P. AERUGINOSA INDUCED TNF-α PRODUCTION IN MACROPHAGES

by

LI MIAO

M.Sc., Shanghai Second Medical University, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Microbiology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 1996

© Li Miao, 1996
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
Vancouver, Canada

Date May 20, 1996
ABSTRACT

*Pseudomonas aeruginosa* has shown itself to be particularly adept at colonizing CF lungs and intractable to treatment even with the most aggressive therapy. Failure to control colonization with *P. aeruginosa* is a major cause of pulmonary debilitation that ultimately leads to almost all of the deaths in CF patients. During bacterial infections, macrophages produce many proinflammatory cytokines, among which TNF-α is believed to be the principal cytokine mediating many of the catastrophic host responses to bacterial infections. Little is known about the virulence factor(s) of *P. aeruginosa* in the pathogenesis of CF lung infections in terms of TNF-α production. The aim of this study was to determine the virulence factor(s) of *P. aeruginosa* with regard to TNF-α production.

The kinetics of TNF-α production by live *P. aeruginosa* coincubated with RAW 264.7 macrophages was studied. RAW 264.7 macrophages were challenged with *P. aeruginosa* at concentrations of $10^4$, $10^6$ and $10^8$ CFU/ml respectively. The production of TNF-α by macrophages increased in a time-dependent fashion with peak levels occurring at about 8 hours, after which the amount of TNF-α decreased, possibly related to TNF-α degradation by proteases from macrophages as well as *P. aeruginosa* itself. However, the data suggested a possible inverse relationship between the peak values of TNF-α produced and the number of bacteria added into macrophages, in that macrophages challenged by lower numbers of *P. aeruginosa* produced higher levels of TNF-α, accompanied by a
slower rate of decline in TNF-α content. To understand what was occurring with both the *P. aeruginosa* and the macrophages during the production of TNF-α, the growth of *P. aeruginosa* and the viability of macrophages during their coincubation were studied in a time course. Depending on the starting bacterial number, the stationary phase of bacterial growth occurred between 8 and 12 hours. Eight hours after challenge with *P. aeruginosa*, macrophage viability started to decrease. The decrease of macrophage viability suggested *P. aeruginosa* had cytotoxic effect on macrophages. TNF-α production appeared to be related to both the growth stage of the *P. aeruginosa* culture and the presence of functioning macrophages. TNF-α production increased during the log growth phase of *P. aeruginosa*, after which the presence of TNF-α in the supernatants as well as macrophage viability decreased. These findings suggested the growth of *P. aeruginosa* and the viability of macrophages were important factors in *P. aeruginosa* induced TNF-α production.

Macrophage-bacteria association is the initial step of macrophage phagocytosis. The relationship between the macrophage-bacteria association and TNF-α production by macrophages is not known. The effect of macrophage-*P. aeruginosa* association on TNF-α production was assessed by increasing their physical contact. The results showed that direct association of RAW 264.7 macrophages with *P. aeruginosa* significantly reduced TNF-α production, indicating the association of macrophages with *P. aeruginosa* may down-regulate the function of macrophage TNF-α production. This hypothesis was supported by
further experiments which used transwell filter inserts. Transwell filter inserts keep the bacteria from direct contact with macrophages while allowing factors released from the bacteria to come into contact with the macrophages. These experiments demonstrated that the production of TNF-α was higher when *P. aeruginosa* were incubated in transwell filter inserts than when incubated directly in association with macrophages. These findings suggested that factors released from *P. aeruginosa* might play a major role in TNF-α production whereas the direct interaction of bacteria with macrophages may suppress TNF-α production.

Presuming that released LPS might be the major virulence factor in the production of TNF-α by *P. aeruginosa*, the inhibition of *P. aeruginosa* induced TNF-α production with different LPS antagonists was investigated. Eight *P. aeruginosa* LPS specific antibodies were acquired, and none of them were able to block TNF-α production by *P. aeruginosa* LPS even though they could bind specific epitopes of *P. aeruginosa* LPS by Western immunoblotting and ELISA. Polymyxin B (PMB) was shown to be cytotoxic to RAW 264.7 macrophages in this study and, therefore, is not a suitable antagonist for LPS-induced TNF-α production. However, *Rhodopseudomonas sphaeroides* diphosphoryl lipid A (RsDPLA) could inhibit *P. aeruginosa* induced TNF-α production by 70-75%. CEME, an cecropin-melittin hybrid, inhibited more than 90% of *P. aeruginosa* induced TNF-α production. In addition, inactivation of the released products of *P. aeruginosa* by heat-treatment resulted in 20% reduction of TNF-α production, indicating that heat stable products such as LPS must be responsible for 80% of TNF-α production by *P.
P. aeruginosa. Taken together the results suggest that LPS is the major virulence factor in the production of TNF-α by P. aeruginosa.

The reasons behind the predilection and chronic persistence of mucoid P. aeruginosa in respiratory tract of CF patients are not completely understood. The ability of mucoid P. aeruginosa to induce TNF-α in murine alveolar macrophage was, therefore, compared with their nonmucoid counterparts. All the mucoid strains studied induced less TNF-α than their nonmucoid counterparts. The reduced TNF-α production seen with the mucoid form of P. aeruginosa may be attributed to the partial obstruction of LPS release by copious alginate coating around bacteria. This is consistent with the clinical findings that mucoid strains of P. aeruginosa are more suited to chronic rather than to acute respiratory infection in that reduced TNF-α as well as other virulence factors could temper the severity of infections.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1 Cystic Fibrosis and <em>P. aeruginosa</em></td>
<td>1</td>
</tr>
<tr>
<td>1.2 <em>P. aeruginosa</em> Virulence Factors</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1 Lipopolysaccharide</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Exopolysaccharide</td>
<td>4</td>
</tr>
<tr>
<td>1.2.3 Pili</td>
<td>5</td>
</tr>
<tr>
<td>1.2.4 Exotoxin A</td>
<td>6</td>
</tr>
<tr>
<td>1.2.5 Exoenzyme S</td>
<td>7</td>
</tr>
<tr>
<td>1.2.6 Proteolytic Enzymes</td>
<td>8</td>
</tr>
<tr>
<td>1.2.7 Phospholipase C</td>
<td>9</td>
</tr>
<tr>
<td>1.2.8 Pyocyanine</td>
<td>9</td>
</tr>
<tr>
<td>1.3 Role of Tumour Necrosis Factor - α in Cystic Fibrosis</td>
<td>10</td>
</tr>
<tr>
<td>1.4 Induction of Tumour Necrosis Factor - α</td>
<td>13</td>
</tr>
</tbody>
</table>
1.5 Objectives of Study

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

2.2 Bacterium Counting

2.3 Cell Maintenance

2.4 Murine Alveolar Macrophage Isolation

2.5 TNF-α Production
   2.5.1 TNF-α Production by RAW 264.7 Macrophages
   2.5.2 TNF-α Production by Alveolar Macrophages

2.6 TNF-α Bioassay

2.7 Assessment of RAW 264.7 Macrophage Viability

2.8 Assessment of Macrophage-Bacterium Association

2.9 LAL Assay

2.10 Inhibition of LPS Induced TNF-α by LPS Antagonists
   2.10.1 Antibody
   2.10.2 Polymyxin B
   2.10.3 RsDPLA
   2.10.4 CEME

2.11 Inhibition of Non-LPS Induced TNF-α Production by Heat-inactivation

2.12 Statistical Analysis
3. RESULTS

3.1 Kinetics of TNF-α Production by *P. aeruginosa* 29

3.2 Growth of *P. aeruginosa* during Coincubation with RAW 264.7 Macrophages 29

3.3 Viability of RAW 264.7 Macrophages during Coincubation with *P. aeruginosa* 30

3.4 Direct Association of Bacteria with RAW 264.7 Macrophages Reduces TNF-α production 30

3.5 TNF-α Production in RAW 264.7 Macrophages Incubated with *P. aeruginosa* in Transwell Filter Inserts 31

3.6 Inhibition of LPS Induced TNF-α by LPS Antagonists

3.6.1 Antibody 38

3.6.2 Polymyxin B 38

3.6.3 RsDPLA 39

3.6.4 CEME 40

3.7 Reduction of *P. aeruginosa* released product induced TNF-α after heat-treatment of bacterial filter 41

3.8 TNF-α Production in Alveolar Macrophages by mucoid and nonmucoid *P. aeruginosa* 53

4. DISCUSSION 56

5. REFERENCES 64
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TNF-α production in RAW 264.7 macrophages by <em>P. aeruginosa</em>.</td>
<td>33</td>
</tr>
<tr>
<td>2. Growth curve of <em>P. aeruginosa</em> in coincubation with RAW 264.7 macrophages.</td>
<td>34</td>
</tr>
<tr>
<td>3. Viability of RAW 264.7 macrophages in coincubation with <em>P. aeruginosa</em></td>
<td>35</td>
</tr>
<tr>
<td>4. Association of RAW 264.7 macrophages with <em>P. aeruginosa</em> and its relation to TNF-α production.</td>
<td>36</td>
</tr>
<tr>
<td>5. TNF-α production in RAW 264.7 macrophages incubated with <em>P. aeruginosa</em> in transwell filter inserts.</td>
<td>37</td>
</tr>
<tr>
<td>6. No inhibition of LPS induced TNF-α by specific antibodies</td>
<td>42</td>
</tr>
<tr>
<td>7. Inhibition of LPS and TSST-1 induced TNF-α by PMB</td>
<td>43</td>
</tr>
<tr>
<td>8. Production of TNF-α induced by LPS in RAW 264.7 macrophages after pretreatment with PMB</td>
<td>44</td>
</tr>
<tr>
<td>9. Cytotoxicity of PMB for RAW 264.7 macrophages</td>
<td>45</td>
</tr>
<tr>
<td>10. Inhibition of LPS induced TNF-α by RsDPLA and No inhibition of <em>S. aureus</em> induced TNF-α by RsDPLA.</td>
<td>46</td>
</tr>
<tr>
<td>11. The production of TNF-α in RAW 364.7 macrophages incubated with <em>P. aeruginosa</em> supernatant and RsDPLA</td>
<td>47</td>
</tr>
</tbody>
</table>
12. Inhibition of LPS induced TNF-α by CEME and No Inhibition of *S. aureus* induced TNF-α by CEME.

13. Inhibition of *P. aeruginosa* induced TNF-α by CEME

14. Inhibition of *P. aeruginosa* induced TNF-α by CEME using transwell filter inserts

15. The effect of boiling on the induction of TNF-α by LPS

16. Reduction of *P. aeruginosa* released product induced TNF-α after heat-treatment of bacterial filtrate

17. TNF-α production in alveolar macrophages by *P. aeruginosa* PAO1 and its mucoid counterparts H328 and H329

18. TNF-α production in alveolar macrophages by mucoid *P. aeruginosa* C96M of CF isolate and its nonmucoid revertant C96NM
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>ETA</td>
<td>Exotoxin A</td>
</tr>
<tr>
<td>exo-S</td>
<td>Exoenzyme S</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide Binding Protein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium blue</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMB</td>
<td>Polymyxin B</td>
</tr>
<tr>
<td>RsDPLA</td>
<td><em>Rhodopseudomonas sphaeroides</em> diphosphoryl lipid A</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic shock syndrome toxin-1</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Niamh M. Kelly, and the members of my supervisory committee for their guidance.

This thesis is dedicated to my parents, Yaqiou Miao and Huiying Ma.
1. INTRODUCTION

1.1 Cystic Fibrosis and *P. aeruginosa*

Cystic fibrosis (CF) is the most common lethal inherited disorder among Caucasians with an incidence of approximately one in every 2500 live births and a carrier frequency calculated at 1 in 20 (Boat, 1989). A large number of population studies have shown that CF is an autosomal recessive disorder (Danks, 1965). However, the identification of the CF gene had to await the development of an entirely new approach known as positional genetics in the study of genetic diseases. It was discovered that the gene responsible for CF, termed cystic fibrosis transmembrane conductance regulator (CFTR) gene, is localized on the long arm of chromosome 7 (Rommens, 1989; Riordan, 1989). More than 170 different mutations have been described in the CFTR gene although the deletion of three bases in exon 10 (Δ508) is responsible for 70 percent of all CF mutations. This most common defective allele Δ508 mutation also gives rise to the most severe symptoms. The other mutations in this gene accounts for the variable symptoms found in CF (Kerem, 1990; Johansen, 1991; Wine 1991).

The CF gene encodes a 1480 amino acid membrane-bound protein called CFTR protein. It has been shown to be the chloride ion channel regulating the transportation of chloride ions across fluid transporting epithelial cell such as are found in exocrine glands (Kartner, 1991). CF, with its basic defect in ion transport, results in the accumulation of sticky tenacious mucus surrounding the epithelial surfaces of the body, particularly in the lungs, but also the gastrointestinal tract,
pancreas, and liver can be affected. The disease is typically characterized by high electrolyte concentrations in sweat, pancreatic insufficiency and production of abnormally viscid bronchial secretions leading to chronic progressive pulmonary disease, exacerbated by microbial infections. Control of gastrointestinal problems can be usually achieved with diet and medication. However, failure to control pulmonary debilitation leads to almost all of the deaths of CF patients.

The luminal surface of airway epithelial cells in normal human body is lined by hair-like cilia which beat in synchrony to sweep inhaled particles and potential pathogens out of the respiratory tract. However, the CF respiratory mucus presents a different environment to potential microbial pathogens from that in non-CF patients. As a direct or indirect consequence of the basic defect in CF, mucociliary clearance is impaired by the viscid, dehydrated nature of the mucosal secretions. The initial pulmonary problem is often pneumonia caused by Staphylococcus aureus or occasionally Haemophilus influenzae. These two pathogens can be effectively treated with antibiotics. As a matter of fact, CF patients with their gastrointestinal symptoms under control can often lead relatively normal lives until the onset of P. aeruginosa colonization of the respiratory tract.

Respiratory colonization by P. aeruginosa is widely accepted as the major microbial challenge in CF lung disease. This organism, virtually nonvirulent in an immunocompetent host, can be found at any given time in the respiratory tracts of over 70% of patients with CF. Once P. aeruginosa takes up residence in the lungs of CF patients, deterioration in pulmonary function accelerates. This appears to be compounded by the emergence of a mucoid form of the organism. The evidence
suggests that mucoid *P. aeruginosa* derive from the original colonizing strain rather than arising by acquisition from an external source (Martin, 1993). Once the mucoid form emerges in the lungs of CF patients the colonizing strain of *P. aeruginosa* is never eliminated from the respiratory tract, nor does it ever spread systemically beyond this site. It remains in the lungs as a persistent pathogen no matter what aggressive therapies are applied and what appears to be a competent systemic immune response to *P. aeruginosa*. The number of *P. aeruginosa* organisms in the sputum can reach $10^8$ organisms/ml of sputum (Seale, 1979). Most CF patients eventually die of pulmonary failure in the face of overwhelming infection with *P. aeruginosa*. It is apparent that this organism is involved in the respiratory failure that ultimately leads to almost all of the deaths of CF patients. Therefore, it is not an overstatement to say that infection with *P. aeruginosa* is the harbinger of death for CF patients (Pier, 1985).

1.2 *P. aeruginosa* Virulence Factors

*P. aeruginosa* is a motile Gram-negative rod. It is generally regarded as an opportunistic pathogen, seldom capable of primary infection in normal, healthy individuals. In those with compromised defences, however, the bacterium can gain a foothold in the host, leading to serious and life-threatening disease in these persons. Patients at particular risk of *P. aeruginosa* infection include those undergoing immunosuppressive therapy. Burn patients are frequently colonized. In CF patients, pulmonary infection, usually involving mucoid strains, occurs with regularity.

*P. aeruginosa* virulence is generally regarded as being multifactorial. Many of its extracellular products and cellular structures may act as virulence determinants
during the initial colonization and subsequent infection with *P. aeruginosa*. The major virulence factors involved in the pathogenesis of *P. aeruginosa* infections are discussed below.

1.2.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a macromolecule bound to the outer surface of bacteria and is an integral part of the bacterial outer membrane. As its term implies, LPS consists chemically of lipid A and a polysaccharide chain composed of an oligosaccharide core and repeating oligosaccharide units which form the O-chain. The polysaccharide chain is not toxic *per se*. However, it renders the toxic lipid part of the molecule soluble, thereby facilitating its biological interaction with host cells (Rudbach, 1990)

LPS is responsible for many of the pathophysiological activities that accompany Gram-negative bacterial infections such as fever, leukopenia and leukocytosis, hemodynamic changes, and lethal shock. One of the mechanisms by which LPS produces its toxic effects is via the activation of monocytes and other cells resulting in the induction of cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-10, IFN-α/β, TGF-β, MIP-1, MIP-2, CSF and others. By communicating with target cells, these molecules induce inflammatory responses. The liberation of TNF-α appears to be of prime importance in LPS-induced pathophysiological states (Arai, 1990; Deuren, 1992). For a further discussion of this topic, see details in Sections 1.3 and 1.4.

1.2.2 Exopolysaccharide
The mucoid phenotype of *P. aeruginosa* is due to the production of copious amounts of alginate, an exopolysaccharide which is composed of 1-4 linked moieties of D-mannuronate and its epimer, L-guluronate (Russell, 1988). This polysaccharide binds a large amount of water and cations, forming a large-volume matrix around the bacteria to give a microcolony in which the bacteria are trapped by the polymer fibrils. Mucoid alginate-producing *P. aeruginosa*, which persist in the CF lung, are major pathogens in debilitating chronic pulmonary infections in patients with CF (Lam, 1980; Govan, 1990). Polyclonal and monoclonal antibodies to alginate inhibit the adherence of mucoid strains of *P. aeruginosa* to tracheal epithelium, indicating that alginate is involved in the attachment of the organisms to ciliated cells (Neil, 1989). Alginate has been shown to inhibit the uptake of bacteria by alveolar macrophages and neutrophils (Oliver, 1985; Meshulam, 1984) which is considered the first and most important defence system against invading pathogens. Alginate has also been reported to suppress the functions of lymphocytes and neutrophils (Mai, 1993). It appears that alginate plays an important role in the virulence of *P. aeruginosa* by evading the host defence system.

1.2.3 Pili

Most *P. aeruginosa* strains possess somatic, retractable, polar pili composed of a single protein termed pilin, which generally has a size of around 15 kD (Paranchych, 1988). Pili are thought to be important as an adhesin in the initial colonization of the respiratory tract by *P. aeruginosa*. Purified *P. aeruginosa* pili can block the attachment of intact *P. aeruginosa* to human buccal cells (Woods, 1980). The attachment of piliated
P. aeruginosa to mouse tracheas was also blocked by anti-pili antibodies raised to the homologous strain (Ramphal, 1984). Piliated strains have been associated with more cases of pneumonia, bacteremia, and mortality than the nonpiliated strains (Tang, 1995). *P. aeruginosa* pili have been shown to bind to asialoGM1 which is increased on the surface of CF epithelial cells due to the genetic defect in CF patients. This renders CF patients more susceptible to bacterial colonization than non-CF individuals (Saiman, 1994). These results illustrate the role of pili in the colonization of *P. aeruginosa* in susceptible host tissue.

1.2.4 Exotoxin A

Exotoxin (ETA) has been identified as the major virulence factor produced by *P. aeruginosa* (Liu, 1974). It is a single-chain polypeptide of 613 amino acids, with a molecular weight of 66,583. ETA has been classified as a member of the adenosine-diphosphate-ribosylating toxins. It is the most toxic product of *P. aeruginosa* and is cytotoxic for a number of mammalian cells (Middlebrook, 1977). The target protein of ETA is eucaryotic elongation factor 2 (EF2) and the result of ADP-ribosylation of EF2 is the loss of protein synthesis in the target cell.

The fact that ETA is a potent inhibitor of eucaryotic protein synthesis is a clear indication of its pathogenic potential. Several studies have compared the virulence of nontoxigenic mutants with parent strains in various experimental infection models, and shown that nontoxigenic strains are less virulent than strains producing toxin (Pollack, 1983; Woods, 1983). Human anti-ETA monoclonal antibody HI-1A4 has been shown to neutralize the toxicity caused by ETA in cell culture and in
experimental *P. aeruginosa* infections in mice (Ohtsuka, 1991). In a recent study, ETA has been shown to inhibit the phagocytosis and killing of *P. aeruginosa* by human and murine polymorphonuclear leukocytes (Miyazaki, 1995).

### 1.2.5 Exoenzyme S

*P. aeruginosa* produces another ADP-ribosyltransferase known as exoenzyme S (exo-S). There are two forms of the proteins, 47 kD and 53 kD, which are encoded by separate genes (Nicas, 1985; Kulich, 1995). Exo-S catalyzes the transfer of ADP-ribose from NAD to a number of eucaryotic proteins. It has been reported that exo-S mono-ADP-ribosylates the intermediate filament protein vimentin, particularly the disassembled form, as well as several membrane-associated GTP-binding proteins, including the p21 product of the proto-oncogene *c-H-ras* (Coburn, 1989 a & b).

The most convincing study to determine whether exo-S plays a role in pathogenesis was the genetic approach using transposon insertional mutagenesis to create mutant strains that have lost the ability to produce exo-S. The virulence of these mutant strains was compared with the wild-type strain in the chronic lung infection model. The evidence indicated that mutants deficient in exo-S were less virulent than the wild-type strain (Nicas, 1985). Additional studies have supported the role of this virulence factor in the lung infection. There were pathological changes observed in lung tissue following exposure to purified preparations of exo-S (Woods, 1988). In the rabbit infection model, alveolar epithelial injury was reduced by utilizing isogenic, exo-S deficient mutants compared with their parent strains (Kudoh, 1994).
Exo-S has been studied recently as a potential adhesin. It has been reported that both exoenzyme S and monoclonal antibody to exoenzyme S inhibits the binding of *P. aeruginosa* to buccal cells (Baker, 1991). These experiments here demonstrates the involvement of exo-S in the adherence of *P. aeruginosa* to susceptible tissue.

1.2.6 Proteolytic Enzymes

*P. aeruginosa* produces and secretes two proteolytic enzymes known as elastase and alkaline protease. Elastase and alkaline protease are metalloproteases with molecular weights of 32 kD and 57 kD respectively. They are most active at alkaline pH of 8-10 and degrade gelatin, casein, laminin and immunoglobulin. Elastase degrades elastin whereas alkaline protease does not (Morihara, 1985).

It is clear that these proteolytic enzymes are capable of contributing to the destruction of both host tissue and elements of the host defence system. Studies using purified proteolytic enzymes have demonstrated the enzymes damaged corneal and pulmonary tissues (Gray, 1975; Gray, 1979). Elastase and alkaline protease have been shown to degrade a number of biologically important molecules including various complement components, the immunoglobulins, INF-γ and TNF-α (Schultz, 1974, Heck, 1990; Horvat, 1989). There is considerable evidence from extensive studies that *P. aeruginosa* proteases inhibit the function of phagocytes, NK cells and T cells (Arsalan, 1989). In animal models, strains producing elastase or alkaline protease were shown to be more virulent than strains deficient in elastase or alkaline protease production (Woods, 1982, Howe, 1984).
1.2.7 Phospholipase C

Phospholipase C, a 78 kD protein with phospholipase and hemolytic activity, is produced by *P. aeruginosa* (Kurioka, 1967; Liu, 1979). Phospholipase C has the ability to degrade phospholipids commonly found in the membranes of eucaryotic cells but not present in membranes of prokaryotic cells. The process of phospholipid degradation also produces diacylglycerol, which can result indirectly in toxic side effects in animals (Berka, 1982; Besterman, 1986; Meyers, 1992). Phospholipase C is also described as hemolytic toxin associated with the virulence of *P. aeruginosa* (Liu, 1979).

1.2.8 Pyocyanine

Pyocyanine (N-methyl-1-hydroxyphenazine) is released by most clinical *P. aeruginosa* isolates (Knight, 1979). It is a blue phenazine pigment with a molecular weight of 210 kD.

Pyocyanine has been shown to be present at concentrations of up to $10^{-4}$ M in sputa from patients with CF who were heavily colonized with *P. aeruginosa*. At physiologically relevant concentrations, it slowed human ciliary beat frequency in vitro and led to disruption of the epithelia (Kanthakumar, 1994). Pyocyanine has been reported to contribute to immunosuppressive action by inhibiting the T-lymphocyte response to antigens and mitogens (Nutman, 1987). On the other hand, pyocyanine at low level showed a concentration-dependent enhancement of IL-2 production by T lymphocytes, of IL-1 and TNF-α production by monocytes-macrophages (Ulmer, 1990) and superoxide production by neutrophils (Miller, 1987).
The dual inhibitory and stimulatory effects of pyocyanine on lymphocytes and monocytes lead to the suppression of specific defense mechanisms and the enhancement of harmful inflammatory reactions of host.

Mucoid *P. aeruginosa* produce fewer virulence factors than do nonmucoid strains. Alginate-producing strains have been shown to produce lower yields of protease than their respective alginate-lacking mutants at all stages of growth in CF sputum medium (Ohman, 1982). In a chronic pulmonary infection rat model, significant decreased levels of exotoxin A, exoenzyme S, phospholipase C, and pyocyanine were seen in the mucoid *P. aeruginosa* rat lung isolates that returned to parental levels after reversion to the nonmucoid phenotype (Woods, 1991). In summary, many factors may come into play and no single factor is primarily responsible for the virulence of *P. aeruginosa*. Nevertheless, some have greater importance than others. Despite extensive advances in the research, this organism still remains one of the most formidable pathogens. Therefore, further investigations in the mechanisms of *P. aeruginosa* virulence factors is necessary for the control and treatment of the infections with *P. aeruginosa*.

1.3 Role of Tumour Necrosis Factor - α in Cystic Fibrosis

The pathogenesis of tissue damage in the lungs of CF patients with *P. aeruginosa* infection is complex. In addition to the pulmonary damage caused by bacterial products, exaggerated host immune responses may also contribute to the pathogenesis of the lung infections in CF patients. It is now accepted that both
bacterial virulence factors and exuberant host inflammatory responses combine to cause the destructive airway disease in CF (Fick, 1992).

Macrophages are among the first cells to respond to bacteria in the lung. Activated macrophages produce proteolytic enzymes, and enzymes that produce superoxide, hydrogen peroxide, hydroxyl radicals, superoxide anions, and nitric oxide anions. All of these could contribute to the inflammation seen in CF lung (Nathan, 1987). More importantly, macrophages produce many inflammatory cytokines, a heterogeneous group of hormone-like peptide compounds acting in an autocrine, paracrine and endocrine fashion (Sherry, 1988). They have been widely recognized as mediators of the inflammatory response (Nathan, 1987). Among them, TNF-α is considered a key mediator in the pathogenesis of bacterial infections (Tracey, 1989; Pass, 1995).

TNF-α exhibits tremendous diversity of cellular responses mediated through membrane receptors present on nearly all cell types with a few exceptions, such as erythrocytes and unstimulated T-lymphocytes. Two types of TNF-α receptors, 55 kD and 75 kD, have been recognized. The p55 receptor is ubiquitous, whereas the p75 receptor is restricted to cells of hematopoietic origin (Smith, 1990; Loetscher, 1990). Following TNF-receptor binding, an increase in membrane fluidity and permeability, subsequent calcium influx into the cytoplasm and enhanced cAMP levels and protein kinase activity have been observed. The activation of protein kinases may modify the phosphorylation of a discrete number of protein substrates which convey TNF-α signals to the nucleus resulting in up- or down-regulation of a number of cellular genes (Anghileri, 1987; Kobayashi, 1987; Zhang, 1988; Stewart, 1995). There is
apparently no intrinsic specific biological activity to TNF-α and its receptors. Rather, various cell types respond differently to TNF-α due to subtle tissue-specific differences in TNF-α signal transmission (Schutze S, 1988). As a consequence, the biological activities of TNF-α cover a broad spectrum of action, including activation of macrophages and granulocytes, activation of T and B lymphocytes and inhibition of hematopoiesis.

Proinflammatory cytokines such as IL-1, IL-8 and TNF-α may contribute to the localized neutrophil-dominated inflammatory state found clinically in CF airways (Schuster, 1993 & 1995). Although critical to host defence, neutrophils can cause progressive airway damage by the release of bioactive lipids, oxygen metabolites, and granule enzymes such as hydrolases, myeloperoxidase, lysozyme and neutral serine proteases. TNF-α may directly induce the influx of neutrophils into lung tissues via a chemotactic effect. The relationships between sputum TNF-α, leukotriene concentration and lung function abnormalities in CF patients were investigated by Greally et al (1993). TNF-α was found in patient sputa at concentrations that have been shown to elicit neutrophil migration, oxidative burst activity and degranulation in vitro. There was a significant correlation between TNF-α concentration and the severity of airflow obstruction. High concentrations of LPS have previously been detected in sputum from CF patients by LAL assay (Kharazmi, 1986). Given that LPS is a potent TNF-α inducer, it is feasible that LPS derived from P. aeruginosa is an important stimulus for the secretion of TNF-α. The TNF-α could, in turn, upregulate the production of the leukotrienes, and thus contribute to the pathophysiology of airways inflammation in CF (Klebanoff, 1986; Raoof, 1995).
High levels of TNF-α in the plasma of CF patients have also been observed. A study was carried out in patients with CF to determine the effect of chronic lung infection with *P. aeruginosa* on the plasma concentration of TNF-α. It was found that the concentration of TNF-α in CF plasma was significantly greater than that of healthy subjects (Norman, 1991). In another study, concentrations of TNF-α in CF plasma increased in the 7 days prior to death when compared with a period of clinical stability during the preceding 6 months (Elborn, 1993). Pfeffer et al (1993) investigated TNF-α production and gene expression by peripheral blood monocyte-derived macrophages from CF patients, compared with normals. The results demonstrated that although both cell populations responded in a dose-dependent manner to LPS; CF macrophages, upon stimulation with LPS at concentrations of 1 to 1000 ng/ml, consistently produced substantially higher amounts of TNF-α than normal macrophages. At the molecular level, CF macrophage TNF-α mRNA expression was 2- to 4-fold greater than that of normal macrophages. High concentrations of circulating TNF-α could account for many of the observed alterations in energy metabolism such as cachexia, anorexia, weight loss in CF patients (Norman, 1991; Suter, 1989; Brown, 1991).

1.4 Induction of Tumour Necrosis Factor - α

TNF-α is produced mainly by monocytes and macrophages but also by a variety of cells such as lymphocytes, natural killer cells, neutrophils, endothelial and epithelial cells, mast cells, fibroblasts, smooth muscle cells, astrocytes and others (Aggarwal, 1985; Aiyer, 1988). TNF-α gene is located on the short arm of human
chromosome 6 (Nedwin, 1985) and murine chromosome 17 (Muller, 1987). It is approximately 3 kilobase pairs long with four exons and three introns (Pennica, 1984; Nedwin, 1985). TNF-α is a 17-kD peptide that is biologically active in the form of a compact trimer of three identical subunits. TNF-α is a 157 amino acid peptide produced as a polypeptide of 233 amino acids and activated by cleavage of 76 amino acid signal peptide (Aggarwal, 1984).

LPS is one of the most potent inducers of TNF-α identified to date (Ulevitch, 1995). Stimulation of macrophages with LPS induces a 3-fold increase in the transcriptional rate of the TNF-α gene, a 100-fold increase in mRNA steady-state level and a 10,000-fold increase in the quantity of synthesized and secreted TNF-α (Han, 1990). Researchers have devoted great efforts to the study of LPS induced TNF-α production. It has been well recognized that LPS binding protein (LBP), a 60 kD serum glycoprotein, binds to LPS via lipid A (Tobias, 1986). Activation of macrophages and other cells by LPS-LBP complexes involves a membrane receptor, which has been identified as CD14 (Wright, 1989 & 1990). CD14 is a 55 kD glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) found in myeloid cells. It is also found as a soluble serum protein (sCD14) lacking the GPI-anchor. A role for mCD14 has been defined in LPS activation in myeloid cells, while sCD14 has been shown to participate in activation of nonmyeloid cell types such as endothelial or epithelial cells that normally do not express mCD14 (Pugin, 1993). Plasma proteins other than LBP, designated septin, have also been proposed as mediating the interaction of LPS with CD14 (Wright, 1992). CD14 appears to be a major factor involved in responses to LPS. However, CD14 is not absolutely
necessary for the activation of macrophages by LPS (Lynn, 1993). Macrophages appears to have CD14-dependent as well as CD14-independent mechanisms for inducing TNF-α production (Lynn, 1993). Anti-CD14 antibody inhibited macrophage response to LPS in serum-free conditions, but this was easily reversed at higher concentrations of LPS, which indicated that CD14 may act primarily as a binding subunit required for high affinity, but not low affinity binding. Also was CD14-negative cells such as the 70Z/3 cell line respond to LPS, although they require as much as 10,000-fold greater LPS concentrations for activation than do CD14-positive cells (Lee, 1992). Despite the evidence for CD14 independent LPS-induced TNF-α production, the mechanisms involved remain ill defined.

Subsequent to binding LPS to the macrophage membrane, intracellular signalling pathways that lead to gene expression and activated macrophage function are induced. Enzyme-mediated phosphorylation and dephosphorylation of cellular proteins is widely recognized as a principal mechanism by which external signals can regulate intracellular responses. These events most likely involve the sequential activation of a complex interactive network of kinases (Hunter, 1987; Krebs, 1989; Chen, 1992; Liu, 1994; Ulevitch, 1995). The complete mechanisms of LPS induced TNF-α production are still waiting to be defined despite considerable advances in the past decade. The delineation of the kinase cascade(s) initiated by LPS will be crucial to unravelling the mechanisms by which LPS mediates its many effects on macrophages.

As a potent TNF-α inducer, LPS has attracted the interests of many investigators in a search for antagonists useful for both fundamental research and
clinical therapy. There are functionally two categories of LPS antagonists. The first category of LPS antagonists is LPS lipid A analogs, which compete with toxic LPS for the physiological binding receptor on target cells. The monosaccharide lipid A precursor, lipid X, was the first such molecule to be investigated. Lipid X inhibited LPS-induced cellular activation and appeared to be protective against LPS injection and Gram-negative bacterial infections in some animal models (Proctor, 1986). The disaccharide lipid A precursor, lipid IVa is more potent as an LPS inhibitor and inhibits LPS-stimulated TNF-α, IL-1 and IL-6 production by human mononuclear cells (Golenbock, 1991). More impressive still is the lipid A from Rhodobacter sphaeroides (RsDPLA). Takayama et al (1989) purified the nontoxic RsDPLA and report that it inhibits LPS-induced macrophage TNF-α secretion. Qureshi et al (1991) extended this finding to the inhibition of LPS-induced TNF-α production in vivo. Other researchers reported that RsDPLA specifically inhibited the biological actions of LPS in a variety of human and murine cells (Golenbock, 1991; Kirkland, 1991; Henricson, 1992; Zuckerman, 1992). The second category of LPS antagonists involve LPS specific antibodies and polycationic antibiotics. These agents bind to LPS and neutralize its biological effects. Burd et al (1993) reported that monoclonal antibodies against both the O-antigen polysaccharide region and deep core/lipid A could inhibit secretion of TNF-α by murine macrophages. Anti-LPS antibodies also protected against endotoxemia in human and murine in vivo models. Enhanced survival was associated with decreased levels of LPS-induced TNF-α (Wortel, 1992; Cornelissen, 1993; Battafarano, 1994). For cationic antibiotics, binding of LPS is one of the mechanisms by which they inhibit or kill bacteria. Polymyxin B (PMB), a polycationic
antibiotic, prevents production of TNF-α by macrophages in response to LPS. It also blocks many of the effects of LPS in vivo including gram-negative bacillary septicemia in several animal models (Stokes, 1989; Coyne, 1993; Corrigan, 1979; Dlynn, 1987). CEME, a cecropin-melittin hybrid is another cationic peptide antibiotic. It has been demonstrated to have a high-binding affinity for either cell-attached or free LPS (Piers, 1994).

In addition to LPS, many other stimuli induce TNF-α production from macrophages. These include bacteria and their products, viruses, protozoa, fungi, immune complexes, interferons, tumour cells and TNF-α itself (Aiyer, 1988; Keller, 1994). The details will not be addressed in this thesis.

As discussed previously, *P. aeruginosa* is a dominant pathogen in the lung infection in CF and this bacterium plays a major role in the pathophysiology of chronic bronchial infection. *P. aeruginosa* has been reported to induce TNF-α production both in vitro and in vivo and increased TNF-α has been observed in both sputa and plasma of CF patients (Nakano, 1991; Pfeffer, 1993; Hirakata, 1993; Buret, 1994). These findings suggest a possible role for TNF-α in the pathogenesis of CF lung disease. However, the virulence factor(s) of *P. aeruginosa* that regulate TNF-α induction is still unclear. *P. aeruginosa* produces a large number of extra-cellular products which may be involved in the induction of TNF-α (Parmely, 1990; Ulmer, 1990; Hirakata, 1993). Studies have shown that LPS isolated from *P. aeruginosa* induced significant amounts of TNF-α (Pfeffer, 1993; Coyne, 1993). However, Staugas et al (1992) studied the induction of TNF-α by formalin-fixed heat-killed *P. aeruginosa* and suggested that bacterial LPS was not involved in the induction of TNF-
α as treatment of *P. aeruginosa* with PMB did not affect its ability to stimulate TNF-α production. There has been no report of the induction of TNF-α in response to live *P. aeruginosa*. Thus, the study of TNF-α production by live *P. aeruginosa* may provide insight into the virulence of *P. aeruginosa* in CF.

1.5 Objectives of Study

*P. aeruginosa* triggers an immune response in the lower respiratory tracts during the infection of the lung in patients. Macrophages are known to be the first and important defence system against *P. aeruginosa* in pulmonary infections. Macrophages produce proinflammatory cytokines, amongst which TNF-α is believed to be a principal cytokine mediating many of the catastrophic host responses to infections. However, the mechanisms of TNF-α induction by *P. aeruginosa* are not clear.

The aim of this study was to determine the virulence factor(s) of *P. aeruginosa* in terms of TNF-α production. Specific objectives were: (1) to study the kinetics of *P. aeruginosa* induced TNF-α production by macrophages; (2) to examine whether the association of macrophage with *P. aeruginosa* is required for the production of TNF-α by macrophages; (3) to explore if LPS release is the main virulence factor involved in the production of TNF-α by *P. aeruginosa*; (4) to investigate the differences between mucoid and nonmucoid *P. aeruginosa* in the production of TNF-α by alveolar macrophages.
2. MATERIALS AND METHOD

2.1 Bacterial Strains and Growth Conditions

*Pseudomonas aeruginosa* PAO1 and its mucoid counterparts H328 and H329 were obtained from Dr. R.E.W. Hancock. Cystic fibrosis (CF) mucoid *P. aeruginosa* isolate C96M and its non-mucoid laboratory revertant C96NM, *P. aeruginosa* mucoid C2383M and non-mucoid C2383NM from the same patient were obtained from Dr. David P. Speert. *Staphylococcus aureus* ATCC 12600 was obtained from ATCC (Rockville, MD). To grow bacteria, a swab of the frozen bacteria was placed on TSA (Difco Laboratories, Detroit, MI) plate and grown at 37°C overnight.

2.2 Bacterial Counting

Bacteria grown on TSA plates were suspended in PBS. The suspension was measured at OD$_{600nm}$ using Hitachi U2000 Spectrophotometer (Hitachi Ltd., Tokyo, Japan) and adjusted to OD$_{600nm}$ of 0.3, which approximates $10^8$ bacteria per ml. Viable counts in the suspension were performed by serial dilutions on TSA plates. The total count in the suspension was also ascertained using Petroff Hausser counting chamber (Hausser Scientific Partnership, Horsham, PA).

2.3 Cell Maintenance

The RAW 264.7 macrophage cell line was obtained from ATCC (Rockville, MD). The L929, TNF-sensitive mouse fibroblast cell line, was obtained from the same source. Both cell lines were maintained in DMEM complete medium, which is
DMEM (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated FBS (GIBCO) or 10% heat-inactivated horse serum in case of L929 cells, 2.4 mM L-glutamine 60 U/ml penicillin and 60 µg/ml streptomycin (GIBCO), $1.2 \times 10^{-4}$ M 2-mercaptoethanol (Sigma, St. Louis, MO) at 37°C and 5% CO₂ in 162 cm² cell culture flask (Costar, Cambridge, MA). Both cell lines were passaged once a week. Confluent monolayers of RAW 264.7 cells were treated with 10 ml cell dissociation medium (Sigma, St. Louis, MO) at 37°C for 10 minutes and L929 cells were treated with Trypsin-EDTA (GIBCO) at 37°C for 5 minutes to dissociate cells from the wall of flasks. Detached cells were transferred to the centrifuge tubes containing 20 ml DMEM complete medium and then centrifuged with IEC HN-SII Centrifuge (International Equipment Company, MA) at 2000 rpm for 10 minutes. The supernatant was discarded and the cells were resuspended in DMEM complete medium. Viable cells were counted by trypan blue exclusion using a hemacytometer (American Scientific Products, McGaw Park, IL). $10^6$ viable cells were seeded in 162 cm² flask containing 50 ml DMEM complete medium and incubated at 37°C in 5% CO₂ for 7 days.

2.4 Murine Alveolar Macrophage Isolation

6-8 weeks CD-1 mice were obtained from Animal Care Centre of University of British Columbia. Mice were first deeply anesthetized with halothane and killed. They were then pinned to a board and swabbed with 75% ethanol for sterilization. After the overlying skin was removed, the abdomen was opened and diaphragm was exposed and pierced. The lung and trachea were carefully dissected from the mouse
and the trachea was then cannulated with a 22-gauge catheter connected to four-way stopcock to which a syringe containing 10 ml PBS (GIBCO) with 0.1% EDTA (Sigma) and an empty syringe for collection of fluid lavage from the lung was mounted. Portions of about 1 ml lavage fluid at a time were introduced and recovered. The lavage fluid was transferred into centrifuge tubes containing 5 ml ice-cold RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO), 2.4 mM L-glutamine (GIBCO), 60 U/ml penicillin and 60 µg/ml streptomycin (GIBCO), 1.2x10^-4 M 2-mercaptoethanol (Sigma) and 1 mM sodium pyruvate (GIBCO) and centrifuged at 400 x g for 10 minutes. The average yield of pulmonary alveolar macrophages was 5 x 10^5 per mouse. Cells were resuspended in the above medium and counted using hemocytometer. 2.5 x 10^5 cells were added to each well of 48-well plates (Costar) and incubated at 37°C in 5% CO2 for 2 hours. All wells were then washed with RPMI 1640 media 3 times. Nonadherent cells were removed and the residual cells were believed to be primarily alveolar macrophages (Alblas, 1979).

2.5 TNF-α Production

2.5.1 TNF-α Production by RAW 264.7 Macrophages

RAW 264.7 macrophages were seeded in 24 well plates (Costar) at a density of 10^6 cells per well in DMEM complete medium and incubated at 37°C in 5% CO2 air for 18 hours. Medium of RAW 264.7 macrophage monolayers was changed with fresh DMEM containing 10% FBS (GIBCO). A suspension of bacteria or solution of PAO1 LPS (Supplied by Dr. H.E.W. Hancock) was then added into each well at the
final concentrations indicated in each experiment. They were coincubated for 6 hours or otherwise indicated, after which the supernatants were removed for the measurement of TNF-α activity. In the case of bacteria, the supernatants were filtered for sterilization using micro centrifuge filter with 0.2 μm nylon filter (Costar). Control experiments indicated that the presence of LPS in supernatants did not interfere with the TNF-α assay (data not shown).

Transwell filter inserts with a porosity of 0.1 μm (Costar) fit in 24 well-plate were applied at the same time in some experiments to examine the difference in TNF-α production between presence and absence of direct physical contact of macrophages with bacteria. The inserts were placed in the wells of 24-well tissue culture plates which were seeded with RAW 264.7 macrophages at 10⁶ well. *P. aeruginosa* PAO1 were added into each insert and the plates were incubated for 6 hours. Supernatants were measured for TNF-α activity. Transwell inserts in the presence of medium alone was performed as a control.

### 2.5.2 TNF-α Production by Alveolar Macrophages

Alveolar macrophages were isolated and seeded as specified in 2.3. After overnight (18 h) incubation at 37°C in 5% CO₂, the medium was changed with fresh RPMI 1640 containing 10% FBS. The bacterial suspension was then added into each well containing alveolar macrophages. Alveolar macrophages and bacteria were coincubated for 6 hours, after which the supernatants were harvested, filter sterilized and measured for TNF-α activity. Medium control was included in each experiment.
2.6 TNF-α Bioassay

TNF-α activity was determined using the murine L929 cytotoxicity assay (Mosmann, 1983). Briefly, duplicates of samples were diluted serially in 96-well microtiter plates (Costar) with DMEM supplemented with 10% heat-inactivated FBS (GIBCO), 2.4 mM L-glutamine 60 U/ml penicillin and 60 μg/ml streptomycin (GIBCO), 1.2x10⁻⁴ M 2-mercaptoethanol (Sigma) and 4 μg/ml actinomycin D (GIBCO). 10⁵ L929 cells in the same medium above were plated in each well. After 2 days of incubation at 37°C in 5% CO₂, the medium was aspirated and replaced with 100 μl of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium blue (MTT) (Sigma) in DMEM without phenol red (GIBCO). Three hours later, 100 μl per well anhydrous ethanol was added to each well to dissolve formazan dye crystals. The plates were left at room temperature for 15 minutes and measured spectrophotometrically with Vₘₐₓ Kinetic Microplate Reader (Molecular Devices Corporation, Menlo Park, CA) at 570 nm with 690 nm reference filter. Cell viability was determined by the ability of viable cells to reduce MTT. Anti-TNF-α antibody was used for the neutralization of TNF-α in the samples in order to ascertain the specificity to TNF-α assay. 1 unit of TNF-α activity was described as the amount of TNF-α producing 50% cytotoxicity in L929 cells.

2.7 Assessment of RAW 264.7 Macrophage Viability

Viability of RAW 264.7 macrophages in the wells of 24-well plates was determined. Supernatants were removed at the times indicated in the experiments and 200 μl cell dissociation medium was added to each well. After a 10 minute
incubation at 37°C, macrophages were lifted from the bottom of the wells by gentle pipetting. Cell viability was assessed by trypan blue exclusion method. Briefly, 1 volume of 0.1 mg/ml trypan blue (BDH, England) was mixed with 9 volumes of cell suspension. The mixture was vortexed and cells were then observed with a hemocytometer. The cells which excluded trypan blue were counted as viable cells.

2.8 Assessment of Macrophage-Bacterium Association

RAW 264.7 macrophages were seeded into 24-well tissue culture plates at the density of 5 x 10^5 cells per well into which acid-treated 12-mm diameter glass coverslips had been placed. After overnight incubation at 37°C in 5% CO_2, macrophages were challenged with 10^7 bacteria per well. The plates were either centrifuged at 1200 g for 10 minutes at room temperature in a Sorvall RT 6000 refrigerated centrifuge or left at room temperature for 10 minutes without centrifugation and then incubated at 37°C in 5% CO_2 for 1 hour. After incubation, wells were gently washed 6 times with PBS. Methanol was then added into all wells and aspirated after 1 minute. The cover slips were air-dried, mounted on microscope glass slides and stained with Diff-Quick Stain Set (American Hospital Supply, del Caribe, Inc., Aguada, Puerto Rico). The association of bacteria with macrophages was assessed by bright field microscopy (Microphot-TXA, Nikon, Japan). Fifty macrophages were observed for each cover slip. Macrophages which ingested or bound more than five bacteria were considered to be macrophages associated with bacteria (Speert, 1992).
2.9 LAL Assay

LPS content was determined using Chromogenic Limulus Amebocyte Lysate Test Kits (Whittaker Bioproducts, Inc., Walkersville, MD). Briefly, 50 μl samples were mixed with 50 μl limulus amebocyte lysate (LAL) in wells of 96-well microtiter plates (Costar), and incubated at 37°C for 10 minutes. During the incubation, LPS in the sample catalyzed the activation of a proenzyme in LAL. 100 μl substrate solution was then added and incubated for an additional 6 minutes. The activated enzyme at this step catalyzed the splitting of p-nitroaniline (pNA) from a colorless substrate Ac-Ile-Glu-Ala-Arg-pNA. The yellow coloured pNA was measured spectrophotometrically at 405 nm after the reaction was stopped with 100 μl acetic acid. The concentration of LPS in a sample was calculated from the absorbance values of solutions containing known amounts of purified *P. aeruginosa* LPS standards ranging from 0.1 ng/ml to 2 ng/ml.

2.10 Inhibition of *P. aeruginosa* LPS Induced TNF-α by LPS Antagonists

2.10.1 Antibody

Anti-LPS monoclonal antibodies F8 and B369, anti-LPS core monoclonal antibodies 3B8 and b11F5 and rabbit anti-outer membrane polyclonal antibodies were obtained from Dr. R.E.W. Hancock. Anti-LPS outer core, anti-LPS inner core and anti-lipid A monoclonal antibodies 5C-101, 7-4 and 177 were obtained from Dr. Joseph Lam. LPS (10-1000 ng/ml) were preincubated with above antibodies, at the ratio (v/v) of 1:1 to 1:100, for 30 minutes in a 37°C water bath before adding into
macrophage seeded wells. TNF-α activity was measured after 6 hour incubation at 37°C in 5% CO₂.

2.10.2 Polymyxin B

Polymyxin B (PMB) was purchased from Sigma. Prior to use, PMB solution at 1 mg/ml was made by dissolving PMB in PBS and then filtered with 0.2 μm pore filter for sterilization. RAW 264.7 preseeded wells received freshly dissolved PMB at the concentrations shown in each experiment, followed by the addition of P. aeruginosa LPS. Supernatants were measured for TNF-α activity after a 6 hour incubation at 37°C in 5% CO₂.

2.10.3 RsDPLA

P. aeruginosa were grown on TSA plates at 37°C overnight and then suspended in PBS. A 0.1 ml suspension at 10⁸/ml was added into wells with 0.9 ml DMEM containing 10% FBS. After a 6 hour incubation at 37°C in 5% CO₂, the suspension was filtered with 0.2 μm pore filter.

RsDPLA was purchased from Advanced Research (Madison, WI). A range of 10 to 1000 ng/ml RsDPLA was examined to determine the optimal concentration of RsDPLA for the inhibition of P. aeruginosa LPS. RAW 264.7 preseeded wells received RsDPLA at the final concentration indicated in the experiments and incubated at 37°C for 30 minutes. Either purified LPS or P. aeruginosa filtrate prepared as above were then added to wells. The plates were incubated at 37°C in 5% CO₂ for 6 hours and the supernatants were assayed for TNF-α activity.
2.10.4 CEME

CEME was a generous gift from Dr. R.E.W. Hancock. It was tested from 2.5 to 50 µg/ml to determine its optimal concentration for the inhibition of TNF-α production by *P. aeruginosa* LPS. CEME at 20 µg/ml was chosen to add into wells of preseeded RAW 264.7 macrophages, which were then challenged with purified LPS or *P. aeruginosa* either with or without transwell filters. After 6 hour incubation at 37°C in 5% CO₂, supernatants were measured for TNF-α activity.

2.11 Inhibition of Non-LPS Induced TNF-α Production by Heat-inactivation

*P. aeruginosa* PAO1 were grown on TSA plate overnight. The bacteria were then suspended in PBS and adjusted to 10⁸/ml. The suspension was diluted 10 times with DMEM containing 10% FBS and added at 1 ml/well to 24 well tissue culture plates. After a 6 hour incubation at 37°C in 5% CO₂, the bacteria were removed with 0.2 µm pore filter. Samples containing the filtrate or purified PAO1 LPS were then heated at 100°C for 1 hour.

RAW 264.7 macrophages preseeded in 24 well tissue culture plates were challenged with either untreated or heat treated *P. aeruginosa* filtrate or LPS and TNF-α production was assayed.

2.12 Statistical Analysis
Data were analyzed by paired Student's t-test using All Stats software program (Department of Health Care & Epidemiology, The University of British Columbia).
3. RESULTS

3.1 Kinetics of TNF-α Production by P. aeruginosa

The kinetics of TNF-α production was studied by performing a time course, whereby P. aeruginosa PAO1 at $10^4$, $10^6$ and $10^8$ CFU were added into RAW 264.7 macrophage seeded wells and supernatants were removed at different time points for TNF-α activity measurements (Figure 1). As shown in Figure 1, TNF-α in samples with $10^8$ CFU bacteria reached the peak at 8 hour and then dropped sharply. TNF-α in samples with $10^6$ CFU bacteria also reached peak at 8 hour, but maintained TNF-α level till 12 hour and then decreased. TNF-α in samples with $10^4$ CFU bacteria reached the peak between 8-12 hour and then decreased at a slower rate. The decrease in TNF-α content after peak may be related to TNF-α molecule degradation by proteases from macrophages as well as P. aeruginosa itself. It appeared that there was an inverse relationship between the peak values of TNF-α produced and the number of bacteria added to macrophages. Although the data shown here were representative of 3 independent experiments, the variation in absolute values between these experiments was high enough that statistical evaluations could not be performed. The experiments may need to be repeated more times in order to obtain a sensible statistical value. These findings suggested macrophages challenged by lower number of P. aeruginosa produced higher levels of TNF-α with a slower rate of decline in TNF-α content.

3.2 Growth of P. aeruginosa during Coincubation with RAW 264.7 Macrophages
Figure 2 shows the growth of *P. aeruginosa* when coincubated with RAW 264.7 macrophages. The bacteria grew exponentially until they reached $5 \times 10^8$ CFU, followed by a stationary phase, which occurred approximately between 8 and 12 hours after incubation depending on the starting bacterial number. It was apparent that the lower the initial concentration of bacteria, the longer the duration of the exponential phase. The beginning of the bacterial stationary phase appeared to related with the TNF-α peak time in Figure 1.

3.3 Viability of RAW 264.7 Macrophages during Coincubation with *P. aeruginosa*

The viability of RAW 264.7 macrophages during coincubation with *P. aeruginosa* was determined by trypan blue exclusion. The results as shown in Figure 3 demonstrate that macrophage viability started to decrease after 8 hour, coincident with the peak TNF-α value seen in Figure 1. There was no decrease of macrophage viability in the wells without *P. aeruginosa*. The decrease in macrophage viability when coincubated with *P. aeruginosa* indicated that *P. aeruginosa* have cytotoxic effects on macrophages.

3.4 Direct Association of Bacteria with RAW 264.7 Macrophages Reduces TNF-α Production

The effect of macrophage-bacterial association on TNF-α production was investigated. In order to increase the direct association of bacteria with macrophages, the macrophage seeded 24-well plates were centrifuged after the addition of bacteria. It was demonstrated by the use of method described in 2.8 that centrifugation
significantly increased the direct association of macrophages with \textit{P. aeruginosa} (P=0.001) (Figure 4a). At the same time, the production of TNF-\(\alpha\) was significantly reduced (P=0.05) when the association of macrophages with \textit{P. aeruginosa} was increased (Figure 4b). This suggests that direct association of bacteria with macrophages reduces TNF-\(\alpha\) production.

3.5 TNF-\(\alpha\) Production in RAW 264.7 Macrophages Incubated with \textit{P. aeruginosa} in Transwell Filter Inserts

To explore the role of factor(s) released from \textit{P. aeruginosa} in TNF-\(\alpha\) production, transwell filter inserts were used to separate bacteria from direct contact with macrophages while allowing factor(s) released by the bacteria to be transported through the filter to macrophages. A time course of TNF-\(\alpha\) production with \textit{P. aeruginosa} incubated in transwell filter inserts was performed (Figure 5). In comparison with those without transwell filter inserts in the same experiment, higher levels of TNF-\(\alpha\) were produced although the peaks of TNF-\(\alpha\) production were delayed (Figure 1). The delayed TNF-\(\alpha\) production may be due to the time required for the transportation of released factors through the filter inserts to macrophages. This is supported by control experiments which showed that LPS at the concentrations of 100 ng/ml and 1000 ng/ml exhibited considerable delay in the induction of TNF-\(\alpha\) when placed in the transwell filter compared with the induction of TNF-\(\alpha\) in the absence of the transwell filter (data not shown). In addition, TNF-\(\alpha\) peak values were directly related with the number of bacteria, which was different.
from those without transwell filter inserts (Figure 1) where the more bacteria added, the less TNF-α produced.

The results of this and the previous experiment, where the direct association of bacteria with macrophages inhibited TNF-α production, suggested that factors released from bacteria may play a major role in the production of TNF-α in macrophages incubated with *P. aeruginosa*. The most likely candidate for the induction of TNF-α was LPS. Accordingly, further investigation was made (see section 3.6).
Figure 1. TNF-α production in RAW 264.7 macrophages by *P. aeruginosa*. *P. aeruginosa* PAO1 at the concentrations indicated were added into wells preseeded with macrophages. Supernatants taken at 2, 4, 8, 12 and 24 hour incubation were measured for TNF-α activity. The data are given as the means of duplicate determinations in a representative experiment from three independent ones.
Figure 2. Growth curve of *P. aeruginosa* in coincubation with RAW 264.7 macrophages. The supernatants of macrophage-bacteria coincubation were removed at 2, 4, 8, 12 and 24 hour. The number of viable *P. aeruginosa* PAO1 in the supernatants were counted with serial dilutions plating on TSA plates. The data are given as the means of duplicate determinations in a representative experiment from three independent ones.
Figure 3. Viability of RAW 264.7 macrophages in coincubation with P. aeruginosa. At 2, 4, 8, 12 and 24 hour post incubation, macrophages were detached by cell dissociation medium from the bottom of the wells and viable cells were counted with trypan blue exclusion method. The data are given as the means of duplicate determinations in a representative experiment from three independent ones.
Figure 4. Association of RAW 264.7 macrophages with *P. aeruginosa* PAO1 (a) and its relation to TNF-α production (b). Values shown are the Mean ± SD of four independent experiments. Statistically significant difference as compared to the controls without centrifugation (* P ≤ 0.05; ** P ≤ 0.01).
Figure 5. TNF-α production in RAW 264.7 macrophages incubated with *P. aeruginosa* in transwell filter inserts. *P. aeruginosa* PAO1 at the concentrations indicated were added into transwell filter inserts fit in the wells preseeded with macrophages. Supernatants taken at 2, 4, 8, 12 and 24 hour incubation were measured for TNF-α activity. The data are given as the means of duplicate determinations in a representative experiment from three independent ones.
3.6 Inhibition of LPS Induced TNF-α Production by LPS Antagonists

LPS antagonists were used to investigate the role of LPS in the production of TNF-α by *P. aeruginosa*.

3.6.1. Antibodies

A range of antibodies against *P. aeruginosa* PAO1 LPS O chain, outer core and inner core, lipid A and outer membrane were tested in their ability to block LPS induced TNF-α production. None of these reagents were able to inhibit LPS induced TNF-α production although they could bind specific epitopes of *P. aeruginosa* LPS by Western immunoblotting and ELISA (Kievit, 1994). One of the antibodies even increased the TNF-α production by more than 2 fold (Figure 6), possibly related to the induction of TNF-α production by immune complexes (Polat, 1993). The reasons that these specific antibodies failed to inhibit LPS induced TNF-α production are not clear, but it is likely that these antibodies do not bind the critical epitopes of LPS which are responsible for the induction of TNF-α in macrophages.

3.6.2 Polymyxin B

Polymyxin B (PMB) is a cationic polypeptide with a unique cyclical configuration and distinct cationic characteristics. It has been reported to bind to the lipid A portion of LPS and block many of the biological effects of LPS including the induction of TNF-α (Stokes, 1989). Figure 7a shows that PMB at 100 μg/ml reduced LPS induced TNF-α production in this study. However, PMB also inhibited TSST-1
induced TNF-α (Figure 7b), suggesting a non-LPS specific mechanism as inhibition, perhaps related to toxicity.

Further experiments were designed to explore the non-specific inhibition of TNF-α production by PMB. First, RAW 264.7 macrophages were treated with PMB for 6 hours, washed thoroughly with DMEM and then incubated with LPS for another 6 hours. LPS induced TNF-α production was completely inhibited after pretreatment with PMB at 100 μg/ml (Figure 8). In a second experiment, RAW 264.7 macrophages were treated with PMB at the concentrations of 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml and 100 μg/ml. After 6 hour incubation at 37°C in 5% CO₂, the viability of macrophages was assessed with trypan blue exclusion. The data showed that the viability of macrophages decreased with concentrations of PMB greater than 20 μg/ml (Figure 9), suggesting that PMB is cytotoxic to macrophages. Therefore, PMB was not a suitable LPS antagonist in this study.

3.6.3 RsDPLA

RsDPLA is a nontoxic LPS isolated from Rhodopseudomonas sphaeroides which blocks induction of TNF-α in macrophages by LPS from other bacterial species (Takayama, 1989). To find the optimal amount of RsDPLA for the inhibition of P. aeruginosa LPS, a range of 10-1000 ng/ml RsDPLA was examined for its ability to inhibit TNF-α production by LPS stimulated macrophages at the concentrations of LPS from 10 to 1000 ng/ml. RsDPLA at 500 ng/ml inhibited LPS at 1 ng/ml, 10 ng/ml, 100 ng/ml by 95%, 80% and 20% respectively. RsDPLA at 1000 ng/ml was not more effective at inhibiting TNF-α production (Figure 10a), however, it induced a
small amount of TNF-α (Data not shown). These results are consistent with another report (Manthey, 1993). There was no inhibition of S. aureus induced TNF-α by RsDPLA (Figure 10b), indicating (i) the specificity of RsDPLA for the inhibition of LPS induced TNF-α and (ii) that RsDPLA was not interfering with the normal production of TNF-α by macrophages.

P. aeruginosa release considerable amounts of LPS while growing in culture (James, 1983). The amount of LPS in the supernatants of P. aeruginosa cultures was about 600 ng/ml as determined by LAL assay (data not shown). However, LPS can only be effectively inhibited by RsDPLA at concentrations below 10 ng/ml, as discussed above. Therefore, in order to inhibit LPS effectively with RsDPLA, P. aeruginosa supernatant filtrates were diluted before addition to RAW 264.7 macrophages which contained RsDPLA (at 500 ng/ml). At dilutions of 1:100 and 1:250 (containing approximately 2-6 ng/ml LPS), induction of TNF-α by P. aeruginosa filtrate was inhibited 70% - 75% by RsDPLA (Figure 11). This suggests that LPS may be responsible for approximately 75-80% (given that RsDPLA neutralization is not 100%) of the TNF-α induced by factors released from P. aeruginosa.

3.6.4. CEME

CEME is a cecropin/melittin cationic hybrid peptide, which demonstrated a high-binding affinity for purified P. aeruginosa LPS and LPS attached to the bacteria (K.L. Piers, 1994). CEME at 20 µg/ml inhibited LPS induced TNF-α in RAW 264.7 macrophages (Figure 12a), but did not inhibit S. aureus induced TNF-α (Figure 12b).
The results demonstrate the specificity of CEME for inhibition of LPS induced TNF-α and also suggested no cytotoxicity of CEME on macrophages.

CEME inhibited more than 90% of *P. aeruginosa* induced TNF-α from macrophages in both direct or indirect contact between macrophages and bacteria (Figure 13, Figure 14), suggesting that *P. aeruginosa* LPS may play a major role in TNF-α production by *P. aeruginosa*.

3.7 Reduction of *P. aeruginosa* Released Product Induced TNF-α after Heat-treatment of Bacterial Filtrate

To further investigate the production of TNF-α by LPS in the released factor(s) from *P. aeruginosa*, heat-treatment was used to inactivate some bacterial products, but not heat-resistant LPS. Compared with the samples without heat-treatment, there was no reduction of TNF-α induction when purified LPS was heated (Figure 15), but a 20% reduction in TNF-α induction was observed when *P. aeruginosa* filtrate was heated (Figure 16). The results suggest that LPS may be responsible for 80% of TNF-α induction by released products from *P. aeruginosa*. This estimate is in agreement with the results of the RsDPLA experiments (see 3.6.3).
Figure 6. No inhibition of LPS induced TNF-α by specific antibodies. Eight LPS specific antibodies were preincubated with LPS (10 ng/ml) at the ratio (v/v) of 1:10 for 30 minutes at 37°C. 0.1 ml mixture of preincubated LPS-antibody was added into the wells preseeded with macrophages. Supernatants were measured for TNF-α activity after 6 hour incubation, in comparison with LPS alone.
Figure 7. Inhibition of LPS and TSST-1 induced TNF-α by PMB. PMB at the concentrations indicated were added into wells preseeded with macrophages, followed by the addition of LPS at 10, 100 and 1000 ng/ml (a) or TSST-1 at 10 µg/ml (b). Supernatants were measured for TNF-α activity after 6 hour incubation. Data represents the means of duplicate determinations in a typical experiment.
Figure 8. Production of TNF-α induced by LPS in RAW 264.7 macrophages after pretreatment with PMB. RAW 264.7 macrophages were treated with PMB for 6 hours, washed thoroughly with DMEM and then incubated with LPS at 100 ng/ml for another 6 hours. Supernatants were measured for TNF-α activity. Data represents means of two independent experiments.
Figure 9. Cytotoxicity of PMB for RAW 264.7 macrophages. The viability of macrophages was determined with trypan blue exclusion method after treatment of PMB at the concentrations indicated for 6 hours. Data represents means of two independent experiments.
Figure 10. Inhibition of LPS induced TNF-α by RsDPLA and No inhibition of *S. aureus* induced TNF-α by RsDPLA. RsDPLA at the concentrations indicated were added into the wells preseeded with macrophages and incubated for 30 minutes, followed by the addition of LPS at 1, 10, 100 and 1000 ng/ml (a) or heat-killed *S. aureus* at $10^6$, $10^7$ and $10^8$ CFU/ml (b). Supernatants were measured for TNF-α activity after 6 hour incubation. Data represents the means of duplicate determinations in a typical experiment.
Figure 11. The production of TNF-α in RAW 264.7 macrophages incubated with *P. aeruginosa* supernatants and RsDPLA. RsDPLA at 500 ng/ml was added into the wells preseeded with macrophages and incubated for 30 minutes, followed by the addition of *P. aeruginosa* PAO1 growth filtrate at the different dilutions indicated. Supernatants were measured for TNF-α activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment from four independent ones.
Figure 12. Inhibition of LPS induced TNF-α by CEME and No Inhibition of *S. aureus* induced TNF-α by CEME. CEME at 20 μg/ml were added into the wells preseeded with macrophages, followed by the addition of LPS at 10, 100 and 1000 ng/ml (a) or heat-killed *S. aureus* at 10⁶ and 10⁷ CFU/ml (b). Supernatants were measured for TNF-α activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment.
Figure 13. Inhibition of *P. aeruginosa* induced TNF-α by CEME. CEME at 20 μg/ml was added to the wells preseeded with macrophages, followed by the addition of *P. aeruginosa* PAO1 at 10^6 and 10^7 CFU/ml respectively. Supernatants were measured for TNF-a activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment.
Figure 14. Inhibition of *P. aeruginosa* induced TNF-α by CEME using transwell filter insert. CEME at 20 μg/ml were added into the wells preseeded with macrophages. *P. aeruginosa* PAO1 at $10^6$ and $10^7$ CFU/ml were added into transwell filter inserts which were then placed into wells. Supernatants were measured for TNF-α activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment.
Figure 15. The effect of boiling on the induction of TNF-α by LPS. LPS was treated at 100°C for 1 hour and then added into the wells preseeded with macrophages at the concentrations indicated. Supernatants were measured for TNF-α activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment.
Figure 16. Reduction of *P. aeruginosa* released product induced TNF-α after heat-treatment of bacterial filtrate. The bacterial growth filtrate was treated at 100°C for 1 hour and then added into the wells preseeded with macrophages at the dilutions indicated. Supernatants were measured for TNF-α activity after 6 hour incubation. Data are given as the means of duplicate determinations in a representative experiment. Statistically significant difference when compared with the non-heat treated *P. aeruginosa* filtrate using Student t-test (* P ≤ 0.05; ** P ≤ 0.01).
3.8 TNF-α Production in Alveolar Macrophages by Mucoid and Nonmucoid *P. aeruginosa*

Murine alveolar macrophages were used to study the production of TNF-α in alveolar macrophages when incubated with mucoid and nonmucoid *P. aeruginosa* laboratory strains and CF isolates. Figure 17 shows TNF-α production in alveolar macrophages when incubated with laboratory strain PAO1 and its mucoid counterparts H328 and H329. Both of the mucoid strains induced significantly less TNF-α than the nonmucoid. Figure 18 shows TNF-α production in alveolar macrophages when incubated with cystic fibrosis isolate mucoid C96M and its nonmucoid revertant C96NM. The results also demonstrate that the mucoid strain induce significantly less TNF-α than the nonmucoid strain. Nonmucoid C2383NM and mucoid C2383M isolated from the same cystic fibrosis patient again shows that the mucoid strain induces less TNF-α than does the nonmucoid (data not shown).
Figure 17. TNF-α production by alveolar macrophages induced by *P. aeruginosa* PAO1 and its mucoid counterparts H328 and H329. Data represents Mean ± SD of three experiments. Statistically significant difference when compared with the non-mucoid counterpart at the same bacterial concentration using Student’s t-test (*P ≤ 0.05*).
Figure 18. TNF-α production by alveolar macrophages induced by mucoid *P. aeruginosa* C96M of CF isolate and its nonmucoid revertant C96NM. Data represents Mean ± SD of three experiments. Statistically significant difference when compared with the non-mucoid counterpart at the same bacterial concentration using Student’s t-test (*P* ≤ 0.05; **P** ≤ 0.01).
Despite the widely recognized importance of cytokines as mediators of inflammatory responses, the role of TNF-α in the pathogenesis of CF lung infections is still unclear. Suter and Norman et al (1989 & 1991) described elevated levels of TNF-α in plasma of CF patients colonized with *P. aeruginosa*, and Greally et al (1993) demonstrated TNF-α in CF patient sputa. Kelly et al (1995) demonstrated that the highest levels of TNF-α were observed in sputa obtained from patients presenting with an exacerbation of their disease. In contrast, Brown et al (1991) reported that they did not find increased levels of circulating TNF-α in either the plasma or airway fluids of CF patients with chronic *P. aeruginosa* lung infection. Study of the kinetics of TNF-α production by *P. aeruginosa* may help to understand the “discrepancy” of these clinical findings.

The kinetics of TNF-α production in RAW 264.7 macrophages showed that macrophages produce TNF-α in a time-dependent fashion upon stimulation with *P. aeruginosa* until about 8 hours, at which point the amount of TNF-α peaked. This was followed by a reduction of TNF-α levels (Figure 1). In addition, the growth of *P. aeruginosa* and the viability of macrophages have been investigated in a time course to gain a better understanding of both *P. aeruginosa* and macrophages during TNF-α production. Interestingly, the growth of *P. aeruginosa* reached a peak between 8-12 hours depending on the starting bacterial number, and the viability of macrophages started to decrease 8 hours after challenge with *P. aeruginosa* (Figure 2 & 3). It appears
that the kinetics of TNF-α production correlates with the growth of *P. aeruginosa* and the viability of macrophages.

Many of the components and extra-cellular products of *P. aeruginosa* have been investigated for their roles in the induction of TNF-α. LPS is widely recognized as the most potent TNF-α inducer (Ulevitch, 1995). TNF-α release from macrophages has been reported as being markedly enhanced by another *P. aeruginosa* product pyocyanine (Ulmer, 1990). On the other hand, other factors from *P. aeruginosa* appear to inhibit TNF-α production in macrophages or degrade TNF-α molecules. *P. aeruginosa* ETA was reported to inhibit the ability of macrophages to produce TNF-α by suppression of immune response. It inhibited the phytohemagglutinin induced TNF-α in a concentration-related manner (Staugas, 1992). *P. aeruginosa* alkaline protease and elastase has been shown to inactivate TNF-α (Parmely, 1990). Thus, the actual amount of bioactive TNF-α present may depend upon both stimulatory and inhibitory effects of *P. aeruginosa* on macrophage TNF-α production and half-life of TNF-α molecules.

*P. aeruginosa* has been showed to be cytotoxic to macrophages leading to the dysfunction or even death of macrophages. McClure et al (1992) reported that *P. aeruginosa* secret two glycolipids, monorhamnolipid and dirhamnolipid, which destroy human monocyte-derived macrophages. Long-term incubation with *P. aeruginosa* resulted in cytolysis and death of these monolayers. The results of the present study also demonstrate the decreased viability of macrophages after coincubation with *P. aeruginosa* (Figure 2). Not only is the production of TNF-α terminated due to macrophage cytotoxicity, but the release of products from the
cytolysis of macrophages may also contribute to the degradation of TNF-α. This hypothesis is supported by the findings that the rate of TNF-α reduction was correlated with the number of bacteria in coincubation with macrophages. The more bacteria coincubated with macrophages, the more cytotoxicity exerted on macrophages, and therefore the greater the potential for TNF-α degradation.

Study of the kinetics of TNF-α production in macrophages by *P. aeruginosa* in relation to growth of bacteria and viability of macrophages may shed some light on what has been observed in clinical patients. The amount of TNF-α detected may represent the combination of TNF-α induction, inhibition or inactivation from both *P. aeruginosa* and macrophages. Other factors may also be involved in vivo. Depending on the clinical conditions, the concentration of TNF-α in either sputum or plasma, therefore, could be different. However, it is clear and unequivocal that *P. aeruginosa* can induce considerable amounts of TNF-α production by macrophages, and that overproduction of TNF-α during the chronic infection could certainly cause inflammatory reactions in the CF host.

Phagocytosis is a primary function of macrophages in the host defense against bacterial infections. The binding of bacteria to specific macrophage receptors is the initial step, followed by ingestion and intracellular killing of bacteria. The relationship between the association of bacteria with macrophages and TNF-α production from macrophages is not known. By increasing the association of macrophages with bacteria, we were able to assess the effect of macrophage-bacteria interaction on TNF-α production. The results showed that direct association of *P. aeruginosa* with macrophages was inversely related with TNF-α production (Figure 4a
& 4b), indicating that bacterial macrophage association may down-regulate macrophage TNF-α production. The results also suggest that factors released by bacteria, as opposed to the bacterial macrophage contact per se, may play the major role in the production of TNF-α.

To determine if factors released from P. aeruginosa play a major role in the induction of TNF-α, transwell filter inserts, which keep bacteria from direct contact with macrophages while allowing bacterial released factors to transport through the filter to macrophages, were used. The results demonstrate that the production of TNF-α is higher when P. aeruginosa were incubated with macrophages in the presence of transwell filter inserts (Figure 5) than when incubated directly with macrophages (Figure 1). It was also noted that peak values of TNF-α with transwell filter are bacterial dose-dependent, rather than inversely dose-dependent as in the case without transwell filter inserts. These findings support the hypothesis that the factors released from P. aeruginosa play a major role in TNF-α production whereas the direct interaction of macrophages with bacteria is a suboptimal stimulus for TNF-α production, perhaps due to interfering effects.

It was of interest to determine what the main virulence factor is in the released products of P. aeruginosa in terms of TNF-α induction. P. aeruginosa spontaneously release LPS at a constant rate during its log-phase and stationary-phase growth (James, 1983), and LPS is the most potent biological inducer of TNF-α (Ulevitch, 1995). However, Staugas et al (1992) indicated that P. aeruginosa LPS was not involved in the induction of TNF-α when treatment of P. aeruginosa with PMB did not affect its ability
to stimulate TNF-α production. In the present study, different LPS antagonists were chosen to investigate the role of LPS in TNF-α production by *P. aeruginosa*.

Anti-LPS antibodies have been reported to inhibit LPS-induced TNF-α from macrophages in vitro (Burred, 1993; Battafarano, 1994) and protect against lethal endotoxemia in vivo (Ziegler, 1991; Jornelissen, 1992). A range of *P. aeruginosa* LPS specific antibodies including anti-O chain, anti-core and anti-lipid A antibodies were used in an attempt to inhibit LPS-induced TNF-α from RAW 264.7 macrophages in this study. Although they could bind specifically with *P. aeruginosa* LPS by Western immunoblotting and ELISA (Kievit, 1994), none of the antibodies were capable of inhibiting LPS induced TNF-α. Similar results where reported by Chia et al (1989) in the evaluation of 13 LPS-reactive monoclonal antibodies, none of which inhibited LPS-induced TNF-α secretion by RAW 264.7 macrophages. The reason that these specific antibodies failed to inhibit LPS induced TNF-α production is unclear. However, it is possible that these LPS reactive antibodies do not bind the critical epitopes which are responsible for the induction of TNF-α and, therefore, are unable to neutralize LPS induced TNF-α production. On the other hand, monoclonal antibody against LPS outer core of *P. aeruginosa* 5c-101 enhanced LPS induced TNF-α production by two fold. This probably related to immune complexes induced TNF-α production via cross-linking of Fc receptors on macrophages (Polat, 1993).

PMB has been recognized as an LPS antagonist based on its ability to bind LPS. However, the cytotoxicity of PMB is often ignored. Stokes et al (1989) reported PMB had 35% and 80% cytotoxicity on macrophages at the concentrations of 10 μg/ml and 100 μg/ml respectively. Similarly, macrophage viability was decreased 50-80% after
treatment with PMB at the concentration of 40-100 μg/ml in the present study (Figure 9). PMB has been known to inhibit protein kinase C activity which is important in the activation of many cell processes including activation of macrophages by lipid A (Mazzei, 1982). Therefore, the ability of PMB to reduce the physiologic effects of LPS would be related to the neutralization of LPS as well as the inhibition of macrophage protein kinase C and to the cytotoxicity. In such case, the inhibition of TNF-α induction by PMB could be the specific inhibition of LPS or nonspecific inhibition of macrophage activation. Therefore, PMB is not a specific antagonist to inhibit LPS induced TNF-α in this study.

Piers et al (1994) demonstrated that CEME has a high-binding affinity for P. aeruginosa LPS. CEME inhibited P. aeruginosa LPS induced TNF-α in this study. A dramatic reduction of up to more than 90% of P. aeruginosa-induced TNF-α production was observed when using CEME at 20 μg/ml.

A nontoxic RsDPLA from Rhodopseudomonas sphaeroides inhibits the induction of TNF-α in macrophages by other toxic LPS molecules (Takayama, 1989; Manthey, 1993; Kirikae, 1994). In the present study, data show an approximate 75% reduction of P. aeruginosa released factor-induced TNF-α by RsDPLA, suggesting LPS was responsible for at least 75% of TNF-α production by released factors from P. aeruginosa.

Heat-treated P. aeruginosa culture filtrate induced 20% less TNF-α from macrophages than did non-heat treated control filtrate (Figure 16), suggesting heat-stable products such as LPS contribute to approximately 80% of TNF-α production.
from released factors of *P. aeruginosa*. On the other hand, heat-labile non-LPS substances may be responsible for approximate 20% of the TNF-α production.

Our results suggest that released LPS contributes to about 80-90% of the TNF-α produced in macrophages when incubated with *P. aeruginosa*, indicating that LPS is the major virulence factor from *P. aeruginosa* in respect to TNF-α production by macrophages. Kelly et al previously demonstrated that LPS release was the main virulence factor for *E. coli* (Kelly, 1991). LPS release may be the general mechanism of TNF-α induction in Gram negative bacterial infections.

The mucoid form of *P. aeruginosa* occurs in up to 90% of the lungs of CF patients. It is the major cause of the deterioration of the patient's condition. However, the predilection and chronic persistence of mucoid *P. aeruginosa* in respiratory tract of CF patients are not completely understood. The ability of mucoid *P. aeruginosa* to induce TNF-α by murine alveolar macrophages was studied, and compared with their nonmucoid counterparts. All mucoid strains studied induced less TNF-α than did nonmucoid ones. Copious viscid alginate could facilitate *P. aeruginosa* to form microcolonies in which the bacteria are trapped. In the environment of the microcolony, not only are bacteria partially blocked from host immune system, but also some released factors from bacteria may be retained by the physical form of alginate capsule. Therefore, LPS release could be partially sequestered by alginate coat around the bacteria. Inhibition of LPS release by alginate may contribute to reduced TNF-α production by mucoid *P. aeruginosa*. This is consistent with the clinical findings that mucoid strains of *P. aeruginosa* are more
suited to chronic rather than to acute respiratory infection in that reduced production of TNF-α as well as other virulence factors may temper the severity of infection.
REFERENCES


Coburn, J., R.T. Wyatt, B.H. Iglewski, and D.M. Gill. 1989b. Several GTP-binding proteins, including p21 c-H-ras, are preferred substrates of Pseudomonas aeruginosa exoenzyme S. J. Biol. Chem. 264:9004-9008


65


