) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, anti-TNF-α antibody was added. TNF-α biological activity was measured at 48 hours pt by biological assay in L929 cells (see Materials and Methods for detail). Data presented are mean of triplicate ± S.D..

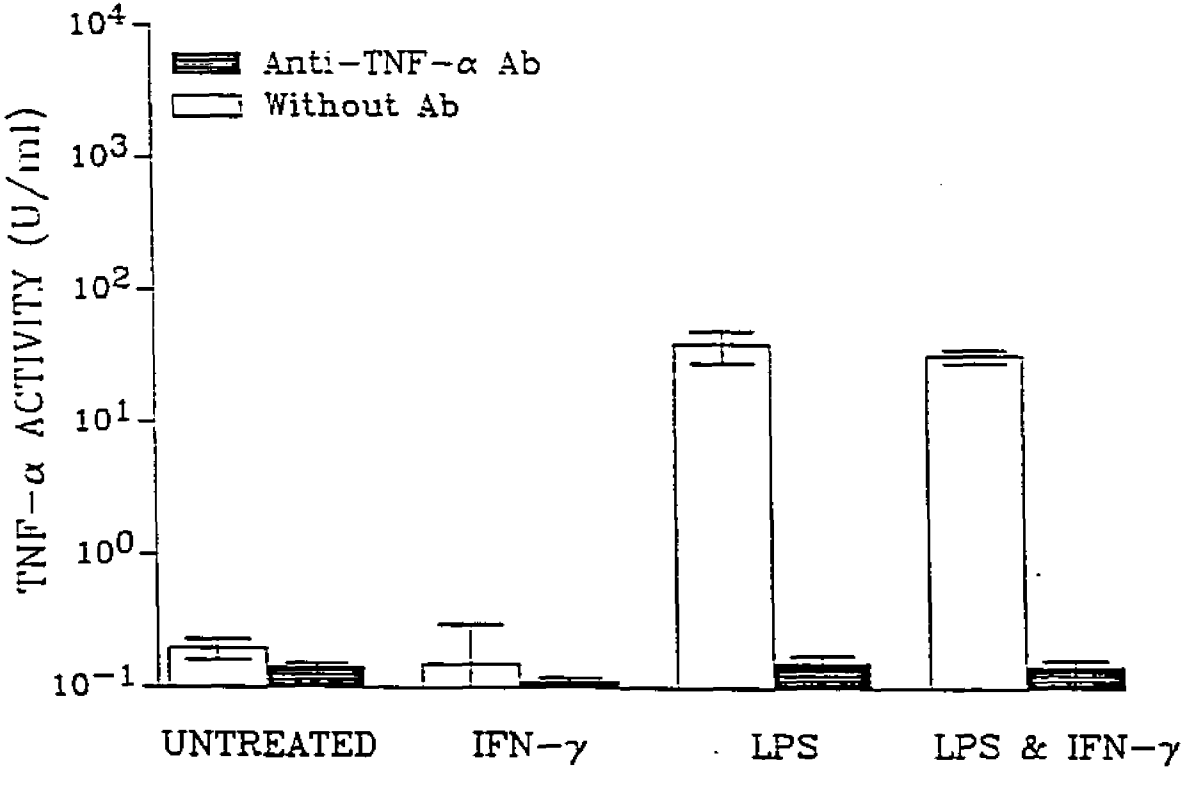


Figure 40

Effect of anti-TNF-α antibody and L-NMMA upon nitrite productionin in CT-infected peritoneal macrophages.CT-inoculated peritoneal macrophages (MOI=2) were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS at 24 hours pi. At the time of treatment, anti-TNF-α antibody, L-NMMA (1 mM) or the antibody and L-NMMA was added. Nitrite production was determined at 48 hours pt by the Griess reagent. Data presented are mean of triplicate ± S.D..

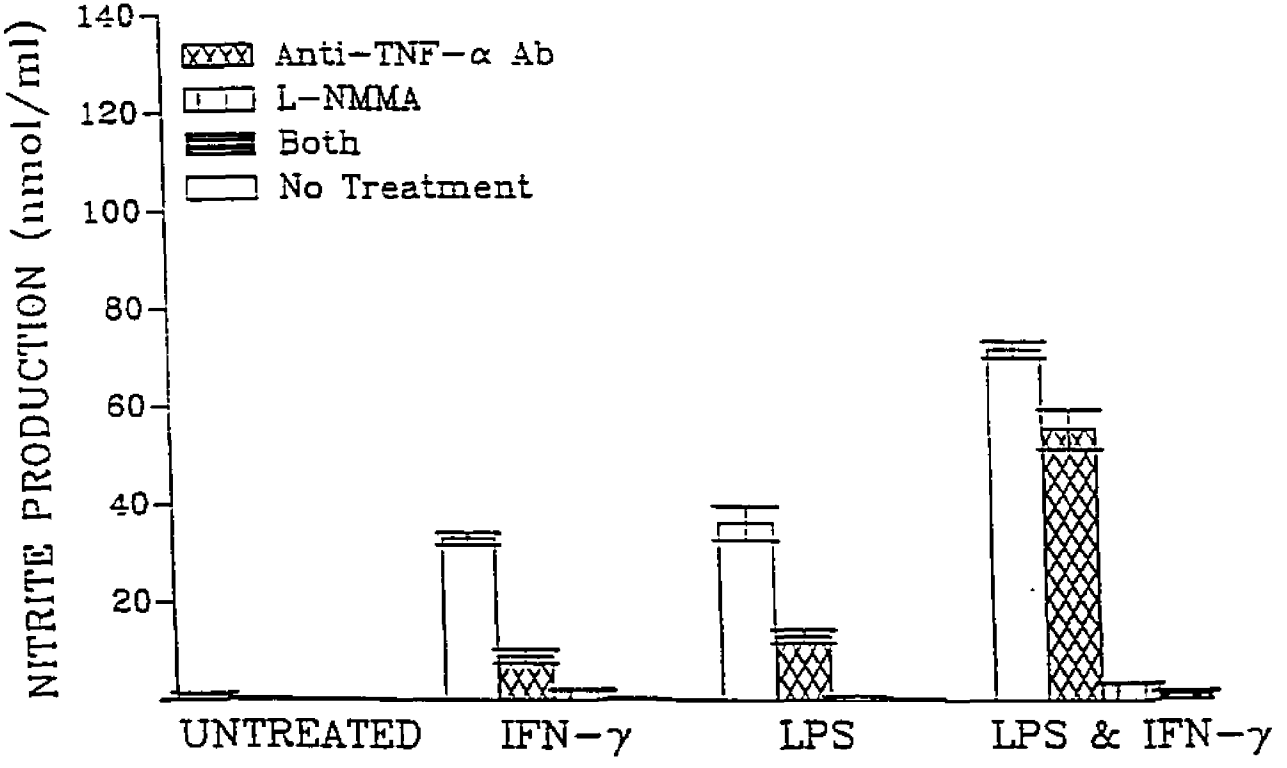
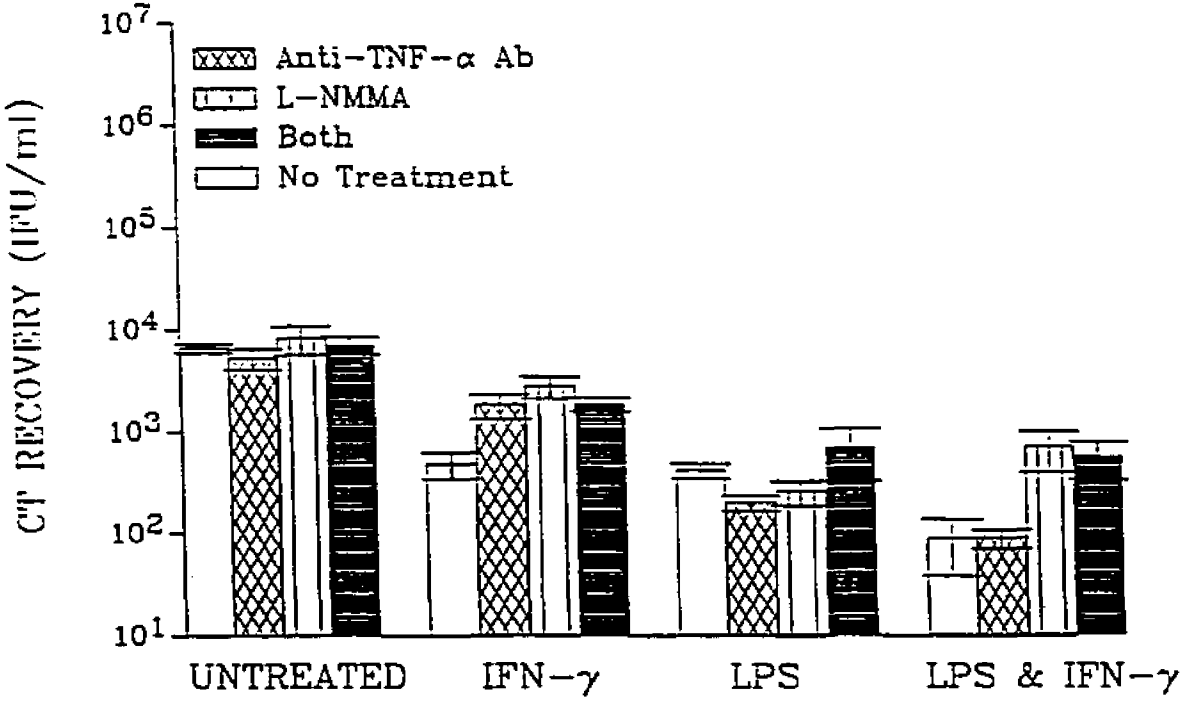


Figure 41

Effect of anti-TNF-α antibody and L-NMMA upon CT replication in CT-infected peritoneal macrophages. CT-inoculated peritoneal macrophages (MOI=2) were treated as described in Figure 40. At 48 hours pt, CT recovery was quantitated in susceptible McCoy cells. Data presented are mean of triplicate ± S.D..



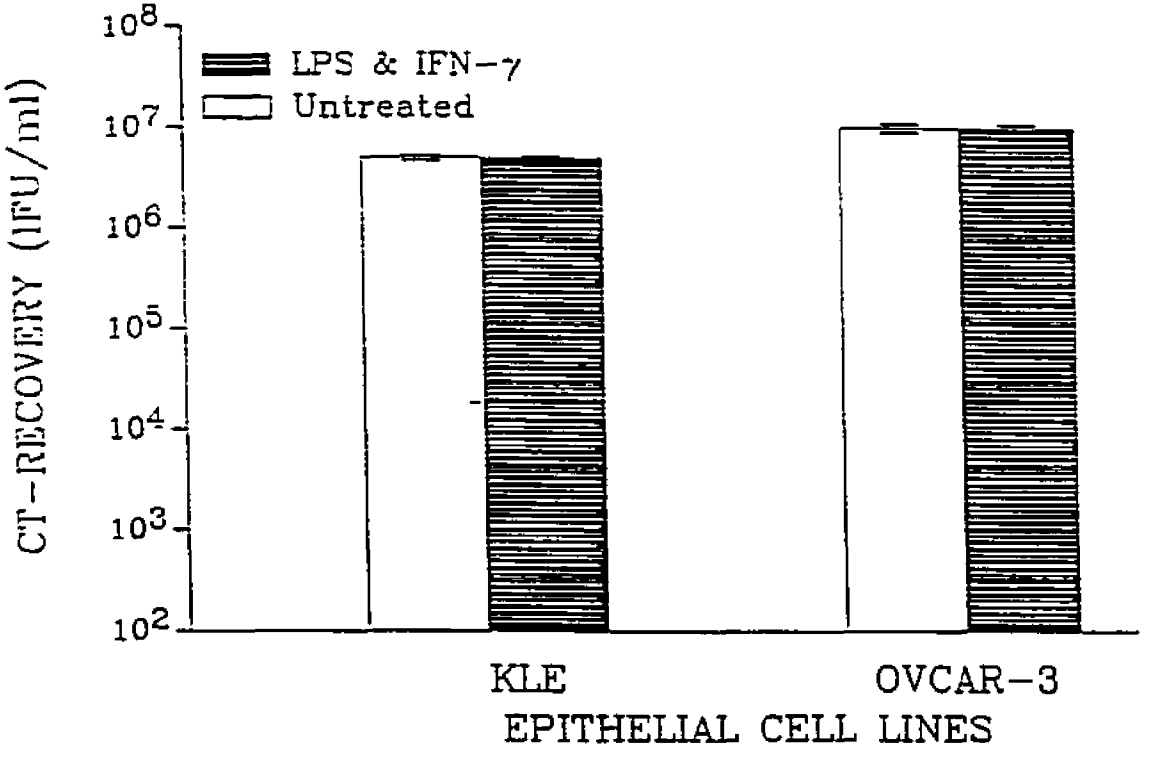
NMMA did not enhance the restoration of either. L-NMMA significantly ($P<0.05$ determined by Student's t test) increased CT replication from 88 to 700 IFU (8.6% restoration) in peritoneal macrophages treated with IFN-γ and LPS, yet anti-TNF-α antibody did not restore replication (Figure 41). In LPS-treated peritoneal macrophages, neither L-NMMA nor the antibody significantly restored CT replication (Figure 41). In contrast to RAW264.7 cells, anti-TNF-α antibody significantly inhibited nitrite production (73%) and partially reversed inhibition of CT replication (34%) in IFN-γ-treated peritoneal macrophages. Anti-TNF-α antibody alone had no effect upon nitrite production or CT replication in RAW264.7 cells. L-NMMA did not reverse inhibition of CT replication in LPS-treated peritoneal macrophages, but it did so in RAW264.7 cells. A final observation was that restoration of CT replication by L-NMMA was less pronounced in peritoneal macrophages treated with IFN-γ than in RAW264.7 cells.

IFN-γ and LPS Did Not Inhibit CT Replication in OVCAR-3 or KLE Cells

OVCAR-3 and KLE cells did not respond to IFN-γ and LPS by producing nitrite or TNF-α (data not shown). These cell lines were used to test the effect of LPS and IFN-γ upon CT replication in susceptible genital epithelial cells. Treatment with LPS and IFN-γ did not effect inhibition of CT replication in these cells (Figure 42). Therefore, studies with L-NMMA or anti-TNF-α antibody were not conducted.

Figure 42

IFN-γ and LPS do not inhibit CT replication in KLE or OVCAR-3 cells. KLE or OVCAR-3 cells were inoculated with CT (MOI=1). At 24 hours pi, infected cells were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) or IFN-γ plus LPS. At 48 hours pt, CT recovery was quantitated in susceptible McCoy cells. Data presented are mean of triplicate ± S.D..



Tryptophan Did Not Abrogate Inhibition of CT Replication

To determine if the tryptophan degradation pathway was involved in the inhibition of CT replication in our system, increasing concentrations (40-1000 μg/ml) were added to CT-infected RAW264.7 cells treated with LPS and IFN-γ. Tryptophan slightly increased TNF-α production (data not shown). However, tryptophan did not affect inhibition of nitrite production by L-NMMA in LPS- and IFN-γ-treated RAW264.7 cells except at a high concentration (1000 μg/ml) where nitrite production was slightly diminished (Figure 43). As expected, CT replication was inhibited by LPS and IFN-γ and was partially restored by the addition of 1 mM L-NMMA (Figure 44). Tryptophan (40-1000 μg/ml) alone did not abrogate inhibition of CT replication nor did tryptophan, when combined with L-NMMA, enhance restoration of CT replication (Figure 44).

Figure 43

Effect of tryptophan upon nitrite production in CT-infected RAW264.7 cells stimulated by IFN-γ and LPS. CT-inoculated RAW264.7 cells (MOI=1) were treated with IFN-γ (100 U/ml) and LPS (100 ng/ml) at 24 hours pi. At the time of treatment, tryptophan (40-1000 μg/ml) or tryptophan plus L-NMMA (1 mM) was added. At 48 hours pt, nitrite production was determined by the Griess reagent. Data presented are mean of triplicate $\pm$ S.D..

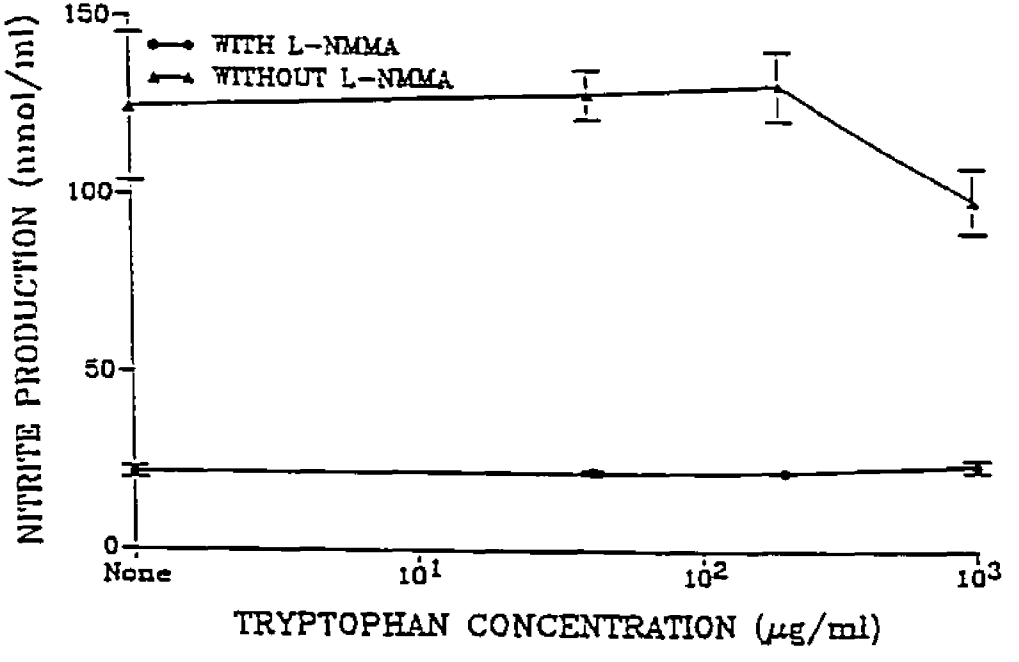
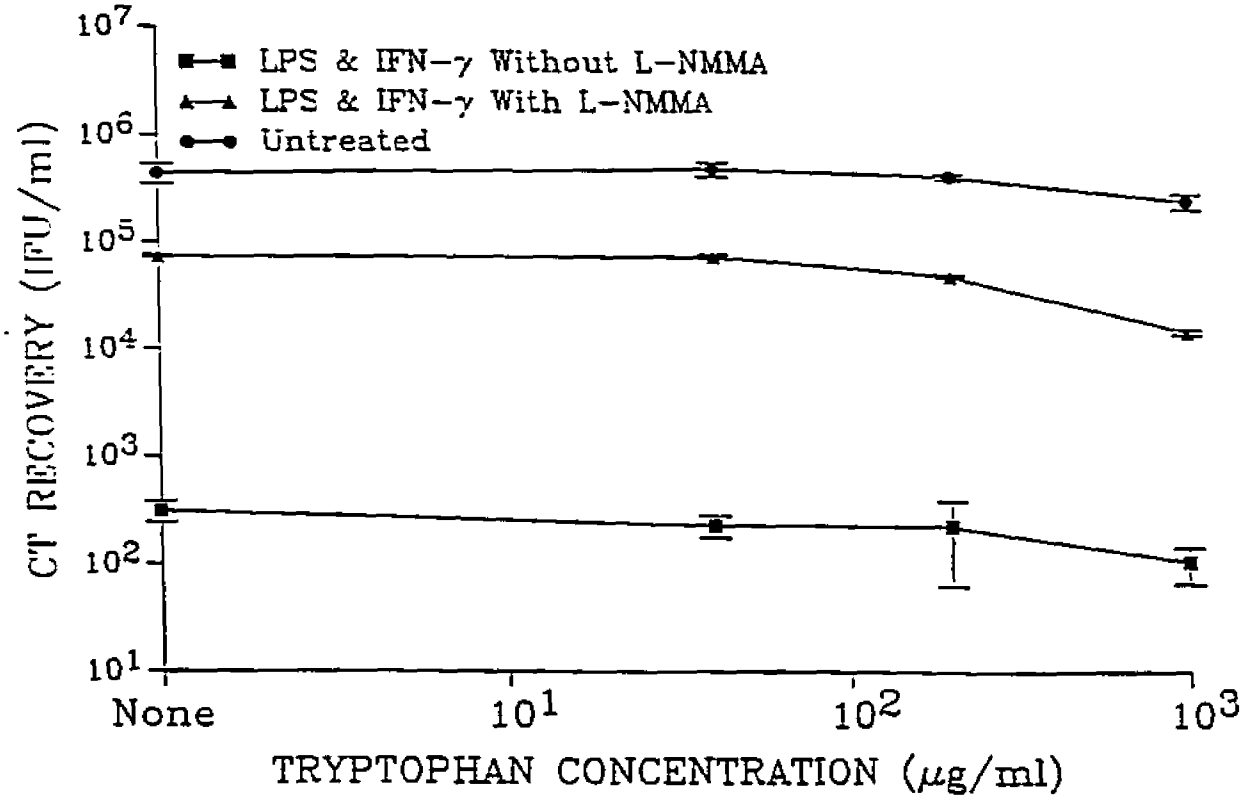


Figure 44

Effect of tryptophan upon CT replication in RAW264.7 cells stimulated by IFN-γ and LPS. CT-inoculated RAW264.7 cells (MOI=1) were treated as described in Figure 43. At 48 hours pt, CT recovery was quantitated in susceptible McCoy cells. Data presented are mean of triplicate ± S.D..



CHAPTER 4

Discussion

CT Infection of Macrophages

Infection of macrophages by CT biovar trachoma has not been confirmed (Kuo 1978, Yong 1987, Ishikawa et al. 1986, Keat et al. 1987). In this study, we showed that CT serovar D infected mouse peritoneal macrophages to form initial inclusions. However, the number of initial inclusions decreased from 4313 at day 1 pi to 340 inclusions per coverslip at day 6 pi (Figure 4). The percentage of infected cells decreased from 0.9% to 0.1% as well (Table 2). In contrast, the number of initial inclusions and percentage of infected cells increased in McCoy cells (Figure 4, Table 2), which presumably was due to secondary infection of new cells by the EBs released from the infected cells. These data indicated that primary infection occurred in macrophages while secondary infection was limited. TNF-α production by macrophages exposed to CT (our unpublished data) may be an explanation for inhibition of secondary infection, but the precise mechanism is unknown. On the other hand, the number of inclusions at 24 hours pi was much higher in macrophages than in McCoy cells (Figure 4), suggesting that chlamydial entry may be more efficient in macrophages than in McCoy cells. Professional phagocytosis by macrophages may mediate entry of the organisms. Since all steps involved in the CT developmental cycle, including attachment, ingestion, reorganization of EB to RB or RB maturation to EB, can affect inclusion development (Moulder 1991), other factors may have

contributed to this phenomenon.

Recovery of CT from infected macrophages was low (Figure 5). A low level of CT recovery also was observed by Kuo (1978). However, it was not clear whether Kuo's result reflected a low level of CT replication or residual inoculum. Our data confirmed that CT replicated in macrophages since (a) CT recovery from macrophages was much higher than that of background (residual inoculum) and (b) a kinetic study demonstrated increase of CT recovery from 48 hr pi through 6 days (Figures 6, 9C). However, the level of CT replication in macrophages was lower than in McCoy cells, as evidenced by lower level of CT recovery and lower yield (Figure 6). CT replicated in macrophages less efficiently than in McCoy cells, indicating that CT replication in peritoneal macrophages was limited at some step of the developmental cycle beyond entry since the number of initial inclusions at 24 hr pi was greater in macrophages than in McCoy cells.

CT Infection of Macrophage Cell Lines

CT biovar trachoma (serovar D) infected mouse macrophage cell lines RAW264.7 and J774A.1 to form initial inclusions (Figure 7). The number of initial inclusions decreased at day 2 pi, then increased at day 3 pi, presumably due to secondary infection as observed in McCoy cells. However, the increase of initial inclusions in macrophage cell lines occurred one day earlier than in McCoy cells (day 4 pi), which suggested that the CT developmental cycle in macrophage cell lines may be shorter than in McCoy cells. The number of cells significantly decreased (Figure 8) and the percentage of infected cells dramatically increased (Table 2) in CT-infected

J774A.1 cell cultures, suggesting that CT infection may result in lysis of J774A.1 cells. As in fresh peritoneal macrophages, the number of initial inclusions at 24 hours pi was higher in J774A.1 than in McCoy cells (Figure 7), again suggesting that professional phagocytosis may mediate entry of the organisms.

Recovery of CT from macrophage cell lines was low at day 1, then increased after day 2 (Figure 9), which demonstrated that CT replicated in the lines. The levels of CT replication in macrophage cell lines were higher than in peritoneal macrophages (Figure 9). The level of the replication in J774A.1 cells was even higher than in McCoy cells (Figure 9), indicating that CT not only entered J774A.1 cells successfully but replicated efficiently. The reason J774A.1 cells were so susceptible to CT infection is not clear. This line was reported to secrete IL-1 continuously (ATCC 1988). IL-1 secretion is enhanced by LPS treatment (Martin and Dorf 1990). Secretion of IL-1 also may be increased in response to CT infection. In this regard, IL-1 production in human monocytes was induced by CT (Manor et al. 1990, Rothermel et al. 1989). A detectable level of TNF-α production in CT-infected J774A.1 cells also was observed in our study. IL-1 and TNF-α should have provided J774A.1 cells some level of protection against CT infection, since these cytokines have been reported to inhibit CT infection (Shemer-Avni et al. 1988, 1989, 1990, Holtmann et al. 1990). It is possible that the level of IL-1 or TNF-α did not reach "threshold" for inhibition of CT replication or that J774A.1 cells did not respond to those cytokines by exhibiting antichlamydial activity. In fact, it has been observed that J774A.1 cells did not respond to IL-1 or TNF-α by producing IL-6, implying that

these cytokines may not be autoregulatory for this macrophage cell line (Martin and Dorf 1990). This line also synthesizes large amounts of lysozyme (ATCC 1988). The significance of the latter during CT infection is unclear.

Medium change every 24 hours significantly increased CT recovery from RAW264.7 and McCoy cells, presumably by providing the infected cells with fresh medium, optimal pH and new nutrients. However, medium change in J774A.1 cells did not increase recovery (Figure 9). This may have contributed to destruction or lysis of the majority of cells after CT infection (Figure 8). The remaining cells were so few that the medium did not become acid and nutrients presumably were sufficient to support CT replication. CT recovery from peritoneal macrophages gradually decreased after day 4. Medium change did not increase CT recovery. Rather, a decrease of CT recovery was observed (Figure 9). This may be due to limited secondary infection of peritoneal macrophages and removal of released EBs by medium change.

Macrophage cell lines RAW264.7 and J774A.1 share several features with fresh macrophages such as phagocytic activity, antibody-dependent cytotoxicity and cytokine production in response to LPS or IFN-γ stimulation (ATCC 1988, Jue et al. 1990, Radolf et al. 1991, Kelly et al. 1991, Oh et al. 1991, Martin and Dorf 1990, Lewis et al. 1990). However, the macrophage cell lines have long life spans in tissue culture and are more susceptible to CT infection than fresh macrophages. Therefore, they may be ideal cell lines for study of interaction between macrophages and CT in vitro.

Inhibition of CT Replication by LPS or IFN-γ

Inhibition of *Chlamydia psittaci*, CT LGV and other microorganisms by IFN-γ-activated macrophages has been demonstrated (Rothermel et al. 1983, de la Maza et al. 1987, Shemer and Sarov 1985, Green et al. 1990, Granger et al. 1990, Adams et al. 1990). In this study, we demonstrated that IFN-γ- or LPS-activated macrophage cell lines RAW264.7 and J774A.1 restricted CT replication (Figure 10). Inhibition induced by LPS was greater than that by IFN-γ in RAW264.7 cells. However, LPS was less effective in inducing inhibition of CT replication than IFN-γ in J774A.1 cells. This result may reflect different mechanisms involved in inhibition in different cell types. Activation of macrophages for microbicidal and tumoricidal activities is thought to be a two signal process (Celada et al. 1984, Pace et al. 1983, Nacy et al. 1988). However, our data and that of others (Rothermel et al. 1983, de la Maza et al. 1987, Shemer and Sarov 1985, Byrne et al. 1989) indicated that IFN-γ or LPS was sufficient to induce antichlamydial activity in CT-infected peritoneal macrophages. Possibly, chlamydial LPS or the infection itself somehow primed the cells. In this case, IFN-γ or LPS served as a second signal to trigger antichlamydial activity in these cells. Two signals such as IFN-γ and LPS did enhance inhibition of CT replication in RAW264.7 and J774A.1 cells (Figure 10). Inhibition of CT replication also was observed in McCoy cells treated with IFN-γ and LPS (Figure 10). However, the inhibition was less pronounced, compared to macrophage cell lines.

Nitrite Production in CT-infected and Uninfected Cells

The mechanism(s) for inhibition of CT replication in activated macrophages have not been defined. In this study, we focused upon nitrite production as a possible mechanism. Our data showed that nitrite production was induced by IFN-γ or LPS in macrophage cell lines RAW264.7 and J774A.1 (Figures 11-12). However, the levels of nitrite production induced by IFN-γ were lower than that induced by LPS, particularly in J774A.1 cells. Lower nitrite production (< 10 nmol/10^6 cells) was observed by others (Stuehr and Marletta 1987b) in IFN-γ-treated J774A.1 cells than in peritoneal macrophages. This may be related to the report that macrophage cell lines expressed fewer receptors for IFN-γ than primary macrophages (Celada at el. 1984). Nitrite production induced by LPS or LPS plus IFN-γ was greater in RAW264.7 cells than in J774A.1 cells. This may be related to a high level of TNF-α production in the treated RAW264.7 cells (Figure 22B-C). IFN-γ and LPS exhibited a synergistic effect upon nitrite production (Figures 11-12). Synergistic effects were exerted by LPS and IFN-γ or by LPS and lymphokines (Stuehr and Marletta 1987b). They also observed that other stimuli such as lymphokines, BCG (bacillus of Calmette and Guerin) or PPD (purified protein derivative of old tuberculin) induced nitrite production in RAW264.7 cells, but not in J774A.1 cells (Stuehr and Marletta 1987b). The peak production of nitrite was 48 hours pt. No detectable nitrite production was observed in untreated cells.

Our data indicated that the presence of IFN-γ and/or LPS was necessary for persistent production of nitrite, since medium change resulted in a significant

reduction of nitrite production (Figure 11B, 12B). But, Stuehr and Marletta (1987a) reported that after peritoneal macrophages were treated with IFN-γ and/or LPS, daily medium change (without IFN-γ or LPS) significantly increased nitrite production, presumbly by providing new nutrients. This discrepancy may reflect differences between macrophage cell lines and fresh peritoneal macrophages. For example, macrophage cell lines can proliferate in vitro, while fresh peritoneal macrophages cannot.

We also observed that a fibroblastic cell line, McCoy cells (a human and mouse hybrid), was induced by IFN-γ and LPS to synthesize nitrite (Figure 13). The levels of nitrite production in McCoy cells were lower than those in the macrophage cell lines. Either IFN-γ or LPS alone induced minimal nitrite production in McCoy cells. Other cell types such as endothelial cells or hepatocytes also were reported to synthesize nitrite (Moncada et al. 1991). This indicated that not only macrophages but a variety of cell types synthesized nitrite in response to IFN-γ and LPS.

CT infection alone was not sufficient to induce nitrite production in RAW264.7 or McCoy cells. However, CT infection enhanced nitrite production induced by IFN-γ in these cells (Figures 15-16). This may be related to chlamydial LPS since LPS has been found in infected cell membranes (Hearn and McNabb 1991) and has been shown to function synergistically with IFN-γ to induce nitrite synthesis. However, BCG or PPD, which did not contain LPS, induced nitrite production in RAW264.7 cells and peritoneal macrophages (Stuehr and Marletta 1987a-b). Therefore, the possibility that CT infection itself primed the infected cells by an

undefined mechanism(s) cannot be ruled out.

Correlation of Nitrite Production and CT Inhibition

A strong correlation between nitrite production and inhibition of CT replication in RAW264.7 (correlation coefficient: -0.93, $p<0.001$) or J774A.1 cells (correlation coefficient: -0.94, $p<0.001$) was observed (Figure 17A-B). This suggested that nitrite production may be a mechanism for inhibition of CT replication. The correlation (correlation coefficient: -0.67, $p<0.05$) was observed in McCoy cells (Figure 17C). However, the slope of the regression curve was smaller (0.522) than those of the macrophage cell lines (2.061-2.453). This indicated that CT replication in McCoy cells was less affected by nitrite production than in the macrophage cell lines.

Inhibition of Nitrite Production and Restoration of CT Replication by L-NMMA

In order to demonstrate that nitrite production is the mechanism of inhibition of CT replication, L-NMMA was employed to block nitrite production. L-NMMA inhibited nitrite production in mouse macrophages and McCoy cells (Figures 19-21), which is consistent with other reports (Mayer et al. 1993, Moncada et al. 1991). Concentrations of L-NMMA varied (0.1-0.5 mM) from laboratory to laboratory (Moncada et al. 1991, Mayer et al. 1993, Hibbs et al. 1987, Murray and Teitelbaum 1992, Lin and Chadee 1992). In our study, the inhibitory effect of L-NMMA was proportional to the concentration used (Figure 18). However, inhibition of nitrite

production was dependent not only upon concentration of L-NMMA, but varied with cell type and stimuli. For example, 1 mM L-NMMA completely inhibited nitrite production in LPS-treated J774A.1 (Figure 20A), but only 77% inhibition was observed in LPS-treated RAW264.7 cells (Figure 19A). 10 mM L-NMMA completely inhibited nitrite production in CT-infected RAW264.7 cells treated with either LPS or IFN-γ, but only 94% inhibition was observed in the cells treated with both IFN-γ and LPS (Figure 24).

LPS is a strong stimulus of TNF-α production in mouse macrophage cell lines and peritoneal macrophages (Jue et al. 1990, Kelly et al. 1991, Martin and Dorf 1990, Lewis 1990). Therefore, TNF-α production was investigated in macrophage cell lines. TNF-α production was higher in LPS-treated RAW264.7 cells (Figure 22) than in the treated J774A.1 cells (Figure 23). L-NMMA did not reduce TNF-α production in these cells, which indicated that inhibition of nitrite production by L-NMMA was specific and that NO did not function as a regulatory molecule for TNF-α production.

L-NMMA abrogated inhibition of CT replication in macrophage cell lines (Figures 25, 27). The abrogation was dose-dependent. Restoration of CT replication induced by L-NMMA (5-10 mM) was 55 or 71% in RAW264.7 cells treated with IFN-γ or LPS, respectively. The restoration in LPS-treated J774A.1 cells (52%) was comparable to that of RAW264.7 cells (55%). However, the restoration induced by L-NMMA was low (<10%) in J774A.1 cells treated with IFN-γ or IFN-γ plus LPS, although CT recovery was increased remarkably (0.9 or 1.4 $\log_{10}$ units, respectively).

The reason for the lower level of restoration in J774A.1 cells is unknown. Continuous IL-1 secretion by J774A.1 cells was reported and secretion increased following treatment with LPS (ATCC 1988, Martin and Dorf 1990, Lewis et al. 1990). IL-1 has been shown to inhibit CT replication in HEp-2 cells by PGE2 production (Shemer-Avni et al. 1990). However, as discussed previously, it is unknown if J774A.1 cells responded to IL-1 by expressing antichlamydial activity. Furthermore, restoration of CT replication induced by L-NMMA in LPS-treated J774A.1 cells was comparable to that in RAW264.7 cells, indicating that IL-1 production may not be involved. These results indicated that (a) nitrite production is one mechanism of inhibition of CT replication in the macrophage cell lines, but that (b) other mechanisms also may be involved in RAW264.7 and J774A.1 cells. It is noteworthy that in the numerous reports that have suggested a role for NO in antimicrobial activities exhibited by activated mouse peritoneal macrophages or murine cell lines, the degree to which L-NMMA or arginase reversed such activity has varied. Restoration of microorganism replication induced by L-NMMA or arginase has ranged from moderate to nearby complete depending upon the test pathogens, the target cells and the cytokines (Granger et al. 1991, James and Glaven 1989, Liew et al. 1990a-c, Green et al. 1990a-b, 1991, Adams et al. 1990, Mauel et al. 1991, Bogdan et al. 1991, Denis 1991).

In McCoy cells, IFN-γ inhibited CT replication but to a lesser extent than in other cells treated with IFN-γ. In contrast to other cells, LPS did not induce NO production or inhibit CT replication in McCoy cells. L-NMMA (0.1 mM) remarkably

inhibited nitrite production, but only slightly increased CT recovery by 0.16 or 0.28 logs IFU/ml in McCoy cells treated with IFN-γ or IFN-γ plus LPS (Figure 29). A high concentration of L-NMMA (5 mM) did not increase CT recovery further. Therefore, nitrite production probably is not the mechanism of inhibition of CT replication in McCoy cells treated with IFN-γ. However, Mayer et al. (1993) recently reported that IFN-γ-pretreated McCoy cells dramatically reduced CT (type H) replication by 4 $\log_{10}$ units. L-NMMA reversed the inhibition. This discrepancy may be due to differences in CT strains or McCoy cells (the McCoy cells used in our laboratory are a human-mouse hybrid, while McCoy cells used by Mayer et al. were a murine-derived cell line). Furthermore, alternate methods of IFN-γ treatment were used in the two studies.

Since all evidence indicated that nitrite production is one of the mechanisms of inhibition of CT replication, it was necessary to determine if nitrite played an essential role in this inhibition. Our data showed that exogenous nitrite (50-200 nmol/ml) did not affect CT replication in RAW264.7 cells (Figure 30), which suggested that the mediator of nitrite production, rather than nitrite itself, functioned as the effector molecule.

Correlation of NOS mRNA Expression with CT Inhibition in RAW264.7 Cells

It has been demonstrated that immunological activation induces NOS at the transcriptional level in RAW264.7 cells (Xie et al. 1992). Few investigators

determined NOS mRNA expression to confirm NO production as a mechanism for antimicrobial or antitumoral activity. However, in this study, we determined NOS mRNA expression (approximately 4.1 kb) in RAW264.7 cells by using Northern Blot and Dot Blot hybridization. IFN-γ or LPS induced significant NOS mRNA expression in CT-infected RAW264.7 cells (Figure 32, 33A, 34A). Combination of IFN-γ with LPS exhibited a synergistic effect upon NOS mRNA expression. The peak of NOS mRNA expression was 6-24 hours pt (Figure 32). No detectable NOS mRNA was observed in untreated RAW264.7 cells. Similar results were observed in uninfected RAW264.7 cells by Lowenstein et al. (1992). These results also were consistent with nitrite production in cells treated with LPS and/or IFN-γ. A strong correlation between NOS mRNA expression and inhibition of CT replication (correlation coefficient: -0.97, $p<0.05$) was observed. This further supported the contention that NO is a mechanism of inhibition of CT replication.

Effect of Anti-TNF-α Ab upon NOS mRNA Expression, Nitrite Production and CT Replication

In vitro, a combination of rIFN-γ and rTNF-α synergistically induced nitrite production in mouse peritoneal macrophages and McCoy cells (Ding et al. 1988, Mayer et al. 1993). However, the effect of endogenuous TNF-α upon nitrite production has not been defined. Anti-TNF-α antibody, which completely neutralized biological activity of secreted TNF-α (Figure 36), inhibited neither NOS mRNA expression (Figures 33A, 34A) nor nitrite production (Figure 37) in LPS- or IFN-γ-

treated RAW264.7 cells. However, the antibody significantly inhibited nitrite production in peritoneal macrophages (Figure 40). One possible explanation for this is that the cell line did not respond to TNF-α by nitrite production, although it did respond to secreted TNF-α by exerting antichlamydial activity (Figure 38). Recently, two distinct TNF-α receptors (TNF-R1 and TNF-R2) have been demonstrated in human and mouse systems (Tartaglia and Goeddel 1992). Binding of TNF-α to TNF-R1 results in cytotoxicity and antiviral activity. However, binding of TNF-α to TNF-R2 results in thymocyte and CTL proliferation. Therefore, there may be different receptors expressed on peritoneal macrophages and RAW264.7 cells. Different receptors may not explain this phenomenon, since the antibody recognized epitopes on TNF-α rather than on receptors. Another possibility is that the membrane-associated TNF-α was not neutralized by the antibody in RAW264.7 cells. In fact, secreted TNF-α was derived from membrane-associated precursors in LPS-treated RAW264.7 cells (Jue et al. 1990). A substantial amount of TNF-α was detected on the treated cell surface (Jue et al. 1990). Since RAW264.7 cells were confluent in cultures, the antibody may not be sufficient to block the activity of membrane-associated TNF-α in these cells. However, peritoneal macrophages were not confluent in cultures. Therefore, the membrane associated TNF-α was sufficiently neutralized by the antibody.

L-NMMA inhibits nitrite production by competitive binding of NOS (Moncada et al. 1991). As expected, L-NMMA did not affect transcription of NOS gene (Figures 33A, 34A), although remarkable inhibition of nitrite production by L-

NMMA was observed (Figure 37).

Restoration of CT replication induced by L-NMMA (5-10 mM) was not complete even in IFN-γ-treated RAW264.7 cells, although nitrite production was almost completely inhibited (Figures 26-27). Furthermore, L-NMMA induced comparable inhibition of nitrite production but restored less of CT replication in LPS-treated than in IFN-γ-treated RAW264.7 cells. The former produced high levels of TNF-α which may be involved in the inhibition. rTNF-α has been shown to inhibit CT replication in HEp-2 cells (Shemer-Avni et al. 1988, 1989). Treatment of McCoy cells with rTNF-α post-inoculation significantly inhibited initial inclusion formation and CT replication (Table 3). This treatment also caused atypical inclusions with less glycogen than those in untreated cells (data not shown). The significance of this is unknown although it has been suggested that glycogen deposition in CT inclusions is not required for complete replication (Matsumoto 1988). The effect of secreted TNF-α upon CT replication remains unclear. An anti-TNF-α neutralizing Ab was employed to investigate this effect in RAW264.7 cells. The Ab, which completely neutralized biological activity of secreted TNF-α, neither inhibited nitrite production (Figure 37) nor reversed inhibition of CT replication (Figure 38) in RAW264.7 cells treated with LPS- and/or IFN-γ. Combination of the Ab with L-NMMA significantly enhanced the restoration of CT replication in the cells treated with LPS or IFN-γ, particularly in cells treated with LPS (Figure 38). These results indicated that (a) secreted TNF-α may be involved in antichlamydial activity, but the mechanism may be distinct from NO production; (b) the antichlamydial effect of secreted TNF-α may be overwhelmed

by NO production in the presence of LPS or IFN-γ.

Inhibition of CT Replication in Peritoneal Macrophages

We have demonstrated that NO and TNF-α production are involved in inhibition of CT replication in LPS- or IFN-γ-activated RAW264.7 cells. We now wished to determine if the same mechanisms were operative in peritoneal macrophages. TNF-α production was induced by LPS in peritoneal macrophages (Figure 36). The level of TNF-α was much lower than in macrophage cell lines. This may suggest that peritoneal macrophages and macrophage cell lines are at different stages of activation. As in macrophage cell lines, high levels of nitrite production were induced in peritoneal macrophages by LPS or IFN-γ (Figure 40) and nitrite production was dramatically inhibited by L-NMMA (Figure 40). However, anti-TNF-α antibody, which did not affect nitrite production in macrophage cell lines, significantly inhibited nitrite production in peritoneal macrophages treated with LPS and/or IFN-γ (Figure 40). This indicated that secreted TNF-α may be an autoregulatory cytokine for nitrite production during activation of peritoneal macrophages.

CT replication was dramatically inhibited in peritoneal macrophages treated with IFN-γ and/or LPS. However, the mechanism(s) of inhibition in peritoneal macrophages may be different from that in the macrophage cell lines. Although L-NMMA partially restored CT replication (34-35%) in the IFN-γ-treated peritoneal macrophages, it did not reverse the inhibition in LPS-treated cells (Figure 41). This

suggested that NO production may be a mechanism of CT inhibition in IFN-γ-treated peritoneal macrophages, but may not be active in LPS-treated cells. Furthermore, anti-TNF-α Ab significantly inhibited nitrite production and partially restored CT replication (34-35%) in peritoneal macrophages treated with IFN-γ. The latter suggested that secreted TNF-α may exert antichlamydial activity by up-regulation of the NO production pathway during activation of peritoneal macrophages by IFN-γ. Neither Ab alone nor in combination with L-NMMA reversed CT inhibition in LPS-treated cells, indicating that NO or TNF-α may not be the mechanism for inhibition of CT replication in peritoneal macrophages activated by LPS.

Direct Effect of IFN-γ and LPS upon CT Replication

In order to examine the direct effect of IFN-γ and LPS upon CT replication, two human epithelial cell lines OVCAR-3 and KLE were employed. These cell lines did not respond to murine IFN-γ and LPS by nitrite or TNF-α production (data not shown). Treatment with IFN-γ and LPS did not affect CT replication in these cells (Figure 42). A direct inhibitory effect of IFN-γ and LPS upon CT replication in highly susceptible cells was thereby refuted. Since IFN-γ used in this study was murine recombinant and response to IFN-γ is species specific, human IFN-γ may elicite an effect upon CT replication in these cell lines.

Effect of Tryptophan upon CT Replication

Tryptophan degradation has been reported to be a mechanism of inhibition of

CT replication in IFN-γ-treated human cell lines (HEp-2 and T24) or human monocytes/ macrophages (Byrne et al. 1986, Murray et al. 1989). However, we demonstrated that exogenous tryptophan (40 to 1000 μg/ml) failed to reverse inhibition of CT replication in IFN-γ- and LPS-treated RAW264.7 cells (Figure 44). In order to exclude the possibility that the effect of tryptophan degradation might have been overwhelmed by NO production, we treated the cells with exogenous tryptophan and L-NMMA. The combination of exogenous tryptophan with L-NMMA did not affect the inhibition (Figure 44). These results indicated that tryptophan degradation may not be the mechanism of inhibition in this macrophage cell line. Antichlamydial effect without involvement of tryptophan degradation in other murine-derived cell lines also was reported by other investigators (Mayer et al. 1993, de la Maza et al. 1985, Murray et al. 1989). Our data supported the hypothesis that the tryptophan degradation mechanism is species-specific, i.e., involved in antichlamydial activity in human cells, but not in murine cells.

Summary and Conclusions

In summary, IFN-γ or LPS induced inhibition of CT replication in peritoneal macrophages and macrophage cell lines. In McCoy cells, only IFN-γ inhibited CT replication. L-NMMA (1 mM) inhibited nitrite production and restored CT replication in RAW264.7 cells treated with IFN-γ or LPS. Inhibition of nitrite production and restoration of CT replication induced by L-NMMA was comparable in LPS-treated J774A.1 cells and IFN-γ-treated peritoneal macrophages. However, L-NMMA (1

mM) inhibited nitrite production but did not significantly restore CT replication in LPS-treated pertoneal macrophages, IFN-γ-treated McCoy or J774A.1 cells. Anti-TNF-α antibody inhibited nitrite production in LPS- or IFN-γ-treated peritoneal macrophages but restored CT replication only in IFN-γ-treated cells. In LPS- or IFN-γ-treated RAW264.7 cells, anti-TNF-α antibody alone neither reduced nitrite production nor restored CT replication. Combination of the antibody and L-NMMA significantly enhanced restoration of CT replication without increase in inhibition of nitrite production. Therefore, we have made the following conclusions: (1) NO production is one of the mechanisms for inhibition of CT replication in IFN-γ-activated peritoneal macrophages and RAW264.7 cells; (2) NO plays a significant role in CT inhibition in LPS-treated macrophage cell lines but not peritoneal macrophages; (3) TNF-α may be associated with inhibition, but the mechanism(s) may not involve NO production; (4) NO production may not be the mechanism for CT inhibition in McCoy cells treated with IFN-γ.

Macrophage activation induced by IFN-γ or LPS is a complicated process which involves many biological and biochemical events including receptor-ligand binding, signal transduction, gene expression, cytokine secretion and feedback regulation (Adams and Hamilton 1992). Although we demonstrated that NO and secreted TNF-α are involved in inhibition of CT replication in peritoneal macrophages and macrophage cell lines activated by IFN-γ or LPS, the whole picture of inhibition in activated macrophages remains unclear. Complete understanding of the interaction between macrophages and CT would have a significant impact on elucidation of the

immune response against CT infection as well as prevention of infection.

BIBLIOGRAPHY

Adams, D. O. and Hamilton, T. A. 1992. Molecular basis of macrophage activation: diversity and its origins. In: Lewis, C. E.; McGee, J. O'D. ed. The Macrophage. The Natureal Immune System. Oxford Press, Oxford, pp. 75-114.

Adams, D. O. and Hamilton, T. A. 1984. The cell biology of macrophage activation. Ann. Rev. Immunol. 2: 283-318.

Adams, L. B.; Hibbs, J. B.; Taintor, R. R. and Krahenbuhl, J. L. 1990. Microbiostatic effect of murine macrophages for Taxoplasma gondii: role of synthesis of inorganic nitrogen oxides from L-anginine. J. Immunol. 144: 2725-2729.

ATCC Catalogue of Cell Lines and Hybridomas 1988 pp 253-254.

Auger, M. J. and Ross, J. A. 1992. Macrophage functions. In: Lewis, C. E.; McGee, J. O'D. ed. The Macrophage. The Natureal Immune System. Oxford Press, Oxford, pp. 31-74.

Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith and J. A. Struhl, K. Short Protocols in Molecular Biology, Second Edition, Greene Publishing Associates and John Wiley & Sons. U.S.A. PP. 1-24-1-25.

Baehr, W.; Shang, Y.-X.; Joseph, T.; Su, H.; Nano, F. E.; Everett, K. D. E.; Caldwell, H. D. 1988. Mapping antigenic domains expressed by Chlamydia trachomatis outer membrane protein genes. Proc. Natl. Acad. Sci. 85: 4000-4004.

Bard, J.; Hall, J. and Levitt, D. 1987. *Chlamydia trachomatis* (L_2 serovar) can be bound, ingested and destroyed by differentiated but not by undifferentiated human promyelocyte cell line HL-60. J. General. Microbiol. 133: 899-910.

Bard, J. and Levitt, D. 1984. Chlamydia trachomatis stimulates human peripheral blood B lymphocytes to proliferate and secrete polyclonal immunoglobulins in vitro. Infect. Immun. 43:84-92.

Bard, J. and Levitt, D. 1985. Binding, ingestion, and multiplication of Chlamydia trachomatis (L2 serovar) in human leukocyte cell lines. Infect. Immun. 50:935-937.

Becker, Y. 1978. The Chlamydiae: molecular biology of procaryotic obligated parasites of eucaryotes. Microbiol. Rev. 42: 274-306.

Billiar T. R.; Ochoa, J. B.; Curti, B. 1991. Increased circulationg nitrogen oxides after human tumor immunotherapy correlate with toxic hemodynamic changes [abstract]. In: Program and abstracts: Biology of Nitric Oxide, 2nd International Meeting (London).

Bogdan C.; Stenger, S.; Rollinghoff, M. and Lolbach, W. 1991. Cytokine ineractions in experimental cutaneous leishmaniasis. Interleukin-4 synergizes with interferon-γ to activated murine macrophage for killing of Leishmania mafor amastigotes. Eur. J Immunol. 21: 327-333.

Bowie, W. R. 1984. Urethritis in males. In: Sexually transmitted diseases. Holmes, K. K.; Mardh, P.-A.; Sparling, P. F.; Wiesner, P. J. eds. New York. McGraw-Hill. PP. 638-650.

Bredt, D. S.; Hwang, P. M.; Glatt, C. E.; Lowenstein, C.; Reed, R. R. and Snyder, S. H. 1991. Nature 351: 714-718.

Brunham, R. C.; Kuo, C.-C. and Chen, W.-J. 1985. Systemic *Chlamydia trachomatis* infection in mice: a comparison of lymphogranuloma venereum and trachoma biovars. Infect. Immun. 48: 78-82.

Brunham, R. C.; Martin, D. H.; Kuo, C.-C.; Wang, S.-P.; Stevens, C. E.; Hubbard, T.; Holmes, K. K. 1981. Cellular Immune response during uncomplicated genital infection with Chlamydia trachomatis in humans. Infect. Immun. 34: 98-104.

Buchmuller-Rouiller, Y. and Mauel, J. 1991. Macrophage activation for intracellular killing as induced by calcium ionophore. Correlation with biological and biochemical events. J. Immunol. 146: 217-223.

Byrne, G. I.; Carlin, J. M.; Merkert, T. P.; Arter, D. L. 1989. Long-term effects of gamma interferon on Chlamydia-infected host cells: microbicidal activity follows microbiostasis. Infect. Immun. 57: 1318-1320.

Byrne, G. I.; Lehmann, L. K. and Landry, G. J. 1986. Induction of tryptophan catabolosm is the mechanism for gamma-interferon-mediated inhibitio of intracellular Chlamudia psittaci replication in T24 cells. Infect. Immun. 53: 347-351.

Byrne, G.I., and Moulder, J.W. 1978. Parasite-specified phagocytosis of Chlamydia psittaci and Chlamydia trachomatis by L and Hela cells. Infect. Immun. 19:598-606.

Byrne, G. I.; Rothermel, C. D. 1983. Differential susceptibility of chlamydiae to exogenous fibroblast inerferon. Infect. Immun. 39: 1004-1005.

Caldwell, H.D., and Perry, fL.J. 1982. Neutralization of Chlamydia trachomatis infectivity with antibodies to the major outer membrane protein. Infect. Immun. 38:745-754.

Cameron, M. L.; Granger, D. L.; Weinberg, J. B.; Kozumbo, W. J. and Koren, H. S. 1990. Human alveolar and peritoneal macrophages mediate fungistasis

independently of L-arginine oxidation to nitrite and nitrate. Am. Rev. Respir. Dis. 142: 1313-1319.

Campbell, W. F.; Dodson, M. G. 1990. Clindamycin therapy for Chlamydia trachomatis in women. Am. J. Obstet. Gynecol. 162:343-347.

Carlin, J. M.; Borden, E. C.; Byrne, G. I. 1989. Inerferon-induced indoleamine 2,3-dioxygenase activity inhibits Chlamydia psittaci replication in human macrophages. J. Interferon Res. 9: 329-337.

Celada, A.; Gray, P. W.; Rinderknecht, F. and Schreiber, R. D. 1984. Evidence for a gamma inerferon receptor that regulates macrophage tumoricidal activity. J. Exp. Med. 160: 55-74.

Centers for Disease Control. 1985. Chlamydia trachomatis infections: Policy guidelines for prevention and control. Morbid. Mortal. Weekly Rep. 34(Suppl.):53S-74S.

Chow, J. M.; Yonekura, M. L.; Richwald, G. A.; Greenland, S.; Sweet, R. L.; Schachter J. 1990. The association between Chlamydia trachomatis and ectopic pregnancy. A matched pair, case-controlled study. JAMA 263:3164-3167.

Cleveland, D. W.; Lopata, M. A.; MacDonald, R. J.; Cowan, N. J.; Rutter, W. J. and Kirschner, M. W. 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell 20: 95-105.

Cunha, F. Q.; Moncada, S. and Liew, F. Y. 1992. Interleukin-10 (IL-10) hinibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. Biochem. Biophysic. Res. Commun. 182: 1155-1159.

Davis, L.G.; Dibner, M. D. and Battey, J. F. 1986. Basic methods in molecular biology. Elsevier Science Publishing Co., New York. pp. 102-104.

de la Maza, L. M.; Gocbel, J. M.; Czarniecke, C. W.; Peterson, E. M. 1984. Untrastructurel analysis of the growth cycle of Chlamydia trachomatis in mouse cells treated with recombinant huma α-interferons. Exp. Mol. Pathol. 41: 227-235.

de la Maza, L. M.; Peterson, E. M.; Burton, L. E.; Gray, P. W.; Rinderknecht, E. and Czarniecki, C. W. 1987. The antichlamydial, antiviral, and antiproliferative activities of human gamma inerferon are dependent on the integrity of the C terminus of the inerferon molecule. Infect. Immun. 55: 2727-2733.

de la Maza, L. M.; Peterson, W. M.; Fennie, C. W.; Czarniecki, C. W. 1985. The antichlamydial and antiproliferative activities of recombinant murine interferon are not dependent on tryptophan concentration. J. Immunol. 135: 4198-4200.

Denis, M. 1991a. Tumor necrosis factorr and granulocyte-macrophage colony-stimulating factor stimulate human macrophages to restrict growth of virulent Mycobacterium avium and to kill avirulent M. avium: killing effector mechanism depends on the generation of reactive nitrogen intermediates. J. Leukoc. Biol. 49: 380-387.

Denis, M. 1991b. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell Immunol. 132: 150-156.

Ding, A. H.; Nathan, C. F. and Stuehr, D. J. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediate from mouse pertoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. 141: 2407-2412.

Ding, A. H.; Nathan, C. F.; Granger, J.; Derynck, R.; Stuehr, D. J. and Srimal, S. 1990. Macrophage deactivating factor and transforming growth factors-β1, β2, and β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. J. Immunol. 145: 940-944.

Drapier, J. C.; Wietzerbin, J. and Hibbs, J. B. 1988. Interferon-γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector, mechanism in murine macrophages. Eur. J. Immunol. 18: 1587-1592.

Eisermann, J.; Odem, R. R.; Gast, M. J.; Collins, J. L. 1988. Tumor necrosis factor in pertoneal fluid of women undergoing laparoscopic surgery. Fertil. Steril. 50: 573-579.

Fitch, F. W.; McKisic, M. D.; Lancki, D. W. and Gajewski, T. F. 1993. Differential regulation of murine T lymphocyte subsets. Annu. Rev. Immunol. 11: 29-48.

Freeman, B. A. 1985. Burrows Textbook of Microbiology. W. B. Saunders Co., pp. 676-684.

Friis, R.R. 1972. Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development. J. Bacteriol. 110:706-721.

Granger, D. L.; Hibbs, J. B.; Perfect, J. R. and Durack, D. T. 1990. Metabolic fate of L-arginine in relation to microbiostyatic capability of murine macrophages. J. Clin.

Invest. 85: 264-273.

Granger, D. L.; Hibbs, J. B.; Perfect, J. R. and Durack, D. T. 1991. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J. Clin. Invest. 81: 1129-1136.

Granger, D. L.; Perfect, J. R. and Durack, D. T. 1986a. Macrophage-mediated fungistasis in vitro: requirements for inrtracellular cytotoxicity. J. Immunol. 136: 672-680.

Granger, D. L.; Perfect, J. R. and Durack, D. T. 1986b. Macrophage-mediated fungistasis: requirements for a macromolecular component in serum. J. Immunol. 137: 693-701.

Green, S. J.; Crawford, R. M.; Hockmeyer, J. T.; Meltzer, M. S. and Nacy, C. A. 1990a. Leishmania mafor amastigotes initiate the L-arginine dependent killing mechanism in IFN-γ-stimulated macrphages by induction of tumor necrosis factor-α. J. Immunol. 145: 4290-4297.

Green, S. J.; Meltzer, M. S.; Hibbs, J. B.; and Nacy, C. A. 1990b. Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine dependent killing mechanism. J. Immunol. 144: 278-283.

Green, S. J.; Nacy, C. A. and Meltzer, M. S. 1991. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular parasites. J. Leukoc. Biol. 50: 93-103.

Hansen, M. B., et al. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119: 203-210.

Hatch, T. P. 1988. Metabolism of Chlamydia. In: Barron, A. L. ed. Microbiology of Chlamydia CRC Press, Florida, pp. 97-109.

Hatch, T. P.; Allan, I.; Pearce. 1984. Structural and ppolypeptide differences between envelopes of infective and reproductive life cycle forms of Chlamydia spp. J. Bacteriol. 157: 13-20.

Hearn, S. A.; McNabb, G. L. 1991. Immunoelectron microscopic localization of chlamydial lipopolysaccharide (LPS) in McCoy cells inoculated with Chlamydia trachomatis. J. Histochem. Cytochem. 39:1067-1075.

Henry-Suchet, J, 1988. Chlamydia trachomatis infection and infertility in women. J. Reprod. Med. 33:912-914.

Hibbs, J. B.; Vavrin, Z. and Taintor, R. R. 1987. L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. 138: 550-565.

Hodinka, R. L.; Davis, C. H.; Choong, J.; Wyrick, P. B. 1988. Ultrastructural study of endocytosis of Chlamydia trachomatis by McCoy cells. Infect. Immun. 56:1456-1463.

Holmes, K. K. 1981. The chlamydia epidemic. JAMA 245:1718-1723.

Holtmann, H.; Shemer-Avni, Y.; Wessel, K.; Sarov, I. and Wallach, D. 1990. Inhibition of growth of Chlamydia trachomatis by tumor necrosis factor is accompanied by increased prodtaglandin synthesis. Infect. Immun. 58: 3168-3172.

Huebner, R. E. and Byrne, G. I. 1988. In vivo-activated mononuclear phagocytes and protective immunity to *Chlamydiae* in mice. Infect. Immun. 56: 1492-1499.

Igietsume, J. U.; Ramsey, K. H.; Magee, D. M.; Williams, D. W.; Theus, S.; Rank, R. G. 1991. The role of MoPn-specific Th1 cell in the control of chlamydial genital infection in mice. Chlamydia trachomatis genital infection. Tri-State Symposium. Johnson City, TN.

Ishikawa, H.; Ohno, O.; Hamasaki, K.; Ikuta, S. and Hirohata, K. 1986. Arthritis presumably caused by Chlamydia in Reiter's syndrome. J. Bone Joint Surg. 68: 777-779.

Iyengar, R.; Stuehr, D. J. and Marletta, M. A. 1987. Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. Proc. Natl. Acad. Sci. USA 84: 6369-6373.

James, S. L. and Claven, J. 1989. Macrophage cytotoxicity against schistosomula of Schistosoma mansoni involves arginine-dependent production of reactive nitrogen intermediates. J. Immunol. 143: 4208-4212.

James, S. L.; Cook, K. W.; and Lazdins, J. K. 1990. Activation of human monocyte-derived macrophages to kill schistosomula of *Schistosoma mansoni* in vitro. J. Immunol. 145: 2686-2690.

Jerrells, T. R. 1983. Association of an inflammatory I region-associated antigen-positive macrophage influx and genetic resistance of inbred mice to *Rickettsia tsutsugamushi*. Infect. Immun. 42: 549-557.

Johnson, A.P. 1985. Pathogenesis and immunology of chlamydial infections of the genital tract. Rev. Infect. Dis. 7: 741-745.

Jue, D.-M.; Sherry, B.; Luedke, C.; Manogue, K. R. and Cerami, A. 1990. Processing of Newly synthesized cachectin/tumor necrosis factor in endotoxin-stimulated macrophages. Biochemistry 29: 8371-8377.

Kazar, J.; Gilmore, J. D. and Gordon, F. B. 1971. Effect of interferon and interferon inducers on infections with a nonviral intracellular microorganism, Chlamydia trachomatis. Infect. Immun. 3: 825-832.

Keat, A.; Thomas, B.; Dixey, J.; Osborn, M.; Sonnex, C. and Taylor-Robinson, D. 1987(1). C. trachomatis and reactive arthritis: the missing link. Lancet: 72-74.

Kelly, N. M.; Young, L. and Cross, A. S. 1991. Differential induction of tumor necrosis factor by bacteria expressing rough and smooth lipopolysaccharide phenotypes. Infect. Immun. 59: 4491-4496.

Koga, T.; Mitsuyama, M.; Handa, T.; Watanabe, Y. and Nomoto, K. 1987. Gamma interferon-mediated increase in the number of Ia-bearing macrophages during infection with *Listeria monocytogenes*. Infect. Immun. 55: 2300-2303.

Kolb, H. and Kolb-Bachofen, V. 1992. Nitric oxide: a pathogenetic factor in autoimmunity. Immunol. Today 13: 157-160.

Kuo, C.-C. 1978. Cultures of *Chlamydia trachomatis* in mouse peritoneal macrophages: factors affecting organism growth. Infect. Immun. 20: 439-445.

Lammert, J. 1982. Cytotoxic cells induced after Chlamydia psittaci infection in mice. Infect. Immun. 35:1011-1017.

Lammont, H. C. and Nichols, R. L. 1981. Immunology of chlamydial infections. In: Nahmias, A. J. and O'Reilly, J. ed. Immunology of Human Infection. New York: Plenum. pp. 441-474.

Lee, C. K.; Moulder, J. W. 1981. Persistent infection of mouse fibroblasts (McCoy cells) with a trachoma strain of Chlamydia trachomatis. Infect. Immun. 32:822-829.

Leinonen, M. 1992. Cell biology. In: Mardh, P.-A.; Placa, M. L.; Ward, M. eds. Proceedings of the European Society for Chlamydia research. Uppsala University Centre for STD Research. PP. 3-5.

Levitt, D.; Danen, R. and Bard, J. 1986. Both species of *Chlamydia* and tow biovars of *Chlamydia trachomatis* stimulate mouse B lymphocytes. J. Immunol. 136: 4249-4254.

Lewis, M. A.; Slater, J. S.; Leverone, L. I. and Campbell, A. E. 1990.

Enhancement of interleukin-1 activity by murine cytomegalovirus infection of a macrophage cell line. Virology 178: 452-460.

Liew, F. Y. and Cox, F. E. G. 1991a. Nonspecific defence mechanism: the role of nitric oxide. Immunol. Today 12: A17-A21.

Liew, F. Y.; Li, Y.; Severn, A.; Millott, S.; Schmidt, J.; Salter, M. and Moncada, S. 1991b. A possible novel pathway of regulation by murine T helper type-2 (Th2) cells of a Th1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. Eur. J. Immunol. 21: 2489-2494.

Liew, F. Y.; Li, Y. and Millott, S. 1990a. Tumor necrosis factor in leishmaniasis: II. TNFα induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology 71: 556-559.

Liew, F. Y.; Millott, S.; Parkinson, C.; Palmer, R. M. J. and Moncada, S. 1990b. Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. J. Immunol. 144: 4794-4797.

Liew, F. Y.; Yun, L. and Millott, S. 1990c. Tumor necrosis facor-α with IFN-γ in mediating killing Leishmania major through the induction of nitric oxide J. Immunol. 145: 4306-4310.

Lin, J.-S.; Ho, Y.; Rice, P. A. 1990. Functions of antibodies and complements in the neutralization of Chlamydia trachomatis infectivity by human sera. In: Bowie, W. R.; Caldwell, H. D.; Jones, R. P., Mardh, P.-A.; Ridgway, G. L.; Schachter, J.; Stamm, W. E.; Ward, M. E. ed. Chlamydial infections. Proceedings of the Seventh International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge, pp. 193-196.

Lin, J. Y. and Chadee, K. 1992. Macrophage cytotoxicity against *entamoeba histolytica* trophozoites is mediated by nitric oxide from L-anginine. J. Immunol. 148: 3999-4005.

Lipkin, E. S.; Moncada, J. V.; Shafer, M.; Wilson, T. E.; Schachter, J. 1986. Comparison of monoclonal antibody staining and culture in diagnosing cervical chlamydial infection. J. Clin. Microbiol. 23:114-117.

Lowenstein, C. J.; Glatt, C. S.; Bredt, D. S. and Snyder, S. H. 1992. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. Proc. Natl. Acad. Sci. USA. 89: 6711-6715.

Lyons, C. R.; Orloff, G. J. and Cunningham, J. M. 1992. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage

cell line. J. Biolog. Chemis. 267: 6370-6374.

Mamat, U.; Lobau, s.; Brade, L.; Schmidt, G.; Brade, H. 1992. Heterogeneity of a gene of chlamydial LPS biosynthesis among the three species. In: Mardh, P.-A.; Placa, M. L.; Ward, M. eds Proceedings of the European Sociey for Chlamydia research. PP. 36-36.

Manor, E.; Sarov, I. 1986. Fate of Chlamydia trachomatis in human monocytes and monocyte-derived macrophages. Infect. Immun. 54: 90-95.

Manor, E. and Sarov, I. 1988. Inhibition of *Chlamydia trachomatis* replication in HEp-2 cells by human monocyte-derived macrophages. Infect. Immun. 56: 3280-3284.

Manor, E.; Schmitz, E. and Sarov, I. 1990. TNF-α, IL-1 and PGE_2 production by human monocyte-derived macrophages infected with C. trachomatis. In: Bowie, W. R.; Caldwell, H. D.; Jones, R. P., Mardh, P.-A.; Ridgway, G. L.; Schachter, J.; Stamm, W. E.; Ward, M. E. ed. Chlamydial infections. Proceedings of the Seventh International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge, pp. 209-212.

Mardh, P.-A.; Paavonen, J.; Puolakkainen, M. 1989. Chlamydia, Plenum Medical Book Co., New York and London, pp. 15-25.

Marletta, M. A.; Yoon, P. S.; Iyengar, R.; Leaf, C. D. and Wishnok, J. S. 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry 27: 8706-8711.

Martin, C. A. and Dorf, M. E. 1990. Interleukin-6 production by murine macrophage cell lines P388D1 and J774A.1: Stimulation requirements and kinetics. Cellular Immunol. 128: 555-568.

Matsumoto, A.; Manire, G. P. 1970. Electron microscopic observations on the effect of penicillin on the morphology of Chlamydia psittaci. J. Bacteriol. 101:278-285.

Matsumoto, A. 1988. Structural characteristics of chlamydial bodies. In: Barron, A. L. ed. Microbiology of Chlamydia CRC Press, Florida, pp. 21-45.

Mauel, J.; Ransign, A. and Buchmuller-Rouiller, Y. 1991. Killing of Leishmania parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen dervatives. J. Leukoc. Biol. 49: 73-82.

Mayer, J.; Woods, M. L.; Vavrin, Z. and Hibbs, J. B. 1993. Gamma interferon-

induced nitric oxide production reduces *Chlamydia trachomatis* infectivity in McCoy cells. Infect. Immun. 61: 491-497.

Mishell, B. B.; Shiigi, S. M. 1980a. Selected Methods in Cellular Immunology W. H. Freeman and Co., San Francisco, Ca., pp. 16-17.

Mishell, B. B. and Shiigi, S. M. 1980b. Selected Methods in Cellular Immunology, W.H. Freeman and Company, San Francisco, PP. 6-8.

Moncada, S.; Palmer, R. M. J. and Higgs, E. A. 1991. Nitric oside: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43: 109-142.

Moulder, J. W. 1991. Interaction of chlamydiae and host cells in itro. Microbiol. Rev. 55:143-190.

Murray, H. W.; Byrne, G. I.; Rothermel, C. D. and Cartelli. 1983. Lymphokine enhances oxygen-independent activity against intracellular pathogens. J. Exp. Med. 158: 234-239.

Murray, H. W.; Granger, A. M. and Teitelbaum. 1991. Gamma interferon-activated human macrophages and *Toxoplasma gondii, Chlamydia psittaci, and Leishmania donovani*: antimicrobial role of limiting intracellular iron. Infect. Immun. 59: 4684-4686.

Murray, H. W.; Szuro-Sudol, A. and Wellner, D. 1989. Role of tryptophan in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. Infect. Immun. 57: 845-849.

Murray, H. W. and Teitelbaum, R. F. 1992. L-Arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. J Infect. Dis. 165: 513-517.

Nacy, C. A.; Belosevic, M.; Meltzer, M. S. and Hoover, D. L. 1988. Interferon and nonviral pathogens. Immunol. Ser. 42: 167-172.

Nakajo, M. N.; Roblin, P. M.; Hammerschlag, M. R.; Smith, P.; Nowakowski, M. 1990. Chlamydicidal activity of human alveolar macrophages. Infect. Immun. 58: 3640-3644.

Nakajo, M. N.; Roblin, P. M.; Hammerschlag, M. R.; Smith, P.; Nowakowski, M. 1988. Interaction of alveolar macrophages (AM) with *Chlamydia trachomatis* (Ct). Pediatr. Res. 23: 377A.

Nathan, C. F.; Murray, H. W.; Wiebe, M. E. and Rubin, B. R. 1983. Identification

of inerferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. 158: 670-686.

Nettelnbreker, E.; Schmitz, E.; Zeidler, H.; Hammer, M.; Wollenhaupt, J. 1992. Persistence of C. trachomatis erovar K in human monocytes (PBMo) after in-vitro infection. In: Mardh, P.-A.; Placa, M. L.; Ward, M. ed. Proceedings of the Europen Society for Chlamydia Research. University of Uppsala, Uppsala, Sweden, pp. 82.

Newhall, W. J. 1987. Biosynthesis and disulfide cross-linking of outer membrane components during the growth cycle of Chlamydia trachomatis. Infect. Immun. 55: 162-168.

Oh, K.-O.; Zhou, Z.; Kim, K.-K.; Samanta, H.; Fraser, M.; Kim, Y.-J.; Broxmeyer, H. E. and Kwon, B. S. 1991. Identification of cell surface receptors for murine macrophage inflammatory protein-1α. J. Immunol. 147: 3978-2983.

Newhall, W. J. 1988. Macromolecular and antigenic composition of chlamydiae. In: Barron, A. L. ed. Microbiology of Chlamydia CRC Press, Florida, pp. 47-70.

Ozanne, G. and Pearce, J. H. 1980. Inapparent chlamydial infection in the urogenital tract of guinea pigs J. Gen. Microbiol. 119: 351-359.

Pace, J. L.; Russel, S. W.; Torres, B. A.; Johnson, H. M. and Gray, P. W. 1983. Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. 130: 2011-2013.

Peeling, R.W. Maclean, I.W. and Brunham, R.C. 1984. In vitro neutralization of Chlamydia trachomatis with monoclonal antibody to an epitope of the major outer membrane protein. Infect. Immun. 46:484-488.

Peterson, E. M.; Cheng, X.; Pal, S. and Maza, L. M. 1993. Effects of antibody isotype and host cell type on in vitro neutralization of Chlamydia trachomatis. Infect. Immun. 61: 498-503.

Qvigstad, E., et al. 1985. Cell-mediated and humoral immune responses to chlamydial and herpesvirus antigens in patients with cervical carcinoma. Gynecol. Oncol. 20:184-189.

Radolf, J. D.; Norgard, M. V.; Brandt, M. E.; Isaacs, R. D.; Thompson, P. A. and Beutler, B. 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. J. Immunol. 147: 1968-1974.

Ramsey, K. H. and Rank, R. G. 1991. Resolution of chlamydial genital infection

with antigen-specific T-lymphpcyte lines. Infect. Immun. 59: 925-

Ramsey, K. H. and Rank, R. G. 1990. The role of T cell subpopulations in resolution of chlamydial genital infection of mice. In: Bowie, W. R.; Caldwell, H. D.; Jones, R. P.; Mardh, P.-A.; Ridgway, G.; Schachter, J.; Stamm, W. E.; Ward, M. E. ed. Chlamydial infections. Proceedings of the Seventh International symposium on Human Chlamydial Infections. Cambredge University Press, Cambridge, PP.241-244.

Ramsey, K. H.; Soderberg, L. S. F.; Rank, R. G. 1988. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. Infect. Immun. 56:1320-1325.

Rank, R. G. and Barron, A. L. 1983. Humoral immune response in acquired immunity to chlamydial genital infection of female guinea pigs. Infect. Immun. 39: 463-465.

Rank, R.G., et al. 1985. Chronic chlamydial genital infection in congenitally athymic nude mice. Infect. Immun. 48: 847-849.

Rank, R. G. et al. 1989. Role of cell-mediated immunity in resolution of secondary chlamydial genital infection in guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. Infect. Immun. 57: 706-710.

Rothermel, C. D.; Byrne, G. I.; Havell, E. A. 1983. Effect of interferon on the growth of Chlamydia trachomatis in mouse fibroblasts (L cells). Infect. Immun. 39: 362-370.

Rothermel, C. D.; Rubin, B. Y.; Jaffe, E. A.; Murray, H. W. 1986. Oxygen independent inhibition of intracellualar Chlamydia psittaci grwth by human monocytes and inerferon-γ-activated macrophages. J. Immunol. 137: 689-692.

Rothermel, C. D.; Rubin, B. Y.; Murray, H. W. 1983. γ-Interferon is the factor in lymphokine that activates human macrophages to inhibit intracellular Chlamyudia psittaci replication. J. Immunol. 131: 2542-2544.

Rothermel, C. D.; Schachter, J.; Lavrich, P.; Lipsitz, E. C. and Francus, T. 1989. Chlamydia trachomatis-induced production of interleukin-1 by human monocytes. Infect. Immun. 57: 2705-2711.

Ruff, M. R. and Gifford, G. E. 1981. Rabbit tumor necrosis factor: mechanism of action. Infect. Immun. 31: 380-385.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989a. Molecular Cloning. A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, U. S. A., pp. 6.24-

6.27.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989b. Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, U. S. A., pp. 7.37-9.55.

Schachter, J. 1978. Chlamydial infections. N. Eng. J. Med. 298:540-549.

Schachter, J.; Moncada, J.; Dawson, C. R.; Sheppard, J.; Courtright P.; Said, M. E.; Zaki, S.; Hafez, S. F.; Lorincz, A. 1988. Nonculture methods for diagnosing chlamydial infection in patients with trachoma: a clue to the pathogenesis of the disease? J. Inf. Dis. 158:1347-1352.

Schaefer, C.; Harrison, R. H.; Boyce, W. T.; Lewis, M. 1985. Illnesses in infants born to women with Chlamydia trachomatis infections. AJDC 139:127-133.

Schumacher, R. H. et al. 1988. Light and electron microscopic studies on the synovial membrane in Reiter's syndrrome. Arth. Rheum. 31: 937-946.

Sessa, W. C.; Harrison, K. J.;Barber, C. M.; Zeng, d.; Durieux, M. E.; D'Angelo, D. D.; Lynch, K. R. and Peach, M. J. 1992. Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. J. Biolog. Chemis. 267: 15274-15276.

Shemer-Avni, Y.; Holtman, H.; Wallach, D.; Sarom, I. 1990. Cytokine-cell interactions resulting in Chlamydia trachomatis inhibition-mechanisms involved. In: Bowie, W. R.; Caldwell, H. D.; Jones, R. P., Mardh, P.-A.; Ridgway, G. L.; Schachter, J.; Stamm, W. E.; Ward, M. E. ed. Chlamydial infections, Proceedings of the Seventh International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge, pp. 173-176.

Shemer-Avni, Y.; Kol, R. and Sarov, I. 1987. TRyptophan reversal of recombinant human gamma-interferon inhibition of Chlamydia trachomatis growth. Curr. Microbiol. 16: 9-13.

Shemer-Avni, Y. and Sarov, I. 1985. Inhibition of growth of Chlamydia trachomatis by human gamma interferon. Infect. Immun. 48: 592-596.

Shemer-Avni, Y., et al. 1989. Reversion of the antichlamydial effect of tumor necrosis factor (TNF) by tryptophan and antibodies to interferon beta. Infect. Immun. 57: 3484-3490.

Shemer-Avni, Y.; Wallach, D.; Sarov, I. 1988. Inhibition of Chlamydia trachomatis growth by recombinant tumor necrosis factor. Infect. Immun. 56: 2503-2506.

Smith, T. F. 1979. Virology Laboratory Procedures. Section of Clinical Microbiology, Mayo Clinic, Rochester, Minnesota, pp. 1-14.

Stephens, R. S.; Wagar, E. A.; Schoolnik, G. R. 1988. High resolution mapping of serovar specific and common antigenic determinants of the major outer membrane protein of Chlamydia trachomatis. J. Exp. Med. 167: 817-831.

Stuehr, D. J. and Marletta, M. A. 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc. Natl. Acad. Sci. USA. 82: 7738-7742.

Stuehr, D. J. and Marletta, M. A. 1987a. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-γ. J. Immunol. 139: 518-525.

Stuehr, D. J. and Marletta, M. A. 1987b. Synthesis of nitrite and nitrate in murine macrophage cell lines. Cancer Res. 47: 5590-5594.

Stuehr, D. J. and Nathan, C. F. 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. 169: 1543-1555.

Tahija, S. G.; Carlin, J. M.; Byrne, G. I.; Miller, S. L. and Rapoza, P. A. 1990. IFN-γ-mediated inhibition of an ocular strain of Chlamydia trachomatis in primary tissue cultures of human conjunctival epithelium. In: Bowie, W. R.; Caldwell, H. D.; Jones, R. P., Mardh, P.-A.; Ridgway, G. L.; Schachter, J.; Stamm, W. E.; Ward, M. E. ed. Chlamydial infections. Proceedings of the Seventh International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge, pp. 213-216.

T'ang, F. F.; Chang, H. L.; Huang, Y. T.; Wang, K. C. 1957. Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. Chin. Med. J. 75: 429-447.

Tartaglia, L. A. and Goeddel, D. V. 1992. Two TNF receptors. Immunol. Today 13: 151-153.

Tau-Cody, K. R. 1992. Dissertation: Molecular and cellular analysis of Chlamydia trachomatis persistance and reactivation. PP. 12-15.

Thelin, I.; Wennstrom, A. M.; Mardh, P.-A. 1980. Contact-tracing in patients with genital chlamydial infection. Br. J. Vener. Dis. 56: 259-262.

Todd, W. J.; Caldwell, H. D. 1985. The interaction of Chlamydia trachomatis with

host cells: ultrastructural studies of the mechanism of release of a biovar II strain from HeLa 229 cells. J. Inf. Dis. 151:1037-1044.

Walters, M. D.; Eddy, C. A.; Gibbs, R. S.; Schachter, J.; Holden, A. E. C.; Pauerstein, C. J. 1988. Antibodies to Chlamydia trachomatis and risk for tubal pregnancy. Am. J. Obstet. Gynecol. 159:942-946.

Wang, S.-P.; Grayston, J. T. 1982. Microimmunofluorescence antibody responses in Chlamydia trachmatis infection. In: Hardh, P.-A.; Holmes, K. K.; Oriel, J. D.; Plot, P.; Schachter, J. eds. A review in chlamydial infections. Elsevier Biomedical Press. Amsterdam. PP. 301-301

Wang, S.-P.; Grayston, J. T. 1991. Three new serovars of Chlamydia trachomatis: Da, Ia, L_2a. J. Inf. Dis. 163:403-405.

Ward, M. E. 1988. Metabolism of Chlamydia. In: Barron, A. L. ed. Microbiology of Chlamydia, CRC Press, Florida, pp. 71-109.

Weisburg, W. G.; Hatch, T. T.; Woese, C. R. 1986. Eubaterial origin of Chlamydiae. J. Bacteriol. 167: 570-574.

Williams, D. M.; Magee, D. M.; Bonewald, L. F.; Smith, J. G.; Bleicker, C. A.; Byrne, G. I. and Schachter, J. 1990. A role in vivo for tumor necrosis factor alpha in host defense against *Chlamydia trachomatis*. Infect. Immun. 58: 1572-1576.

Wynn, T. A.; Nicolet, C. M. and Paulnock, D. M. 1991. Identification and characterization of a new gene family induced during macrophage activation. J. Immunol. 147: 4384-4392.

Wyrick, P. B. and Brownridge, E. A. 1978. Growth of *Chlamydia psittaci* in macrophages. Infect. Immun. 19: 1054-1060.

Wyrick, P. B.; Choong, J.; Davis, C. H.; Knight, S. T.; Royal, M. O., Maslow, A. S.; Bagnell, C. R. 1989. Entry of Chlamydia trachomatis into polarized human epithelial cells. Infect. Immun. 57:2378-2389.

Xie, Q.-W.; Cho, H. J.; Calaycay, J.; Mumford, R. A.; Swiderek, K. M.; Lee, T. D.; Ding, A.; Troso, T.; Bathan, C. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256: 225-228.

Yong, E. C.; Chi, E. Y.; Kuo, C.-C. 1987. Differential antimicrobial activity of human mononuclear phagocytes against the human biovars of Chlamydia trachomatis. J. Immunol. 139: 1297-1302.

Zhang, Y.-X.; Stewart, S.; Joseph, T.; Hayler, H. R.; Caldwell, H. D. 1987. Protective monoclonal antibodies recognise epitopes located on the major outer membrane prtein of Chlamydia trachmatis/ J. Immunol. 138: 575-581.

Zhong, G. and de la Maza, L.M. 1988. Activation of mouse peritoneal macrophages in vitro or in vivo by recombinant murine gamma interferon inhibits the growth of Chlamydia trachomatis serovar L1. Infect. Immun. 56:3322-3325.

Zhong, G. et al. 1989. Role of endogenous gamma interferon in host defense against *Chlamydia trachomatis* infections. Infect. Immun. 57: 152-157.

VITA

Bojun Chen

Personal Data:	Date of Birth:	Nov. 4, 1955
	Place of Birth:	Jiangxi, China
	Marital Status:	Married

Education:

Yushan High School, Jiangxi, China
1973

Jiangxi Medical College, Nanchang, China,
Medicine
M.D., 1982

Peking Union Medical College, Beijing, China,
Infectious Disease
M.S., 1985

East Tennessee State University, Johnson City,
Tennessee, Biomedical Science
Ph.D., 1993

Professional Experience:

Graduate Assistant, Division of Infectious Disease, Department of Internal Medicine, Peking Union Medical College, Beijing, China, 1982-1985

Research Assistant & Resident, General Internal Medicine, Peking Union Medical College Hospital, Beijing, China, 1986-1988

Graduate Assistant, Department of Microbiology, James H. Quillen College of Medicine, East Tennessee State University, 1989-present

Publications:

Chen, B.; Wang, S.; Wang, A.; Li, B.; Yang, S.; Wang, X. and Liu, C. 1988. Clinical Significance of Anti-HBc and Anti-HBc IgM. Acta Academiae Medicinae Sinicae 10 (2): 104-109.

Chen, B.; Wang, S.; Wang, A.; Li, B.; Yang, S.; Wang, X. and Liu, C. 1988. Significance of Anti-EBV IgM and Anti-CMV IgM in Acute or Chronic Viral Hepatitis. Chinese J Infec. Dis. 6 (4): 217-219.

Papers Presented at Regional and National Meetings

Chen, B.; Tau-Cody, K.R.; Wang, H.; Stout, R.D. Campbell, W.F. 1991. Cellular Immune Responses of Mice infected by Chlamydia trachomatis. 91st General Meeting of the American Society for Microbiology, Dallas, TX

Chen, B.; Stout, R.D. and Campbell, W.F. 1991, the effects of Cytokines upon Chlamydia trachomatis Infection In Vitro, Tri-State Chlamydia Symposium, Johnson City, TN

Tau-Cody, K.R.; Wang, H.; Chen, B. and Campbell, W.F. 1991. Chlamydia trachmatis Produces Persistent Genital Infection in Mice, Tri-State Chlamydia Symposium, Johnson City, TN

Chen, B.; Stout, R.D. and Campbell, W.F. 1992. Effects of Macrophages and Cytokines upon Chlamydia trachmatis Infection In Vitro. 92nd General Meeting of the American Society for Microbiology, New Orleans, LA

Tau-Cody, K.R.; Wang, H.; Chen, B.; Suttles, J. and Campbell, W.F. 1992. Reactivation of Persistent Chlamydia trachomatis Genital Infection in Mice. 92nd General Meeting of the American Society for Microbiology, New Orleans, LA