

**Effects of Sub-Lethal Concentrations of Pneumolysin on
the Proinflammatory Activities of Human Neutrophils *In
Vitro***

by

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Summary

The *Streptococcus pneumoniae*-derived toxin, pneumolysin, has been reported to augment neutrophil-mediated inflammatory responses in murine models of experimental infection of the airways, and to favour invasive pneumococcal disease. The laboratory research presented in this thesis has been designed to investigate the possible proinflammatory interactions of pneumolysin with human neutrophils *in vitro*, as well as the underlying mechanisms of these.

Addition of pneumolysin (0.0167 – 41.75 ng/ml) to neutrophils caused dose-related enhancement of the following proinflammatory activities of these cells: superoxide generation, elastase release, expression of the β_2 -integrin CR3, phospholipase A_2 activity and production of leukotriene B_4 and prostaglandin E_2 , oxidative inactivation of α -1-proteinase inhibitor, and synthesis and release of interleukin-8. Pneumolysin-mediated enhancement of these neutrophil activities was observed in the absence of detectable cytotoxicity and was most striking when the toxin was added together with the bacterial chemoattractant N-formyl-L-methionyl-L-leucyl-L-pnenylalanine (FMLP, 1 μ M).

Treatment of neutrophils with pneumolysin also resulted in uncontrolled influx of Ca^{2+} into the cells in the setting of membrane depolarisation and efflux of K^+ , which appeared to be a consequence of the pore forming actions of the toxin. Importantly, the proinflammatory interactions of pneumolysin with neutrophils were completely attenuated by exclusion of Ca^{2+} from the cell-suspending medium.

These observations identify novel proinflammatory properties of pneumolysin which result from pore formation in the plasma membrane, influx of Ca^{2+} and augmentation of Ca^{2+} -activatable neutrophil functions.



Samevatting

Daar is vasgestel dat die *Streptococcus pneumoniae* verkrygte toksien, pneumolisien, die neutrofiel-bemiddelde inflammatoriese response in die muismodel met eksperimentele lugweg infeksie verhoog het en ook die indringende pneumokokkale siekte bevoordeel het. Die laboratoriumnavorsing wat in hierdie proefskrif beskryf word, is daarop gemik om die moontlike proinflammatoriese interaksies van pneumolisien en mensneutrofiel *in vitro*, asook die onderliggende meganismes van sodanige interaksies, te ondersoek.

Die byvoeging van pneumolisien (0.0167-41.75ng/ml) by neutrofiel het 'n dosis- verwante verhoging van die volgende proinflammatoriese aktiwiteite van hierdie selle veroorsaak naamlik: superoksied generasie, elastase vrystelling, uitdrukking van die β_2 integrien CR3, fosfolipase A_2 aktiwiteit en produksie van leukotriene B_4 en prostaglandiene E_2 , oksidatiewe inaktivering van α -1 protease inhibeerder asook die vervaardiging en vrystelling van interleukien-8. Pneumolisien-bemiddelde verhoging van hierdie neutrofiel aktiwiteite is waargeneem in die afwesigheid van enige waarneembare sitotoksiteit en is die treffendste wanneer die toksien en die bakteriese leukolokmiddel N-formiel-L-metioniel-L-leusiel-L-feniellalanien (FMLP, $1\mu\text{M}$) in kombinasie gebruik is.

Blootstelling van neutrofiel aan pneumolisien het tot 'n onbeheerde invloed van Ca^{2+} in die sel in die raamwerk van membraan depolarisasie gelei en die uitvloeï van K^+ , tot gevolg gehad. Dit blyk dat die voormelde gevolge uit die porievormende aksie van die toksien voortspruit. Dit is van belang om daarop te let dat die proinflammatoriese interaksie van pneumolisien met neutrofiel geheel en al verswak is deur die uitsluiting van Ca^{2+} in die selsuspenderende medium.

Hierdie waarnemings het nuwe proinflammatoriese eienskappe van pneumolisien geïdentifiseer, wat uit die porie-vorming in die plasma membraan, die invloed van Ca^{2+} en die verhoging van Ca^{2+} -aktiveerbare neutrofiel funksies voortspruit.

Publications

Part of this thesis has been published in the following papers:

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Cockeran R., Durandt C., Feldman C., Mitchell T.J. & Anderson R. (2002) Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils *in vitro*. *Journal of Infectious Diseases* **186**:562-565.

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List of Abbreviations

ADP	adenosine diphosphate
AIF	apoptosis inducing factor
AMP	adenosine monophosphate
API	α -1 proteinase inhibitor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BPI	bactericidal/permeability increasing protein
Ca ²⁺	calcium
⁴⁵ Ca ²⁺	radiolabelled calcium-chloride
cAMP	cyclic adenosine monophosphate
CB	cytochalasin B
cPLA ₂	cytosolic phospholipase A ₂
cpm	counts per minute
CPPD	calcium pyrophosphate dihydrate
CRP	C-reactive protein
Cyclo	cycloheximide
DAG	diacylglycerol
di-O-C ₅ (3)	3,3-dipentyloxacarbocyanine
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EGTA	ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIC	elastase inhibitory capacity
ELISA	enzyme-linked immunosorbent assay
FcR	Fc region
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
H ₂ O ₂	hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HIV	human immunodeficiency virus



HMP	hexose monophosphate
HOCl	hypochlorous acid
Hyl	hyaluronate lyase
ICAM	intercellular adhesion molecule
IFN- γ	interferon- γ
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IP ₃	inositol triphosphate
K ⁺	potassium
KCl	potassium chloride
LDH	lactate dehydrogenase
LECL	lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTB ₄	leukotriene B ₄
MAPk	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MgCl ₂	magnesium chloride
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MSU	monosodium urate monohydrate
Na ⁺	sodium
NAD ⁺	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP ⁺	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Na ⁺ ,K ⁺ ATPase	sodium, potassium, adenosine triphosphatase
NaOH	sodium hydroxide
NK	natural killer
NO	nitric oxide
O ₂ ⁻	superoxide
PAF	platelet activating factor



PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDE4	type 4 phosphodiesterase
PGE ₂	prostaglandin E ₂
PHA	phytohemagglutinin
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
Pln	pneumolysin
PMA	4- α -phorbol 12-myristate 13-acetate
PR3	proteinase 3
PsaA	pneumococcal surface antigen A
PspA	pneumococcal surface protein A
⁸⁶ Rb ⁺	radiolabelled rubidium-chloride
rlu	relative light units
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLPI	secretory leucocyte protease inhibitor
sPLA ₂	secretory phospholipase A ₂
TLC	thin layer chromatography
TNF- α	tumour necrosis factor- α
UV	ultra-violet
ZmpB	zinc metalloprotease B
Zn ²⁺	zinc

Chapter 1:

Literature Review

Topics reviewed in this literature review include the role of pneumolysin in pneumococcal virulence, as well as the functions of human neutrophils in host defence, with emphasis on those which may be altered by pneumolysin.

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae, also known as the pneumococcus, was first described in 1881, independently by George Miller Sternberg and Louis Pasteur, and although the organism has been studied extensively, it still intrigues biomedical scientists today (as cited in Austrian, 1981; Watson *et al.*, 1993).

Many important scientific/biomedical discoveries originated from these early studies. These include:

- The pneumococcus was one of the first pathogenic bacteria observed by Christian Gram in the 1880's, while developing the Gram-stain (as cited in Watson *et al.*, 1993).
- The protective effect of antibodies was demonstrated in 1891 by the Klemperers, who showed that the offspring of immunized rabbits were resistant to challenge with the homologous strain of pneumococcus (as cited in Austrian, 1981).
- In 1928 Griffith demonstrated that when heat-killed encapsulated and non-encapsulated live strains of the pneumococcus were injected into mice simultaneously, the non-encapsulated strains could be transformed into capsulated strains with the capsule-type of the encapsulated strain (as cited in Watson *et al.*, 1993).
- Penicillin was discovered in 1929 by Fleming, and the third patient treated with this new drug, and the first to show any clinical benefit, was suffering from pneumococcal conjunctivitis (as cited in Watson *et al.*, 1993).



- Inspired by the work of Griffith, bacterial DNA was first described as the fundamental unit in the “transforming principle” by Avery and colleagues in 1944. This was the first report of biologic activity of a nucleic acid.
- Felton and colleagues (1955) confirmed that resistance to phagocytosis was due to the pneumococcal polysaccharide capsule. They also showed that mice immunized with different types of pneumococcal capsular polysaccharides developed protective antibodies.
- The antibody response to the capsular polysaccharide of *S. pneumoniae* is regulated in a positive and negative manner by thymus-derived lymphocytes (Baker *et al.*, 1970; Baker, 1975).
- The first penicillin-resistant strain of *S. pneumoniae* was described in an adult (Hansman, 1976), while Jacobs and colleagues (1978) reported the first multidrug resistant strain in children - in whom resistance occurs more frequently (Klugman, 1990).
- Asplenic patients have an increased susceptibility to pneumococcal septicaemia. This observation established the protective role of the spleen in clearing the pneumococcus by phagocytic mechanisms, as well as by production of anti-capsular antibodies of the immunoglobulin (Ig) G₂ subclass (Wara, 1981).
- In the absence of specific antibody, clearance of the pneumococcus may be facilitated by C-reactive protein (CRP)-mediated complement activation (Kilpatrick & Volanakis, 1985). The cell wall is a potent activator of the alternate complement pathway, and binds acute-phase CRP (Tuomanen *et al.*, 1987). CRP, at concentrations comparable to those of acute phase human sera, can protect *xid* mice, which naturally lack antibodies to *S. pneumoniae*, from pneumococcal infection (Horowitz *et al.*, 1987). During the exposure of *xid* mice to a higher inoculum of bacteria, CRP prolonged life, but did not eradicate pneumococci from the blood. However, in normal mice, that have anti-phosphocholine (a constituent of the teichoic acids and lipoteichoic acids of the cell wall and membrane of pneumococcus) antibodies, infection with the higher bacterial inoculum did not result in fatal infection, due to the protective properties of CRP (Mold *et al.*, 1981; Yother *et al.*, 1982).

- Pneumococcal capsular polysaccharides can activate the alternative complement pathway (Mitchell & Andrew, 1997).
- Complement proteins, in isolation, cannot lyse the rigid capsule of *S. pneumoniae* and therefore require the deposition of opsonic C3b and C3bi (which bind to the CR1 and CR3 receptors on phagocytes, respectively) and antibody on the bacterial surface to promote phagocytosis and intraphagocytic killing (Hostetter, 2000). This extracellular pathogen is only able to cause disease outside of phagocytes (Johnston, 1991).

S. pneumoniae is present in the upper respiratory tract of many healthy individuals. The translocation of the pneumococci from the nasopharynx to the lungs may be attributed to the failure of specific (secretory IgA), or non-specific (filtration, cough reflex, mucus secretion and ciliary transport) host defences (Boulnois, 1992; Musher, 1992). It has been suggested that the immune status of the host, as well as the virulence of the particular strain, determines whether the pneumococcus will remain confined to the nasopharynx, or become invasive (Alonso de Velasco *et al.*, 1995).

Infections caused by the pneumococcus include otitis media, pneumonia, bacteraemia and meningitis, which carry high morbidity and mortality worldwide (Mufson, 1990). The pneumococcus has been documented as being one of the major causes of bacterial meningitis and otitis media in children (Dagan *et al.*, 1994). Even though antibiotics are widely available, the mortality attributed to pneumococcal disease is high, and has remained stable, between 25% and 29%, in the last four decades (Musher *et al.*, 2000). The incidence of pneumococcal disease was considered to be highest in infants under 2 and adults over 60 years of age. However, in sub-Saharan Africa, in particular, this situation has changed with the emergence of human immunodeficiency virus (HIV). HIV-infected individuals have significantly more infections with those pneumococcal serotypes commonly found in children, and they also harbour more penicillin-resistant strains than HIV-seronegative individuals (Feldman *et al.*, 1999). It was also reported by Feldman and colleagues (1999) that pneumococcal infection in HIV-seropositive individuals occurs at a mean age of 32 years.

1.1.1 Virulence factors of *S. pneumoniae*

The pneumococcal surface consists of three major layers, namely the plasma membrane, cell wall and capsule, the latter being the thickest of the three (Sørensen *et al.*, 1988). The capsule, composed of polysaccharides, promotes escape from phagocytosis, and therefore contributes to pneumococcal survival. As such, it is believed to be the most important virulence factor of the microbial pathogen because encapsulated strains are 50% more lethal than non-encapsulated strains, as well as being 10^5 times more virulent (Watson & Musher, 1990). Differences in the capsular type also influence pneumococcal colonization of the nasopharynx, trachea and lungs (Kadioglu *et al.*, 2002). The association between capsular type and disease is well known, as well as the protection afforded by capsular antibodies against disease (Boulnois, 1992).

Other virulence factors include cell wall components and pneumococcal proteins. Pneumolysin, pneumococcal surface protein A (PspA), hyaluronate lyase (Hyl), neuraminidase, pneumococcal surface antigen A (PsaA), autolysin and metalloproteases are some examples of the proteins that influence pneumococcal virulence (Jedrzejewski, 2001).

The cell wall of the pneumococcus consists of a triple-layered peptidoglycan backbone, that anchors the capsular polysaccharides, the cell wall polysaccharides, and possibly proteins (Sørensen *et al.*, 1990). It has potent proinflammatory properties (Tuomanen *et al.*, 1985a; Tuomanen *et al.*, 1985b; Tuomanen *et al.*, 1987). The symptoms of experimental administration of cell wall components mimic those of infection with live bacteria in animal models (Tuomanen *et al.*, 1985a; Tuomanen *et al.*, 1987; Ripley-Petzoldt *et al.*, 1988). Cell wall components activate the alternative pathway of the complement cascade, they bind to the acute phase reactant CRP, activate procoagulant activity on the surface of endothelial cells, and induce cytokine and platelet-activating factor (PAF) production upon binding to epithelia, endothelia, and macrophages. They also initiate the influx of leucocytes and initiate an extremely strong interleukin (IL)-1 response (Winkelstein & Tomasz, 1978; Riesenfeld-Orn

et al., 1989; Tomasz & Saukkonen, 1989; Tuomanen & Sande, 1989; Cabellos *et al.*, 1992; Geelen *et al.*, 1992; Geelen *et al.*, 1993; Heumann *et al.*, 1994).

Pneumolysin, a thiol-activated toxin, is a cytoplasmic enzyme which subverts the physical defences of the host (Paton *et al.*, 1986). Pneumolysin slows ciliary beat frequency, disrupts tight junctions between epithelial cells, as well as the integrity of the bronchial epithelial monolayer (Steinfort *et al.*, 1989; Feldman *et al.*, 1990). This toxin, found in all clinically significant isolates of the pneumococcus, is described in more detail in section 1.1.2.

PspA has been found on every pneumococcus strain to date, and appears to protect the bacteria against the host complement system by reducing complement-mediated clearance and phagocytosis (Yother & Briles, 1992). PspA has a highly polar electrostatic charge and this results in a capsular charge stabilization through the electropositive end of the polypeptide; complement activation is prevented by the predominantly electronegative region of PspA (Jedrzejewski *et al.*, 2000).

The majority of *S. pneumoniae* strains produce Hyl, which is directly involved in host invasion (Berry *et al.*, 1994). The surface protein Hyl, a member of the group of enzymes called hyaluronidases, facilitates tissue invasion by breaking down extracellular matrix components, resulting in increased tissue permeability which plays a role in wound infections, pneumonia, meningitis and bacteraemia (Paton *et al.*, 1993). Hyaluronan, the primary substrate of Hyl and an important component of the extracellular matrix, is known to play a role in embryonic development, cell migration and differentiation, wound healing and inflammation (Kreil, 1995; Jedrzejewski, 2001). It interacts with, and binds proteins on cell surfaces, with CD44 being the surface receptor molecule for hyaluronan (Aruffo *et al.*, 1990). The CD44 receptor, a member of a family of cell surface adhesion molecules, is present on the surface of macrophages, neutrophils, T-cells, B-cells and various epithelial cells (Miyake *et al.*, 1990), indicating the importance of Hyl in the pathogenesis of pneumococcal infections.

Neuraminidase, like hyaluronidase, is an enzyme that may promote pneumococcal colonization by facilitating tissue invasion through the breakdown of extracellular matrix components; it may therefore destroy ciliated epithelium and play a role in the pathogenesis of pneumococcal disease (Kirvan *et al.*, 1988). Neuraminidase cleaves the terminal sialic acid residues from a wide variety of glycolipids, glycoproteins and oligosaccharides on cell surfaces or body fluids (Kirvan *et al.*, 1988). O'Toole and colleagues (1971) reported that both coma and bacteraemia occur more often amongst patients with pneumococcal meningitis, when concentrations of N-acetylneuraminic acid in cerebrospinal fluid are elevated. Immunization of mice with neuraminidase resulted in limited protection against subsequent challenge with virulent pneumococci, indicating that neuraminidase *per se* is not a major virulence factor (Lock *et al.*, 1988).

The function of PsaA is likely to be in the transport of manganese and zinc ions into the bacterial cytoplasm (Dintilhac *et al.*, 1997). This virulence factor elicits protective immunity in mice against *S. pneumoniae*, while PsaA-negative mutants were avirulent in the mouse model of experimental pneumococcal infection (Jedrzejewski, 2001).

Autolysin is a cell wall-degrading enzyme which cleaves the peptidoglycan backbone of the pneumococcus, leading to cell lysis. This enzyme is located in the cell envelope and may play a role in physiological cell functions associated with cell wall growth (Jedrzejewski, 2001).

Hydrogen peroxide (H₂O₂), a reactive oxidant, is produced by all pneumococci and is also considered to be a virulence factor. It has been shown that H₂O₂ slows ciliary beating and disrupts epithelial integrity of human respiratory epithelium (Feldman *et al.*, 1990; Feldman *et al.*, 1994). H₂O₂ induces apoptosis in cultured human retinal pigment epithelial cells (Jin *et al.*, 2001), and also causes early-occurring deoxyribonucleic acid (DNA)-damage to ciliated respiratory epithelium (Feldman *et al.*, 2002). In combination, pneumolysin and H₂O₂ were found to have an additive, rather than a synergistic, adverse effect on

the ciliary beat frequency and structural integrity of ciliated epithelium (Feldman *et al.*, 2002).

Immunoglobulin A proteases, described as early as 1978, are defined as unique extracellular microbial enzymes whose only known substrate is human IgA of the IgA₁ subclass. These proteases cleave IgA specifically at the internal prolyl-threonyl peptide bond in the heavy chain to yield intact Fab α and Fc α fragments (Plaut, 1978). Mulks *et al.* (1980) showed that *S. pneumoniae* produces an IgA protease, a property that distinguishes it from other microbial pathogens which cause infections of the upper respiratory tract.

Hase and Finkelstein (1993) have shown that zinc metalloproteases play an important role in the virulence of several pathogens. In *S. pneumoniae*, the gene *zmpB*, encoding for the production of zinc metalloprotease B (ZmpB), has been identified as a potential virulence factor (Wizemann *et al.*, 2001; Marra *et al.*, 2002). This protease has been shown to be important in the surface expression of certain choline-binding proteins. Mutant pneumococci, lacking ZmpB, showed alterations in the ability of the bacteria to traffic proteins to the cell surface, affecting morphology, physiology and the ability to colonize (Novak *et al.*, 2000).

1.1.2 Pneumolysin

The first report of production of a haemolysin by the pneumococcus was made in 1905 (extracted from: Mitchell & Andrew, 1997). Shumway (1958) subsequently suggested that haemolysin plays a role in the pathogenesis of pneumococcal infections. This argument was based on the observations derived from experiments in which rabbits were exposed, intradermally, to either a cell-free pneumococcal extract or viable pneumococcal cells. The time course of the occurrence of spherocytosis and the rapid onset of anaemia, were similar in rabbits injected with either the bacterial extract or with live bacteria. This cell free extract was purified and the biological activity attributed to a single protein species, namely pneumolysin (Shumway & Klebanoff, 1971).

1.1.2.1 *Origin of pneumolysin*

Pneumolysin is located in the cytoplasm of the pneumococcus. This toxin lacks an N-terminal secretion signal sequence resulting in the widely accepted view that pneumolysin is released upon autolysin-induced autolysis of the pneumococcus. This may be true for the majority of *S. pneumoniae* strains, which release pneumolysin in the late log phase of growth. However, this contention has recently been challenged by Balachandran and colleagues (2001) who reported that release of pneumolysin by several strains of the pneumococcus, including a virulent type 3 clinical isolate, occurs during the early log phase of bacterial growth and is independent of autolysin. Furthermore, in mouse virulence studies, the loss of autolysin function in mutant strains did not greatly impair the ability of the mutated pneumococcal strain to cause an infection, in contrast to the marked loss of virulence of strains which lack pneumolysin (Balachandran *et al.*, 2001). From this study, it appears that pneumolysin and autolysin have different effects on virulence and that autolysin function is not a requirement for pneumolysin virulence. The authors concede that it is possible that different strains of the pneumococcus may have evolved different mechanisms of pneumolysin release (Balachandran *et al.*, 2001).

1.1.2.2 *Structure and pore-forming actions*

Tettelin and colleagues sequenced the complete genome of *S. pneumoniae* in 2001. They determined that 64% of the coding regions could be assigned biological roles, 16% of the predicted proteins matched proteins of unknown function and the residual 20% had no database match. They also identified several surface-exposed proteins that could be possible vaccine candidates.

Pneumolysin is a 35kDa protein which consists of 471 amino acids (shown in Figure 1.1, extracted from Walker *et al.*, 1987), is a member of the family of thiol-activated cytolysins and is produced by all clinical isolates of *S. pneumoniae* (Alcantara *et al.*, 2001; Baba *et al.*, 2001; Balachandran *et al.*, 2001; Jedrzejewski, 2001). Antibodies to pneumolysin, located free in serum, have been identified, but free pneumolysin in serum, has not been found (Kalin *et al.*, 1987). The lytic action of pneumolysin on lipid bilayers is a complex combination of events

including pore formation within the membrane, extraction of the lipid into free oligomeric complexes, aggregation and fusion of the membranes and membrane destabilization, all of which contribute to the formation of small vesicles. These observations suggest that cholesterol-binding toxins such as pneumolysin, may not only permeabilize cell membranes, but also cause the cells to form large vesicles, aggregate and fuse (Bonev *et al.*, 2001).

Pneumolysin consists of four domains. The upper surface of the first domain, which contains the N-terminal region, consists of negatively charged amino acids, and may play a role in the orientation of the molecule with respect to the membrane. Deletion of domain 1 results in a marked reduction in haemolytic activity, while binding to the cell membrane and cholesterol is unaffected (Baba *et al.*, 2001). The reduction in haemolytic activity is a result of impaired oligomerization after binding to the membrane. Although domain 1 is structurally associated with domain 3, these two domains never bind to the cell membrane. Domain 2 is a β -sheet structure, and forms a junction between domains 1 and 4.

Domain 4 accounts for approximately 25% of the length of pneumolysin and is the site for membrane binding and self-association (Baba *et al.*, 2001). It is capable of binding cholesterol and binds to erythrocyte membranes, this initial binding being essential to initiate the lytic process (Baba *et al.*, 2001). Deletion of the C-terminal region of pneumolysin results in loss of cytolytic activity, which may also be attributable to conformational changes in domain 4 resulting in a loss of binding activity (Baba *et al.*, 2001).

Pneumolysin interacts with the outer membrane of all eukaryotic cells and its cytolytic actions are unaffected by P-glycoprotein (Johnstone *et al.*, 2001). In the case of alveolar epithelial cells and pulmonary endothelial cells, the toxin disrupts the alveolar capillary boundary, producing alveolar flooding, as well as providing nutrients for bacterial growth and facilitating the penetration through epithelium into the bloodstream (Jedrzejewski, 2001).

1.1.2.3 *Pneumolysin in the pathogenesis of pneumococcal infections*

The pathogenesis of pneumococcal infections is complex and involves interactions between multiple possible virulence determinants and the immune response of the host. Two recent comprehensive reviews (Gillespie & Balakrishnan, 2000; Jedrzejewski, 2001) have provided a summary of new information that has emerged about the processes by which pneumococci may cause severe disease.

In vitro studies have demonstrated that pneumolysin possesses proinflammatory properties. Exposure of human monocytes to the toxin results in increased release of proinflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and IL-1 β (Houldsworth *et al.*, 1994). Some of the proinflammatory effects of pneumolysin may also be due to cytolysis of various host cell types, including ciliated respiratory epithelium (Feldman *et al.*, 1990), endothelial cells (Rubins *et al.*, 1992) and type II alveolar epithelial cells (Rubins *et al.*, 1993). An increase in the activity of phospholipase A₂ (PLA₂) in pneumolysin-treated pulmonary endothelial cells has been reported. However, effects of the toxin on the production of arachidonic acid-derived mediators of inflammation have not been described (Rubins *et al.*, 1994). The proinflammatory effects of pneumolysin are summarized in Table 1.1.

As mentioned earlier (page 5), *S. pneumoniae* culture filtrates, as well as pneumolysin alone, slowed human ciliary beating and damaged respiratory epithelium *in vitro* (Feldman *et al.*, 1990). In these studies, eight different culture filtrates were used, producing significant ciliary slowing which correlated with simultaneous pneumolysin activity, reaching maximal effects at 18 hours. Pneumolysin at clinically significant concentrations (>50 ng/ml) caused significant ciliary beat slowing and epithelial damage (Feldman - personal communication).

Pneumolysin has also been reported to induce apoptosis in a number of cell types, including neutrophils, macrophages and neuronal cells (Zysk *et al.*, 2000; Dockerell *et al.*, 2001; Braun *et al.*, 2002), an activity which appears to be related to uncontrolled influx of calcium into toxin-treated cells (Braun *et al.*, 2002), and

which may enhance persistence and invasiveness of the pneumococcus. Human lung epithelial cell and monocyte viability were decreased in a dose-dependent manner when these cells were exposed to increasing concentrations of pneumolysin (Hirst *et al.*, 2002). The uncontrolled influx of calcium into pneumolysin-treated mammalian cells was originally reported by Cockeran *et al.*, (2001) and these experiments are described in Chapter 2.

Sub-lethal damage to the plasma membrane resulting in uncontrolled influx of calcium is, however, not the only mechanism by which the pneumococcus causes apoptosis of eukaryotic cells. Pneumococci are the only invasive human pathogens that release H₂O₂ (Duane *et al.*, 1993), which may also be involved in the induction of apoptosis, as mentioned on page 6. Rapid apoptosis, induced directly by pneumococcus, in human microglial and neuronal cell lines, is due to massive mitochondrial damage, resulting in the release of apoptosis inducing factor (AIF), rather than being caspase dependent (Braun *et al.*, 2001; Braun *et al.*, 2002). Pneumococcus-derived H₂O₂, itself a permeable oxidant, appears to mediate a rapid increase in intracellular oxidative stress, which together with pneumolysin results in increased cytosolic calcium concentrations, the latter being important for inducing mitochondrial damage and apoptosis (Braun *et al.*, 2002).

In early studies, pneumolysin was reported to interfere with the protective functions of human neutrophils *in vitro*. Paton and Ferrante (1983) confirmed the findings of Johnson *et al.* (1981), demonstrating that pneumolysin inhibits the chemotactic response and random migration of neutrophils. Pneumolysin was also found to inhibit the respiratory burst, by causing inhibition of both hexose monophosphate (HMP) shunt activity, as well as H₂O₂ production, leading to the inability of neutrophils to kill *S. pneumoniae in vitro* (Paton & Ferrante, 1983). In apparent contrast to these findings, Clark (1986) showed that pneumolysin could be oxidatively inactivated by the myeloperoxidase (MPO) system of neutrophils, using either purified MPO and reagent H₂O₂, or stimulated, intact neutrophils.

Alcantara *et al.* (1999) showed that the complement-activating activity of pneumolysin appears to be of extreme importance in reducing pneumococcal

clearance from the bloodstream. The same authors undertook a further study (2001) on the effects of pneumococcal bacteraemia on complement levels of cirrhotic and control rats. Cirrhotic hosts were studied because of the known association of cirrhosis with hypocomplementaemia. The authors demonstrated that the complement-depleting activity of pneumolysin had only minimal effects on host defence in animals with a normal complement-generating system, but was detrimental in hypocomplementaemic cirrhotic hosts, being associated with impaired phagocytosis and bloodstream clearance. This may underpin the mechanism by which cirrhotic patients are at risk for pneumococcal bacteraemia.

Otitis media, most commonly caused by the pneumococcus, is an important medical condition, which occurs at least once in more than 80% of children below 5 years of age. The infection is associated with intense inflammation and is a significant cause of acquired hearing loss. In a recent short review, current understanding of the pathogenesis of the inflammatory process in pneumococcal otitis media was highlighted (Tuomanen, 2001). While release of cell wall components plays a major role in generating inflammation, the hair cells in the cochlea have been shown to be highly sensitive to the cytotoxic effects of pneumolysin. In the experimental situation, pneumolysin causes severe ultrastructural damage to the organ of Corti. This has not been shown with other virulence determinants of the pneumococcus, such as neuraminidase.

The pneumococcus is also the main cause of meningitis in both adults and children. Two mechanisms are thought to be responsible for the brain damage that may occur with pneumococcal meningitis. The first of these is direct inflammatory neurotoxicity due to the release of bacterial products and of host mediators. The second is brain oedema occurring as a consequence of loss of integrity of the blood–brain barrier. Zysk *et al.* (2001) used an *in vitro* blood–brain barrier model, represented by a human brain microvascular endothelial cell line. Both pneumolysin *per se* and pneumolysin-producing pneumococci induced severe damage in the cell line. In contrast, neither a pneumolysin-deficient strain of the pneumococcus, nor pneumococcal cell wall components, had any effect. The authors suggested that pneumolysin may be important in causing damage to

the blood–brain barrier in pneumococcal meningitis and may contribute to the entry of pneumococci into the cerebral compartment, with subsequent development of cerebral oedema.

Ferrante and colleagues (1984) investigated the effects of the pneumolysin on the proliferative responses of human lymphocytes *in vitro*. They showed that pneumolysin caused a marked inhibition of the response of lymphocytes to the mitogens concanavalin A, phytohemagglutinin (PHA), pokeweed mitogen and protein A, measured by ³H-thymidine incorporation. This inhibitory effect of pneumolysin was not a direct cytotoxic effect, but related to early events in the lymphocyte response, which was attributed to modification of mitogen receptors on the plasma membrane, thereby preventing mitogen binding.

A single case study of necrotizing fasciitis occurring in association with blunt trauma infection due to penicillin-resistant *S. pneumoniae* was reported by Ballon-Landa and colleagues (2001). The authors presented a review of the literature of 11 previously documented cases of deep or necrotizing soft tissue infections associated with this organism. It was suggested, that several virulence factors of the pneumococcus, including pneumolysin, may play a role in the pathogenesis of this condition.

In 1986, Paton *et al.* were able to clone the gene encoding pneumolysin in *Escherichia coli*, using the recombinant plasmid technique. Berry and colleagues (1989) subsequently demonstrated that by insertion-duplication mutagenesis, pneumolysin-negative mutants could be produced. Mice infected with these mutants intranasally were found to have longer survival times and more efficient blood clearance when compared to the corresponding wild-type strains. They also showed that after back-transformation, full virulence was restored, and therefore concluded that the reduction in virulence was a direct result of inactivation of the gene encoding pneumolysin (Berry *et al.*, 1989).

Several differences between pneumolysin-negative and pneumolysin-producing strains have been noted.



- Pneumolysin-negative strains are more susceptible to *in vitro* killing by phagocytes (Benton *et al.*, 1995).
- Pneumolysin-producing strains initiate higher production of IL-6 in murine models of experimental infection (Benton *et al.*, 1995).
- Circulating interferon- γ (IFN- γ) was only detected in mice infected with pneumolysin-producing strains, and only when the mice were extremely septic or just prior to death (Benton *et al.*, 1995).
- Amino acid changes in the pneumolysin-producing gene resulted in reduced cytotoxic activity and complement activation by the pneumococcus (Alexander *et al.*, 1998).
- Pneumolysin-deficient strains caused less mucosal damage than pneumolysin-producing strains (Rayner *et al.*, 1995).
- Pneumolysin-sufficient strains caused a lowering of ciliated beat frequency, with a loss of ciliated epithelium in an organ culture (Rayner *et al.*, 1995).
- Infection of mice with pneumolysin-deficient strains was associated with significantly lower numbers of pneumococci in the nasopharynx, trachea and lungs, when compared to mice infected with pneumolysin-sufficient strains (Kadioglu *et al.*, 2002).
- In murine-models of experimental infection of the airways, pneumolysin-producing strains caused exaggerated pulmonary inflammatory responses that were characterized by an excessive influx of neutrophils into the lungs (Kadioglu *et al.*, 2000).
- Alveolar macrophages are responsible for the clearance of bacteria from the alveoli, they accomplish this mainly through the production of nitric oxide (NO) and oxygen radicals, both of which have potent anti-bacterial activity. Large amounts of NO are produced during bacterial sepsis. This second messenger causes hypotension which is a critical pathological feature of septic shock. Braun and colleagues (1999) showed that pneumolysin is the main inducer of NO production in murine macrophages, while a pneumolysin-deficient mutant induced low or undetectable levels of NO production. They also showed that IFN- γ is an essential cofactor for pneumolysin-mediated expression of inducible NO synthase (iNOS).

These results suggest that pneumolysin is the primary component of the pneumococcus involvement in NO production.

1.1.2.4 Immunogenicity of pneumolysin

Pneumolysin evokes a T cell-dependent antibody response, leading to the production of secretory IgA (Simell *et al.*, 2001) and circulating IgG (Musher *et al.*, 2001a; Simell *et al.*, 2001) antibodies. Because pneumolysin is of intracellular origin, these antibodies (in the case of IgG) do not possess opsonophagocytic properties and their role in the prevention and/or containment of pneumococcal infection has been difficult to ascertain.

1.1.2.4.1 Antibodies to pneumolysin in host defence

Rapola *et al.* (2001) reported that infants with culture-confirmed acute otitis media caused by *S. pneumoniae* mounted specific antibody responses to pneumolysin, the magnitude of which was determined by previous pneumococcal contact, but the protective efficacy of these antibodies was not evaluated. However, Jero *et al.* (2000) reported that those children who developed an antibody response to pneumolysin had a higher frequency of recurrence of disease and middle ear effusions after 1 month than that observed in pneumolysin non-responders, and concluded that circulating antibodies to pneumolysin play no significant role in preventing otitis media. It is noteworthy that in both of these studies meaningful increases in the levels of circulating antibodies to pneumolysin were detected in only 7% (Jero *et al.*, 2000) and 23% (Rapola *et al.*, 2001) of patients.

In contrast, circulating antibodies to pneumolysin measured in adult patients with pneumococcal pneumonia at the time of hospital admission were highest in those patients with non-bacteraemic disease and lowest in those with bacteraemic disease. The protective activities of antibodies to pneumolysin were underscored in experiments in which passive administration of purified human IgG antibodies to pneumolysin protected mice against subsequent intraperitoneal challenge with *S. pneumoniae*, by preventing dissemination of the bacteria (Musher *et al.*, 2001a). The authors raised the interesting possibility that IgG antibodies to pneumolysin may function primarily to restrict the invasiveness of the

pneumococcus by neutralizing the cytotoxic and proinflammatory actions of the toxin, both of which may favour extra-pulmonary dissemination through disruption of mechanical barriers (Musher *et al.*, 2001a).

In the case of HIV infection, it is noteworthy that Sullivan and colleagues (2001), in contrast to the findings of an earlier study (Amdahl *et al.*, 1995), were unable to detect differences in baseline, circulating concentrations of antibodies to pneumolysin (measured on average 5 months prior to the development of infection) in HIV-infected injectable drug users (HIV infection and drug usage are both significant predisposing factors to pneumococcal infection) with bacteraemic pneumococcal disease, in comparison with HIV-seropositive and HIV-seronegative injectable drug users who remained free from severe pneumococcal disease. However, these findings should be interpreted cautiously, since the mean increment in pneumolysin antibody levels prior to onset of disease and at follow-up (3–9 months after infection) was trivial in comparison to that reported by Musher *et al.* (2001a) for their group of HIV-seronegative individuals with bacteraemic and non-bacteraemic pneumococcal disease, suggesting that antibody production may have been compromised as a consequence of HIV infection. This contention may be supported by the apparent failure of a pneumococcal conjugate vaccine in HIV-infected African subjects (French *et al.*, 2000).

1.1.2.4.2 Vaccine potential of pneumolysin

Vaccines in which the capsular polysaccharide of *S. pneumoniae* has been conjugated to non-pneumococcal carrier polypeptides (to promote T-cell recruitment and immunological memory) represent an important advance, principally because of their improved efficacy in children. Nevertheless, they do have several limitations (Lee *et al.*, 2001), which have stimulated interest in pneumococcal proteins as potential vaccine candidates in their own right, or as carriers of polysaccharide antigens. In addition to pneumolysin, several surface proteins, particularly pneumococcal surface adhesin A, PspA and C, and choline binding protein A have been identified as potential candidates for a pneumococcal protein vaccine (Briles *et al.*, 2001; Lee *et al.*, 2001; Obaro, 2001; Ogunniyi *et al.*,

2001; Simell *et al.*, 2001). The advantages of using these molecules *per se* as vaccines, or as carriers in glycoconjugate vaccines, is that they are immunogenic and induce protection in animal models of experimental pneumococcal infection (Paton *et al.*, 1983; McDaniel *et al.*, 1991; Brooks-Walter *et al.*, 1999). Moreover, these protein antigens are common to virtually all pneumococcal isolates and would therefore be expected to provide cross-immunity regardless of serotype.

In murine models of experimental infection, glycoconjugate vaccines consisting of pneumococcal polysaccharide coupled to pneumolysin have been reported to generate broader protective immunity, associated with the production of high avidity antibodies with opsonophagocytic activity, than conjugates which utilize non-pneumococcal carriers (Lee *et al.*, 2001). However, in the case of experimental otitis media, the only pneumococcal protein that has provided evidence of protection is the surface protein PspA (Briles *et al.*, 2001); this is consistent with data from the clinical study by Jero *et al.* (2000) which failed to show a protective effect of circulating antibodies to pneumolysin in children with acute otitis media. Inclusion of pneumolysin with PspA in a combined vaccine has, however, been reported to restrict the dissemination of the pneumococcus in experimental cavity infections, which is in agreement with the findings of Musher *et al.* (2001a) in both the clinical and experimental infection settings.

Surface proteins such as PspA, as opposed to intracellular proteins such as pneumolysin, appear to be the preferred pneumococcal polypeptide vaccine candidates, although pneumolysin-based vaccines may have a role in protecting against invasive disease.

1.1.2.4.3 Pneumolysin-based laboratory procedures in the diagnosis of pneumococcal diseases

Serological procedures (usually enzyme-linked immunosorbent assay) based on the detection of circulating IgG antibodies, including those sequestered in immune complexes, appear to have little or no role in the laboratory diagnosis of acute pneumococcal infections. In several well controlled studies they have been shown to be of low sensitivity, varying from 7% to around 50%, depending on type

of disease and age (Jero *et al.*, 2000; Bobes *et al.*, 2001; Musher *et al.*, 2001b; Rapola *et al.*, 2001; Simell *et al.*, 2001; Sullivan *et al.*, 2001).

Recent innovations in pneumolysin gene-based polymerase chain reaction (PCR) technology have, however, provided a seemingly rapid and robust assay for the improved non-culture diagnosis and case ascertainment of meningitis and septicaemia caused by the pneumococcus. The highly sensitive and specific procedure described by Corless *et al.* (2001) is a real-time PCR procedure which enables the simultaneous detection of *S. pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* in clinical samples of cerebrospinal fluid, plasma, serum and whole blood taken from patients with suspected meningitis and septicaemia.



1	TTACAAGACCAACCT <u>ATG</u> ATTGACTTAGATAAGGTATTTATGTTGGATAATACGGTTATTC	60	
61	CGACTTCTTATCTAGCCAGACGGCGACGCAATGCTCAGAAGAAATTGTACGAGGAAATTT	120	<u>Nucleotides</u>
121	TGGATCACTTAGTCCAACCCAGGCTGATTTCCGCTGAACAAGTCTGAGTTTATGCAACTCA	180	A = Adenine
181	ATCCAGGAACTTATTT <u>ATGAGG</u> ATGAGAAGATGSCAAATAAAGCAGTAAATGACTTTATACTA	240	G = Guanine
			T = Thymine
241	GCTATGAATTACGATAAAAAGAAACTCTTGACCCATCAGGGGAGAAAGTATTGAAAATCGT	300	C = Cytosine
	A M N Y D K K K L L T H Q G E S I E N R		
301	TTCATCAAAGAGGGTAAATCAGCTACCCGATGAGTTTGTGTTATCGAAAGAAAGAAGCGG	360	
	F I K E G N Q L P D E F V V I E R K K H		
361	AGCTTGTGACAAATACAAGTGATATTTCTGTAACAGCTACCAACGACAGTCCGCTCTAT	420	
	S L S T N T S D I S V T A T N D S R L Y		
421	CCTGAGACCTTCTCSTAGTGGATGAGACCTGTGTAGAGAAATAATCCCACTCTCTTCCG	480	<u>Amino acids</u>
	P G A L L V V D E T L L E N N P T L L A		A = Alanine
481	GTTGATCGTGCCTCCGATGACTTATAGTATTGATTTGCCGTTGGCAAGTACGGATAGC	540	R = Arginine
	V D R A P M T Y S I D L P G L A S S D S		N = Asparagine
541	TTTCTCAAAGTGGAAAGACCCAGCAATTCAGTGTTCGCGGAGCGGTAACGAITTTGTTG	600	D = Aspartic acid
	F L Q V E D P S N S S V R G A V N D L L		C = Cysteine
601	GCTAAGTGGCATCAAGATTATGGTCAGGTCAATAATGTCGCCAGCTAGAAATGCAATGAA	660	Q = Glutamine
	A K W H Q D Y G Q V N N V P A R M Q Y E		E = Glutamic acid
661	AAAATAACGGCTCACAGCATGGAACAACCTCAAGSTCAAGTTGTTCTGACTTTGAAAAG	720	G = Glycine
	K I T A H S M E Q L K V K F G S D F E K		H = Histidine
721	ACAGGGAATTCCTTGATATTGATTTTAACTCTGTCCATTCAGGTGAAAAGCAGATTTCAG	780	I = Isoleucine
	T G N S L D I D F N S V H S G E K Q I Q		L = Leucine
781	ATTGTTAATTTAAGCAGATTTATTATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGA	840	K = Lysine
	I V N F K Q I Y Y T V S V D A V K N P G		M = Methionine
841	GATGTGTTTCAAGATACTGTAACGGTAGAGGATTTAAAACAGAGAGGAATTTCTGCAAG	900	F = Phenylalanine
	D V F Q D T V T V E D L K Q R G I S A E		P = Proline
901	CGTCCCTTGGTCTATATTTCSAGTGTTCCTATGGGCGCCCAAGTCTATCTCAAGTTGSA	960	S = Serine
	R P L V Y I S S V A Y G R Q V Y L K L E		T = Threonine
961	ACCACGAGTAAGAGTGATGAAGTAGAGGCTGCTTTTGAAGCTTTGATAAAAAGGAGTCAAG	1020	W = Tryptophan
	T T S K S D E V E A A F E A L I K G V K		Y = Tyrosine
1021	GTAGCTCCTCAGACAGAGTGGAAAGCAGATTTGGACAATACAGAAGTGAAGCGGTTATT	1080	V = Valine
	V A P Q T E W K Q I L D N T E V K A V I		
1081	TTAGGGGGCGACCCAAGTTCGGGTGCCCGAGTGTGAACAGGCAAGGTGGATATGGTAGAG	1140	
	L G G D P S S G A R V V T G K V D M V E		
1141	GACTTGATTCAGAAGGCGAGTCCGCTTTACAGCAGATCATCCAGGCTTGCCGATTTCCCTAT	1200	
	D L I Q E G S R F T A D H P G L P I S Y		
1201	ACAACCTCTTTTACGTCACAATGTAGTTGCCAGCTTTCAAACAGTACAGACTATGTT	1260	
	T T S F L R D N V V A T F Q N S T D Y V		
1261	GAGACTAAGGTTACAGCTTACAGAAACGAGATTTACTGCTGGATCATAGTGGTGCCTAT	1320	
	E T K V T A Y R N G D L L L D H S G A Y		
1321	GTTGCCCAATATTATATTACTTGGGATGAATTATCCTATGATCATCAAGGTAAGGAAATC	1380	
	V A Q Y Y I T W D E L S Y D H Q G K E V		
1381	TTGACTCCTAAGGCTTGGGACAGAAATGGGCAGGATTTGACGGCTCACTTTACCACTAGT	1440	
	L T P K A W D R N G Q D L T A H F T T S		
1441	ATTCCCTTAAAGGGGAATGTTTCGTAATCTCTCTGTCAAAATAGAGAGTGTACCGGGCTT	1500	
	I P L K G N V R N L S V K I R E C T G L		
1501	GCCTGGGAATGGTGGCGTACGGTTATGAAAAAACCGATTTGCCACTAGTGCCTAAGCGG	1560	
	A W E W W R T V Y E K T D L P L V R K R		
1561	ACGATTTCTATTTGGGGAACAACCTCTATCCTCAGGTAGAGGATAAGGTAGAAAATGAC	1620	
	T I S I W G T T L Y P Q V E D K V E N D		
1621	TAGCAGAGGAGAATGCTTGCACAAAAAGA 1650		

Figure 1.1: Sequence of the pneumolysin gene and flanking DNA with the predicted amino acid sequence. The possible promotor sequence upstream of the ATG initiation codon is underlined, and the putative Shine-Dalgarno sequence is boxed. The two in-frame ATG triplets are also underlined (Walker *et al.*, 1987).

Table 1.1: The proinflammatory effects of pneumolysin *in vitro*

Effect	Cell Type	Reference
Inhibits respiratory burst	human neutrophils, monocytes	Paton & Ferrante, 1983; Nandoskar <i>et al.</i> , 1994
Inhibits chemotaxis	human neutrophils	Paton & Ferrante, 1983
Inhibits random migration	human neutrophils	Paton & Ferrante, 1983
Inhibition of intraphagocytic killing of opsonized pneumococci	human neutrophils	Paton & Ferrante, 1983
Decreases HMP-shunt activity	human monocytes	Nandoskar <i>et al.</i> , 1986
Decreases H ₂ O ₂ production	human monocytes	Nandoskar <i>et al.</i> , 1986
Decreases degranulation	human monocytes	Nandoskar <i>et al.</i> , 1986; Houldsworth <i>et al.</i> , 1994
Slows ciliary beating	human respiratory epithelium	Feldman <i>et al.</i> , 1990
Increases production of IL-1 β	human monocytes	Houldsworth <i>et al.</i> , 1994
Activates PLA ₂	bovine pulmonary artery endothelial cells	Rubins <i>et al.</i> , 1994
Activates inducible NO synthase	murine macrophages	Braun <i>et al.</i> , 1999
Activates IL-6 production	murine macrophages	Braun <i>et al.</i> , 1999
Activates TNF- α production	murine macrophages	Braun <i>et al.</i> , 1999



1.2 Neutrophils

Neutrophils are the first line of defence against invasive microorganisms. They originate from stem cells in the bone marrow and are transported to the various tissues by the blood. The signals that mobilize neutrophils from the bone marrow include; IL-1, IL-3, TNF- α , granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), and other cytokines, as well as complement factors C3e and C5a (Mary, 1985). Neutrophils have a lifespan of 24-48 hours, after which they undergo apoptosis, but their lifespan can be prolonged when they migrate out of the circulation to sites of inflammation, or when exposed to anti-apoptotic cytokines, such as GM-CSF and IL-8 (Watson *et al.*, 1999).

1.2.1 Neutrophil adhesion and migration

In pulmonary capillaries, the slow flow rate of blood is further reduced by localized blood vessel dilation caused by regional inflammation. This allows for a loose and somewhat transient adhesion, also known as 'tethering', resulting in the rolling of neutrophils along the endothelium. During this stage, the neutrophils respond to ligands, mainly chemokines, PAF and PAF-like agents, which are presented on the endothelium surface (Springer, 1994). The rolling step is mediated by neutrophil L-selectin, as well as by E- and P-selectins expressed on activated endothelial cells (Phillips *et al.*, 1995).

L-selectin, on the surface of neutrophils permits interaction, not only with endothelial cells, but also with other neutrophils, via the P-selectin glycoprotein ligand (Diacova *et al.*, 1996; McEver & Cummings, 1997). The L-selectin molecule is localized to neutrophil microvilli and is cleaved or "shed" during neutrophil activation, possibly permitting neutrophils to detach during subsequent stages of transmigration. Metalloprotease inhibitors, which block shedding, cause slower rolling, increased adherence and accumulation of neutrophils *in vitro* (Walcheck *et al.*, 1996). Crosslinking of L-selectin on neutrophils not only stimulates adhesion, but also increases intracellular calcium concentrations due,

in part, to release from intracellular stores, thereby priming the cells for increased superoxide (O_2^-) production in response to chemoattractants (Waddell *et al.*, 1994). Sulphatide ligands for L-selectin have been shown to increase neutrophil expression of messenger ribonucleic acid (mRNA) for TNF- α and IL-8, and to promote tyrosine phosphorylation and activation of mitogen-activated protein kinase (MAPK) (Laudanna *et al.*, 1994; Waddell *et al.*, 1995). Frohlich *et al.*, (1998) showed that L-selectin triggers the respiratory burst of rolling neutrophils, and that CD11b/CD18 was not required for O_2^- generation under these conditions.

P-selectin is concentrated along endothelial borders, where discontinuities of the tight junctions are observed, targeting neutrophil adhesion and facilitating transendothelial migration (Burns *et al.*, 1999). Binding of neutrophils to P-selectin facilitates and supports degranulation, O_2^- production and membrane depolarization, through a priming event that is directly mediated by PAF and bacterial formylated peptides such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Lorant *et al.*, 1993). The architecture of endothelial cell-to-cell junctions is modified during the firm adhesion between neutrophils and endothelial cells (Del Maschio *et al.*, 1996).

The firm adhesion of neutrophils to endothelial cells involves the exclusive interaction of leucocyte integrins of the β_2 subfamily, composed of variable α subunits CD11a, CD11b and CD11c, and a common β subunit namely CD18 (Burg & Pillinger, 2001). These subunits have cytosolic domains that allow for the stabilization of cell adhesion and provide a framework for signaling proteins (Burg & Pillinger, 2001). The two important β_2 integrins on neutrophils are CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1, CR3). Counter-ligands for the above-mentioned integrins are intercellular adhesion molecule (ICAM)-1 and -2 for LFA-1, and ICAM-1 and -2, fibrinogen, heparin and factor X for CR3 (Sligh *et al.*, 1993).

The β_2 integrins cannot interact with their physiological ligands in unstimulated neutrophils. Binding activity is acquired by activating signals that lead to integrin

clustering and the transition of the β_2 integrin sub-population to a high affinity state (Stewart & Hogg, 1996). A variety of calcium-mobilizing agonists trigger CR3 calcium-dependent activation, such as chemoattractants (PAF, FMLP, C5a and IL-8), cytokines and growth factors (TNF- α , GM-CSF), as well as bacterial products (N-formylated peptides and lipopolysaccharides [LPS]) (Witko-Sarsat *et al.*, 2000). IL-8 is a selective chemoattractant for neutrophils which does not affect monocytes, although it does mobilize eosinophils (Premack & Schall, 1996; Rollins, 1997). At sites of experimental inflammation in animal models, intravenous administration of anti-IL-8 monoclonal antibodies inhibited neutrophil recruitment completely, confirming the importance of this chemokine in mobilizing neutrophils from the bloodstream (Sekido *et al.*, 1993; Folkesson *et al.*, 1995; Matsumoto *et al.*, 1997).

LFA-1-deficient mouse neutrophils have also been reported to show a marked reduction in firm attachment to purified ICAM-1 and to endothelial cells (Ding *et al.*, 1999), while CR3-deficient mice showed impaired degranulation and O_2^- production in experimental peritonitis, and a slight decrease in neutrophil adhesion, as well as blocking neutrophil apoptosis induced by the phagocytosis of opsonized particles (Coxon *et al.*, 1996; Ding *et al.*, 1999).

Neutrophils possess multiple receptors for chemoattractants that can trigger adhesion, direct cell migration, and promote degranulation and oxidative responses. These receptors bind complement C5a, formylpeptides, PAF, leukotriene B₄ (LTB₄), and chemokines such as IL-8 (Yokomizo *et al.*, 1997). The ligation of chemoattractants to these receptors, which belong to the G-protein coupled seven-transmembrane receptor family, activates phospholipase C (PLC) via heterodimeric G-proteins, which in turn results in intracellular calcium release, calcium channel opening and serial activation of protein kinases (Bokoch, 1995; Premack & Schall, 1996).

Adherent neutrophils migrate into tissue through a multistep process, consisting of repeated attachment/detachment processes which involve a series of diverse chemoattractants, adhesion molecules and substrates. Neutrophil locomotion

requires the continuous formation of new adhesive contacts at the cell front, while the rear of the cell detaches from the adhesive substrate (Lauffenburger & Horwitz, 1996).

Neutrophils are often required to migrate across polarized epithelium in order to accumulate in the lumen (Parkos, 1997). This migration involves the reversible disruption of intracellular tight junctions, controlled by complex signaling which leads to the cortical restructuring of epithelial F-actin (Hofman *et al.*, 1996). These are resealed after neutrophil passage (Nash *et al.*, 1987; Parsons *et al.*, 1987). If the neutrophils are activated in the lumen by bacterial products, they release 5'-adenosine monophosphate (AMP) which has been shown to be rapidly converted to adenosine, resulting in vectorial secretion of chloride ions into the luminal compartment, thereby modifying the epithelial electrolyte secretion and facilitating the hydration of luminal surfaces (Strohmeier *et al.*, 1995; Parkos, 1997).

1.2.2 Neutrophil phagocytosis and degranulation

Neutrophil phagocytosis depends on direct contact with bacteria or other targets that have been opsonized by immunoglobulin receptors and/or complement. Receptors for the Fc region (FcR) of IgG play essential roles in the effector functions of neutrophils, including the antibody-mediated clearance of microbes. Low affinity IgG receptors (Fc γ Receptors), Fc γ RIIA (CD32) and Fc γ RIIIB (CD16), predominate on resting cells with upregulation of high affinity receptors, Fc γ RI (CD64), induced by incubation of the cells with IFN- γ or by crosslinking of β_2 integrins (Takano *et al.*, 2000). The complement receptors involved are CR1 (CD35) and CR3. The adhesion of neutrophil CR1 and CR3 to particles coated with C3b/iC3b, is not sufficient in isolation to promote phagocytosis unless the neutrophils are activated by activators such as 4- α -phorbol 12-myristate 13-acetate (PMA) or by formyl-peptides and contact with fibronectin or laminin (Brown, 1986; Wright & Meyer, 1986).

The ultimate stimulus for neutrophils is phagocytosis, leading to release of granule contents, O_2^- production, as well as LTB_4 and PAF formation (Bazzoni *et al.*, 1991). LTB_4 and PAF are bioactive lipids, generated within seconds to minutes of stimulation of the cells and act as chemotactic agonists which function as amplifiers of the defence response (Bazzoni *et al.*, 1991).

Cytoplasmic granules, found in neutrophils, contain an array of proteases, antimicrobial peptides and polypeptides, which in turn participate in the migration and antimicrobial activities of neutrophils. The granules can be classified into four different groups: primary, secondary, and tertiary granules, as well as secretory vesicles (Witko-Sarsat *et al.*, 2000). The primary granules store the majority of neutrophil proteases and antimicrobial peptides/polypeptides, including bactericidal/permeability increasing protein (BPI), defensins, MPO and serine proteases such as elastase, proteinase 3 (PR3) and cathepsin G. The secondary granules contain membrane-associated adhesion molecules, such as CR3, the nicotinamide adenine dinucleotide phosphate - reduced form (NADPH) oxidase component, cytochrome b_{558} , and receptors for chemoattractants and cytokines. Tertiary granules and secretory vesicles also act as reservoirs of membrane polypeptides, involved in neutrophil activation and function – although to a lesser extent than secondary granules (Witko-Sarsat *et al.*, 2000).

Neutrophil stimulation not only results in granule fusion with the plasma membrane, but also allows for fusion of specific and primary granules with phagocytic vacuoles. This fusion accounts for the delivery of NADPH oxidase-derived reactive oxidants into the vacuole, as well as intravacuolar release of a range of cationic proteinases that interact with and disrupt negatively charged bacterial surfaces (Burg & Pillinger, 2001).

Serine proteases are a large family of enzymes occurring in the primary granules of neutrophils, including cathepsin G, elastase, PR3, and the enzymatically inactive CAP-37 (Witko-Sarsat *et al.*, 2000).

PR3 is found in both primary granules and secretory vesicles (Witko-Sarsat *et al.*, 1999). PR3 enhances the cleavage and activation of TNF- α and IL-1 β , from LPS-stimulated THP-1 (a human monocytic cell line) cells, in the presence of activated human neutrophils, suggesting that PR3 may play an important role in the amplification of the inflammatory response (Coeshott *et al.*, 1999).

BPI is cytotoxic to many Gram-negative bacteria at nanomolar concentrations. Its N-terminal allows binding to LPS, resulting in a selective and potent antibacterial effect and suppression of LPS bioactivity, while its C-terminal promotes bacterial attachment to neutrophils, leading to phagocytosis (Iovine *et al.*, 1997). The binding of BPI to live bacteria via LPS causes immediate growth arrest, although actual killing coincides with damage to the inner bacterial membrane, increasing membrane permeability and the activity of hydrolyzing bacterial phospholipases (Elsbach, 1998).

Defensins, also known as human neutrophil peptides, are a major component of the primary granules and are found in phagocytic vacuoles. Their functions vary from being antimicrobial, antiviral and cytotoxic to being chemotactic, opsonic and modulating hormonal responses (Ganz *et al.*, 1990). These broad-spectrum antimicrobial agents preferentially permeabilize microbial membranes, attacking multiple molecular targets to cooperatively penetrate and disrupt microbial surfaces and membrane barriers (Ganz & Weiss, 1997). Defensins also enhance the effect of BPI towards Gram-negative bacteria, probably by altering the activity of BPI or by inducing signaling (Levy *et al.*, 1994). The β -sheet defensins, small cationic antimicrobial peptides, induce microbial membrane permeabilization of both Gram-positive and -negative bacteria. Defensins interact with protease inhibitors, such as α -1 proteinase inhibitor (API, a major circulating inhibitor of serine proteinases) and α 1-antichymotrypsin (Panyutich *et al.*, 1995). Preincubation of API and α 1-antichymotrypsin with increasing concentrations of defensins resulted in a progressive decrease of antiprotease activity of these inhibitors against cathepsin G, as well as of the antiprotease activity of API against human neutrophil elastase. Conversely, both of these inhibitors inhibited

defensin-mediated cytotoxicity towards a human lung carcinoma cell line, whereas the elastase inhibitor, antileukoprotease, did not (Panyutich *et al.*, 1995).

Elastase causes lung matrix destruction and experimental emphysema; its proteolytic activities include the cleaving of insoluble elastin, as well as a variety of matrix proteins (Rao *et al.*, 1993). Plasma API is the main physiologic defence against elastase. These two proteins coexist within the primary granule population of human neutrophils, the elastase tends to be localized in the periphery of the granules, whereas the API is usually diffusely present in the matrix of the granules (Mason *et al.*, 1991). API is also present in the alveolar lining fluid in milligram concentrations, which increases to even higher levels during the acute phase response (Perlmutter *et al.*, 1989), while elastase *per se* and LPS, both individually and in combination, cause increased synthesis of API (Perlmutter & Punsal, 1988).

Deficiency of API is characterized by unopposed proteinase activity, predisposing to infection, and culminating in pulmonary emphysema (Crystal *et al.*, 1989; Lieberman, 2000). In individuals with hereditary deficiency of API, replacement therapy results in a marked reduction in the frequency and severity of pulmonary infections (Lieberman, 2000). Interestingly, it has been reported that API inhibits the haemolytic and cytotoxic activities of pneumolysin (Rubins *et al.*, 1992), but this was subsequently attributed to cholesterol contamination of the API preparation (Rubins & Freiberg, 1994).

Although elastase is mainly known for its proinflammatory and tissue destructive properties, Belaaouaj *et al.* (1998) reported that mice deficient in neutrophil elastase were predisposed to Gram-negative sepsis. In a further study, the same authors showed that elastase degrades the outer membrane protein A, localized on the surface of Gram-negative bacteria, identifying a novel mechanism of non-oxidative bacterial killing by elastase, and showing that the outer membrane protein A is a bacterial target in host defence (Belaaouaj *et al.*, 2000).

Taken together, these reports suggest that while elastase may possess direct antimicrobial properties, that uncontrolled activity of the protease predisposes to pulmonary dysfunction and microbial infection, and that optimum host defense is dependent on a balanced relationship between elastase and API.

The protective activity of API is reinforced by the secretory leucocyte protease inhibitor (SLPI) which is present in neutrophils and is also produced by cells on mucosal surfaces, including the respiratory epithelium in the human lung. SLPI can act as a barrier to tissue destruction mediated by neutrophil serine proteases (elastase, cathepsin G and PR3). SLPI is also important in the anti-elastase protection of the lung, but it is not active against PR3 (Rao *et al.*, 1993).

1.2.3 NADPH oxidase system

In the defence against microorganisms, neutrophils produce potent antimicrobial reactive oxidants from O_2^- . Control of reactive oxidant production is essential, and therefore the system that generates O_2^- - NADPH oxidase (involved in the respiratory burst and activation of oxidative metabolism), is only assembled upon cell activation (Burg & Pillinger, 2001). This oxidase consists of at least six components: p47^{phox} (for phagocyte oxidase), p67^{phox} and p40^{phox} (both found as a cytosolic complex in the resting neutrophil), rac-2 (a cytosolic ras-related protein), p22^{phox} and gp91^{phox} (membrane components that comprise cytochrome b₅₅₈). The classification of these components is as follows: *p* represents a protein or polypeptide, *gp* is a glycoprotein, *phox* stands for phagocyte oxidase, and the numbers represent the molecular weight in kilo Daltons. During assembly and activation of NADPH oxidase, the cytosolic protein components translocate to the plasma membrane, where they assemble around a central membrane bound flavohaemoprotein, known as cytochrome b₅₅₈ (DeLeo & Quinn, 1996; Babior, 1999; Clark, 1999).

Electrons are transferred from NADPH to O_2 to create O_2^- in the following reaction: $2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$ (Burg & Pillinger, 2001). The newly produced O_2^- is rapidly converted to H_2O_2 by superoxide dismutase or by spontaneous dismutation. H_2O_2 is reduced to O_2 and water by catalase (Babior,

1984; Nathan, 1987), but, MPO converts H_2O_2 to hypochlorous acid (HOCl) (Weiss, 1989). MPO is released from the primary granules, into the phagolysosome or extracellular space, upon cell activation, where it amplifies the toxic effect of H_2O_2 . HOCl is the most bactericidal of oxidants produced by neutrophils, and can also indirectly damage host tissue by inactivating the elastase-inhibitory capacity of API (Wasil *et al.*, 1987). HOCl also mediates the activation of pro-collagenase and pro-gelatinase, and mediates the conversion of cholesterol to chlorohydrins (Weiss *et al.*, 1985; Heinecke *et al.*, 1994). MPO can also utilize nitrite and H_2O_2 as substrates to catalyze tyrosine nitration of proteins (Sampson *et al.*, 1998).

1.2.4 Phospholipase A₂

The activation of neutrophils is accompanied by the calcium-dependent activation of PLA₂, which cleaves the integral membrane protein phosphatidylcholine (PC), to produce arachidonic acid and lysophosphatidylcholine (LPC) (Cassatella, 1999). This enzyme, produced not only by activated neutrophils, but also by other types of immune and inflammatory cells, possesses potent bactericidal activity, and can be classified as either secreted or cytosolic PLA₂ (sPLA₂ or cPLA₂, respectively). In the case of Gram-negative bacteria such as *E. coli*, BPI synergizes with PLA₂ in the intraphagocytic killing of these bacteria (Wright *et al.*, 1990; Weiss *et al.*, 1994). Neutrophils primed with TNF- α and stimulated with FMLP, showed a calcium-dependent increase in both sPLA₂ and cell-associated sPLA₂.

Arachidonic acid and LPC are the primary products of PLA₂ cleavage of membrane phospholipids. The former is the precursor of eicosanoid mediators of inflammation including leukotrienes, thromboxanes, prostaglandins and PAF (Scott *et al.*, 1990). Prostaglandin E₂ (PGE₂) possesses a range of proinflammatory activities and prolongs the lifespan of neutrophils by inhibiting apoptosis (Rossi *et al.*, 1995; Alonso *et al.*, 1998). LTB₄ also possesses a range of proinflammatory actions, which include the mobilization of intracellular calcium, and upregulation and synthesis of pro-adhesive and chemotactic factors (Alonso *et al.*, 1998). PAF, a potent and versatile biologically active lipid is produced by

neutrophils in a PLA₂-dependent manner. PAF can amplify inflammatory responses by interaction with G-protein/PLC-coupled PAF receptors on target cells, particularly eosinophils and neutrophils (Prescott, 1999). LPC possesses a range of proinflammatory activities, including pro-oxidative and pro-adhesive effects on phagocytes and vascular endothelial cells respectively, as well as the induction of degranulation, resulting from the activation of protein kinase C (PKC) by this lysophospholipid (Oishi *et al.*, 1988).

1.2.5 Calcium fluxes and homeostasis

Receptor-mediated activation of neutrophil functions requires the transient elevation of cytosolic calcium. These calcium-dependent functions include the activation of β_2 integrins and adhesion to vascular endothelium, O₂⁻ production, granule release and activation of cytosolic nuclear transcription factors, such as NF κ B (Lew *et al.*, 1986; Thelen *et al.*, 1993; Dolmetsch *et al.*, 1997).

In neutrophils, intracellular calcium is stored in either calciosomes – localized in the juxtannuclear space and mobilized by FMLP, or in a site located proximal to the plasma membrane which is possibly involved in the activation of β_2 integrins. (Favre *et al.*, 1996; Pettit & Hallett, 1996). The hydrolysis of membrane phosphatidylinositol is catalysed by PLC and results in the formation of inositol triphosphate (IP₃) and diacylglycerol (DAG). Inositol triphosphate induces calcium release from intracellular stores, while DAG, in conjunction with calcium, allows for PKC activation (Bokoch, 1995).

The abrupt increase (within 1 second) in cytosolic calcium, following FMLP exposure, is an exclusive result of calcium mobilization from the intracellular stores, with little or no contribution from extracellular calcium, during which the basal cytosolic calcium levels increase from around 100nM to $\pm 1\mu$ M (Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997; Geiszt *et al.*, 1997). Influx of extracellular calcium is delayed and can be detected at approximately 1 min after FMLP addition, terminating at around 5 min, which is compatible with a store-

operated calcium influx, the incoming cation being used primarily for refilling of empty stores (Anderson & Goolam Mahomed, 1997).

Restoration of calcium homeostasis in activated neutrophils is essential to prevent calcium overload and hyperactivity of these cells. This is accomplished through the action of the plasma membrane and endomembrane calcium adenosine triphosphatases (ATPases) operating in unison, while extracellular cation is excluded from the cells through the membrane depolarizing activity of NADPH oxidase (Anderson & Goolam Mahomed, 1997; Tintinger *et al.*, 2001).

1.2.6 Neutrophil cytokines

Neutrophils are a source of various cytokines. These cytokines can be divided into various groups, such as proinflammatory and antiinflammatory cytokines, C-X-C and C-C chemokines, as well as growth factors (Cassatella, 1999; Witko-Sarsat *et al.*, 2000). Neutrophils, the major infiltrating cells in inflamed tissue, possess 10-20 times less ribonucleic acid (RNA) per cell and synthesize 10 to 300-fold less RNA in comparison with peripheral mononuclear cells (Cassatella, 1999). However, this is balanced to some extent by the predominance of neutrophils, particularly in the early stages of the inflammatory response.

Chemokines are chemotactic cytokines that are able to recruit discrete leucocyte populations during inflammation. Chemokines are classified, according to the position of the first two cysteine residues on the N-terminal, into four closely related subfamilies, the C-X-C, C-C, C and CX₃C subfamilies. The first two subfamilies have been extensively characterized (Rollins, 1997; Baggiolini, 1998; Luster, 1998). The intercrine α subfamily or C-X-C subfamily predominantly activates the chemotactic responses of neutrophils, while the intercrine β subfamily or C-C cytokines are predominantly chemotactic for monocytes, eosinophils, basophils and certain T lymphocyte subsets (Oppenheim *et al.*, 1991). The C subfamily belongs to lymphotactin, and is a potent attractant for lymphocytes, while the CX₃C subfamily belongs to fractalkine or neutrotactin, an

integral membrane protein with a chemokine domain at its N terminus (Rollins, 1997; Baggiolini, 1998; Luster, 1998).

1.2.6.1 *Interleukin-8*

IL-8 is a member of the C-X-C cytokine family and is a key mediator in the recruitment of circulating neutrophils. It is expressed in response to inflammatory stimuli and secreted by a variety of cell types, of which neutrophils are the most abundant - suggesting that neutrophils, at the site of inflammation, can recruit more neutrophils to that specific site (Samanta *et al.*, 1990). Neutrophils respond to IL-8 by rapidly increasing intracellular calcium levels, undergoing chemotaxis, releasing granule enzymes, upregulating CR1 and CD11a,b,c/CD18 surface expression, increasing adherence to unstimulated endothelial cells, migrating across the endothelium, delaying apoptosis and acquiring increased resistance to corticosteroids (Dewald *et al.*, 1988; Thelen *et al.*, 1988; Djeu *et al.*, 1990; Thornton *et al.*, 1990; Oppenheim *et al.*, 1991; Strickland *et al.*, 2001).

FMLP, C5a, PAF, LTB₄, thrombopoietin, and other chemotactic factors act as triggers for neutrophils to release IL-8 (Cassatella *et al.*, 1992; Arnold *et al.*, 1994; Ember *et al.*, 1994; McCain *et al.*, 1994; Brizzi *et al.*, 1997). IL-8 production, activated by chemoattractants (such as FMLP, PAF and C5a), was found to be preceded by an enhanced expression of IL-8 mRNA, and was dependent on *de novo* protein synthesis (Cassatella *et al.*, 1992). Other activators of IL-8 secretion by neutrophils are TNF- α , which stimulates IL-8 mRNA secretion in a time and dose-dependent manner (Strieter *et al.*, 1992; Cassatella *et al.*, 1993a; Fujishima *et al.*, 1993; Hachicha *et al.*, 1995), as well as GM-CSF, the activity of which is potentiated somewhat paradoxically by the immunosuppressive drugs, cyclosporin A and rapamycin (Hachicha *et al.*, 1995; Hilger *et al.*, 1995).

Neutrophils (isolated from human peripheral blood) also release IL-8 following stimulation with calcium mobilizing stimuli such as the calcium ionophore A23187, or with thapsigargin, an inhibitor of the endomembrane calcium-ATPase which increases cytosolic calcium concentrations by promoting leakage of the divalent cation from intracellular stores. IL-8 was localized by sucrose gradient

centrifugation to a subcellular fraction of heterogeneous, light membranous organelles. The accumulation of IL-8 within these organelles was inhibited by cycloheximide, but not actinomycin D, suggesting that IL-8 accumulation is under translational rather than transcriptional control. This indicates that peripheral blood neutrophils are capable of synthesizing large amounts of IL-8, with subsequent release of IL-8 during exudation, which may amplify neutrophil migration into sites of inflammation (Kuhns & Gallin, 1995).

In addition to the aforementioned activators of IL-8 production by neutrophils, the inflammatory microcrystals monosodium urate monohydrate (MSU) and calcium pyrophosphate dihydrate (CPPD), the major mediators of gout and pseudogout, enhance the TNF- α - and GM-CSF-induced production of IL-8 synergistically (Hachicha *et al.*, 1995). IL-8 mRNA levels are also highly expressed when neutrophils are stimulated with PMA, and staurosporine (a nonspecific kinase inhibitor), in a time- and dose-dependent manner (Cassatella *et al.*, 1993b; Fujishima *et al.*, 1993; Arnold *et al.*, 1994; Au *et al.*, 1994; Kuhns & Gallin, 1995; Kuhns *et al.*, 1998). Pretreatment of neutrophils with the LTB₄ inhibitor MK-886 was reported to partially block IL-8 production, suggesting that the 5-lipoxygenase pathway is involved in the synthesis and/or release of this chemokine, possibly by autocrine augmentation of calcium mobilization (Riedel & Kaufmann, 1997).

The production and release of IL-8 by neutrophils can also be initiated by exposure of the cells to bacteria and their products, as well as to viruses. These include:

- *Listeria monocytogenes* (invasive and noninvasive strains) and *Yersinia enterocolitica* (Arnold & Konig, 1998).
- Bacterial toxins:
 - Erythrogenic toxin A from *Streptococcus pyogenes*
 - Panton-Valentine leukocidin from *Staphylococcus aureus*
 - Alveolysin from *Bacillus alvei* (Konig *et al.*, 1994; Cassatella, 1999).
- Products of *Pseudomonas aeruginosa*, but not lipopolysaccharides (Inoue *et al.*, 1994).

- *Salmonella typhimurium* and *S. aureus* strongly induce IL-8 secretion, but *S. pneumoniae*, *Staphylococcus epidermidis* and *Candida albicans* are less potent IL-8 stimulators (Hachicha *et al.*, 1998).
- *Mycobacterium tuberculosis*, with the ratio between neutrophils and microorganisms being important, as IL-8 release critically depends on it, with a 1:1 ratio being optimal (Friedland *et al.*, 1992).
- Respiratory syncytial virus (RSV), both native and heat- or ultra-violet (UV)-inactivated (Konig *et al.*, 1996).
- Epstein-Barr virus (EBV) which increases the accumulation of IL-8 specific mRNA in the intracellular compartments (McColl *et al.*, 1997).

During the inflammatory response, the magnitude of IL-8 production is correlated with the intensity of neutrophil accumulation at the inflammatory site. These neutrophils exhibited 100-fold greater levels of cell-associated IL-8, and spontaneously released up to 50-fold more IL-8 than resting cells, suggesting that this chemokine plays a role in the autocrine regulation of the neutrophil-mediated inflammatory response (Kuhns & Gallin, 1995).

1.2.6.2 *Tumour necrosis factor- α*

TNF- α , is a member of the superfamily of membrane-anchored and soluble cytokines that are centrally involved in T cell immunity. It is a paracrine and endocrine mediator, has potent immunomodulatory and proinflammatory properties and plays an important role in host defence (Tracey & Cerami, 1994; Bazzoni & Beutler, 1996). It contributes significantly to vasodilation, thrombosis, leucocyte recruitment, bone resorption, matrix degradation in cartilage, changes in liver metabolism, pannus formation and cachexia. Cellular targets of TNF- α include monocytes, macrophages, lymphocytes, eosinophils and neutrophils; it is also a potent priming agent for neutrophil functions (Tracey & Cerami, 1994; Edwards & Hallett, 1997).

Haziot and colleagues (1993) showed that the surface molecule CD14, a receptor for LPS and LPS-binding protein, mediates the ability of LPS to induce TNF- α .

secretion in neutrophils, suggesting that this pathway is a major contributor in the pathogenesis of endotoxic shock. Other stimuli of TNF- α production include sulphatides, thapsigargin, the mannoprotein fraction 2 of *C. albicans*, *L. monocytogenes*, *Y. enterocolitica*, *Cryptococcus neoformans* and extracts thereof, LPS from various periodontopathic bacteria, and *Plasmodium falciparum*-infected erythrocytes (Arnold *et al.*, 1993; Laudanna *et al.*, 1994; Wahlgren *et al.*, 1995; Retini *et al.*, 1996; Torosantucci *et al.*, 1997; Yoshimura *et al.*, 1997; Kuhns *et al.*, 1998).

Neutrophils plated on fibrinogen and treated with FMLP, released TNF- α (within 45 min, with maximal production after 90 min), as well as H₂O₂ and lactoferrin (a specific granule component), with similar kinetics. In this experimental model, the neutralization of TNF- α with anti-TNF- α antibodies led to the attenuation of H₂O₂ and lactoferrin release stimulated by FMLP, while anti-CD14 and anti-IL-8 antibodies showed no effect (Balazovich *et al.*, 1996). Actinomycin D and cycloheximide-treated neutrophils released 33% less H₂O₂ and lactoferrin than untreated cells, suggesting that protein synthesis is required for this FMLP-mediated activation of adherent neutrophils. This supports the authors' contention that TNF- α release and ligation of TNF- α receptors are central for prolonged FMLP-stimulated oxidant release from neutrophils adherent to fibrinogen (Balazovich *et al.*, 1996).

The latter observations were contested by Derevianko *et al.* (1996), who reported that FMLP antagonised the production of TNF- α by neutrophils adherent to fibronectin, laminin or plastic. They also reported that LPS was a potent stimulator of TNF- α production and that PMA did not induce release of this cytokine.

Vulcano and colleagues (1998) reported that FMLP did not induce, but rather inhibited the production of TNF- α by neutrophils. They found that neutrophil CD14 and LPS binding sites were unaltered after FMLP exposure, but that the CD11a/CD18 complex (a LPS receptor) was upregulated (Vulcano *et al.*, 1998).

The ability of neutrophils to release TNF- α in response to a variety of stimuli suggests that the role of the granulocyte in host defence goes beyond the killing of microorganisms. Moreover, TNF- α production may represent a mechanism whereby neutrophils can be activated in an autocrine or paracrine fashion (Kowanko *et al.*, 1996). Nevertheless, the role of the neutrophil as a major TNF- α -producing cell, as well as the relevance of this, if any, to autocrine activation of these cells remains to be established.

1.2.6.3 *Interleukin-1*

IL-1 is an important mediator of the host defence response to injury and infection, it has both protective and proinflammatory effects. Monocytes, macrophages, dendritic cells, lymphocytes, neutrophils, keratinocytes, natural killer (NK) cells, endothelium, fibroblast and glial cells can secrete IL-1. There are two forms of IL-1, IL-1 α and IL-1 β , in most studies their biological activities are indistinguishable (Dinarello, 1997).

IL-1 affects the hematopoietic system at various levels. It costimulates T cell proliferation in the classic costimulator assay, it induces cytokine production in monocytes, prolongs the *in vitro* survival of neutrophils, activates the adhesive prothrombotic activities of endothelial cells, and stimulates the release of corticotropin-releasing hormone by the hypothalamus, causing a release of corticosteroids into the bloodstream by the adrenals (Dinarello, 1997). It also induces hypotension, fever, weight loss, neutrophilia, and acute-phase response *in vivo* (Dinarello, 1996).

The production of IL-1 by human neutrophils can be triggered by a variety of stimuli. These stimuli include cytokines and growth factors, activators of surface molecules/receptors, bacteria and fungi and their related products, viruses, as well as other agents such as PMA, concanavalin A, synovial fluid from the affected joints of patients with rheumatoid arthritis and matrix proteins (fibronectin and laminin) (Cassatella, 1999).

1.2.6.4 *Interleukin-6*

IL-6 is a multifunctional cytokine; it is a terminal differentiation factor for B cell maturation, a growth factor for myeloma, plasmacytoma and hybridoma cells, T cells, mesangial cells and megakaryocytes, as well as possessing both pro- and antiinflammatory properties (Hirano, 1998). IL-6 is synthesized by a wide variety of cells, including fibroblasts, T and B cells, macrophages, endothelial cells, astrocytes and keratinocytes (Hirano, 1998). The ability of neutrophils to express IL-6 is a controversial matter. Several authors claim that it cannot be expressed by neutrophils (Takeichi *et al.*, 1994; Wang *et al.*, 1994), while others contend that it is (Mianji *et al.*, 1996).

1.2.6.5 *Interleukin-12*

Mononuclear phagocytes, B cells, neutrophils and dendritic cells produce IL-12. The biological activities of IL-12 are mainly directed towards NK cells and T lymphocytes. It induces the production of other cytokines, enhances cell-mediated cytotoxicity, and is required for the optimal differentiation of cytotoxic T lymphocytes (Gately *et al.*, 1998; Trinchieri, 1998). It is possible that IL-12, produced by neutrophils in response to bacterial or parasitic infections, induces IFN- γ production by T and NK cells, which in turn activate macrophages, favouring Th1 immune responses (Trinchieri *et al.*, 1993). If this does happen to any meaningful extent *in vivo*, then neutrophils may play an important role in the early, innate immune response to microbial pathogens which are subsequently eradicated by cellular immune mechanisms. In this context, neutrophils may provide an important link between the innate and adaptive host defence mechanisms involved in the eradication of intracellular microbial pathogens such as *M. tuberculosis*.

1.2.6.6 *Interferon- γ*

The glycoprotein IFN- γ is mainly secreted by T cells and NK cells, and has weak antiviral and antiproliferative activity. It is responsible for inducing non-specific cell-mediated mechanisms in host defence (Farrar & Schreiber, 1993). Yeaman and colleagues (1998) showed that IFN- γ is produced by neutrophils. It is a

macrophage-, as well as a neutrophil-activating factor (Berton & Cassatella, 1992). The functions of IFN- γ include the induction of surface expression of Fc γ RI, the enhancement of neutrophil antibody-dependent cell cytotoxicity, the potentiation of the generation of reactive oxygen intermediates and granule release in response to different stimuli, as well as the modulation of neutrophil microbicidal activity (Berton & Cassatella, 1992). It is also widely used in the immunoprophylaxis of chronic granulomatous disease, although the molecular/biochemical mechanism of protective action has not been established (Segal *et al.*, 2000).

1.2.6.7 *Granulocyte/macrophage colony-stimulating factor*

GM-CSF is an inducer of proliferation and functional activation of myeloid cells. Exposure of neutrophils to GM-CSF results in mRNA expression for different cytokines, including TNF- α , macrophage colony-stimulating factor (M-CSF) and G-CSF. However, only the latter two were translated into secretory proteins (Lindemann *et al.*, 1989). G-CSF enhances the proliferation of granulocyte precursors in bone marrow, it primes mature neutrophils for superoxide production, phagocytosis and antibody-mediated cytotoxicity against targets such as tumour cells, and also delays neutrophil apoptosis (Demetri & Griffin, 1991; Savill, 1997).

1.2.7 Apoptosis

The termination of neutrophil emigration from blood vessels, is achieved by at least three different mechanisms. Firstly, changes in the pattern of inflammatory/antiinflammatory cytokines and cytokine antagonists secreted by tissue cells and infiltrated leucocytes. Secondly, the progressive return of endothelial cells to their resting state in terms of down-regulation of expression of membrane adhesion molecules and displayed chemoattractants, and thirdly, the inactivation of chemoattractants by specific enzymes or via receptor-mediated endocytosis (Ayesh *et al.*, 1995; Hofman *et al.*, 1998).

The major mechanism of removal of neutrophils and resolution of inflammation is apoptosis and ingestion of apoptotic neutrophils by macrophages (Cox *et al.*,

1995; Savill, 1997). Inflammatory mediators, such as LPS, GM-CSF, and IL-8 can delay apoptosis by increasing mitochondrial stability, reducing caspase 3 activity, and by down-regulating the gene expression of Bax (a pro-apoptotic member of the Bcl-2 family) (Dibbert *et al.*, 1999; Watson *et al.*, 1999).

The Fas or the Fas ligand system represents an important cellular pathway that is responsible for the induction of apoptosis in various tissues (Chinnaiyan & Dixit, 1997), and has also been suggested to function as an autocrine pathway to T cell suicide (Dhein *et al.*, 1995). Neutrophils are highly susceptible to Fas-induced death, and the Fas system plays a fundamental role in the regulation of spontaneous neutrophil apoptosis (Iwai *et al.*, 1994; Hsieh *et al.*, 1997; Larochelle *et al.*, 1998). The release of soluble Fas ligand, by macrophages, can be promoted by the ingestion of opsonized particles or apoptotic neutrophils, as well as by the killing of bystander neutrophils (Brown & Savill, 1999). This could represent a negative feedback loop, accelerating the resolution of inflammation by eliminating recruited leucocytes through apoptosis (Witko-Sarsat *et al.*, 2000).

A reduction of approximately 50% in spontaneous neutrophil death *in vitro*, during a 72 hour period, was observed when a soluble receptor for Fas ligand was added to the medium (Liles *et al.*, 1996). It has been suggested that the Fas system could represent a key element controlling the rapid spontaneous turnover of neutrophils, but could also implicate a possible role for the Fas ligand in neutrophil-mediated cytotoxicity (Liles & Klebanoff, 1995).

Phagocytosis of apoptotic neutrophils actively inhibits the production of IL-1 β , IL-8, IL-10, GM-CSF, TNF- α , leukotriene C₄ and thromboxane B₂ by human macrophages, suggesting that the resolution of inflammation depends not only on the removal of apoptotic cells, but also on active suppression of inflammatory mediator production (Fadok *et al.*, 1998).

1.3 Hypothesis tested:

The laboratory research described in the following chapters has been designed to test the hypothesis that pneumolysin at low, pore-forming concentrations causes sub-lethal damage to the plasma membrane of human neutrophils, resulting in altered cation fluxes (particularly influx of calcium) and consequent enhancement of the proinflammatory activities of these cells.

1.4 Objectives:

The primary objectives of this study were to investigate the effects of sub-cytolytic concentrations of purified pneumolysin on:

- the proinflammatory activities (O_2^- production, elastase release, CR3 expression, PLA₂ activity, production of PGE₂, LTB₄ and IL-8) of human neutrophils *in vitro* and to relate changes in these to altered membrane permeability to univalent (potassium, sodium) and divalent (calcium) cations,
- the vulnerability of API to neutrophil-mediated, oxidative functional inactivation.

Chapter 2:

Proinflammatory interactions of pneumolysin with human neutrophils

2.1 Introduction

As described in Chapter 1, pneumolysin is considered to be intimately involved in the pathogenesis of infections caused by *S. pneumoniae* (Mitchell & Andrew, 1997). Although the involvement of the toxin in the pathogenesis of pneumococcal meningitis (Friedland *et al.*, 1995) and otitis media (Sato *et al.*, 1996) is less compelling, evidence derived from animal models of experimental infection and inflammation clearly supports a primary role for pneumolysin in the etiology of inflammatory events and tissue damage in pneumococcal pneumonia, septicaemia and ocular infections (Johnson & Allen, 1975; Feldman *et al.*, 1991; Canvin *et al.*, 1995; Rubins *et al.*, 1995; Rubins *et al.*, 1996). Interestingly, pneumolysin-mediated ocular damage in rabbits is attenuated by prior induction of leukopenia (Harrison *et al.*, 1983).

The proinflammatory potential of pneumolysin is supported by *in vitro* studies in which treatment of human phagocytes with this microbial toxin resulted in increased release of granule enzymes (Johnson *et al.*, 1981) and proinflammatory cytokines (Houldsworth *et al.*, 1994). Although some of the proinflammatory effects of pneumolysin may be due to cytolysis of various host cell types, including ciliated respiratory epithelium (Feldman *et al.*, 1990), endothelial cells (Rubins *et al.*, 1992) and type II alveolar epithelial cells (Rubins *et al.*, 1993), as well as the activation of PLA₂ in endothelial cells (Rubins *et al.*, 1994), relatively little is known about the proinflammatory actions of sub-lytic, pathologically-relevant concentrations of the toxin.

In this study, I investigated the effects of pneumolysin on several proinflammatory activities of human neutrophils and related changes in these to alterations in the permeability of the outer membrane of these cells to calcium (Ca²⁺) and potassium (K⁺).

2.2 Materials and Methods

2.2.1 *Pneumolysin*

Recombinant native and attenuated preparations of pneumolysin were kindly provided by Prof T. Mitchell, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland, UK. Recombinant pneumolysin was expressed in *E. coli* and purified from cell extracts as described by Saunders and colleagues (1989). Protein homogeneity was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The haemolytic activity of this preparation of pneumolysin was evaluated and confirmed in a series of preliminary experiments, the results of which are shown in Appendix 1 (page 106). The same preparation of pneumolysin was used in all the studies presented in this thesis. An attenuated form of the toxin was generated by oligonucleotide-mediated, site-directed mutagenesis (Berry *et al.*, 1989). This mutant contains a tryptophan to phenylalanine substitution at position 433 in the protein. The stock concentrations of the biologically active and mutant toxins contained 0.21 and 0.30 mg/ml of protein respectively, which corresponded to 1.3×10^6 and 1.4×10^2 haemolytic units/ml. These pneumolysin preparations were essentially free of contaminating bacterial endotoxin (< 2 pg/ml) and were diluted in the same endotoxin-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM CaCl_2 , Highveld Biological (Pty) Ltd, Johannesburg, RSA), which was added to the pneumolysin-free control systems described below.

2.2.2 *Chemicals and reagents*

Unless indicated these were obtained from the Sigma Chemical Co, St Louis, MO, USA, while radiochemicals were purchased from DuPont NEN Research Products, Boston, Mass, USA.

2.2.3 *Neutrophils*

Purified neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood from healthy, adult volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma

Diagnostics) cushions at 400 x g for 25 min at room temperature. The resultant pellets were resuspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) containing 3% gelatin to sediment and remove most of the erythrocytes. After centrifugation, the remaining erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1×10^7 cells/ml in PBS and held on ice until used.

2.2.4 Cytotoxic activity of pneumolysin

This was determined by measurement of intracellular adenosine triphosphate (ATP) levels using a sensitive luciferin/luciferase chemiluminescence procedure (Holmsen *et al.*, 1972), as well as by spectrophotometric measurement of lactate dehydrogenase (LDH) in cell-free supernatants of neutrophils (1×10^6 /ml) which had been incubated with active pneumolysin (0.0167 - 41.75 ng/ml) for 10 min at 37°C in Ca^{2+} -free HBSS, or Ca^{2+} -replete HBSS without and with the Ca^{2+} -chelating agent, ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 5 mM, final).

2.2.5 Superoxide production

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) procedure (Minkenberg & Ferber, 1984). Neutrophils (1×10^6 /ml) were preincubated for 5 min at 37°C in 800 μ l of indicator-free HBSS containing 0.2 mM lucigenin, followed by addition of native (0.0167 - 41.75 ng/ml, final) or mutant (1.62 - 8077 ng/ml, final) pneumolysin. In some experiments the neutrophils were suspended in HBSS which was nominally free of Ca^{2+} ($\sim 2 \mu\text{M}$ according to the manufacturer) or in Ca^{2+} -replete HBSS to which the extracellular calcium-chelating agent, EGTA (5 mM, final), was added 1 min prior to pneumolysin. The vials were then reincubated for 10 min at 37°C after which basal chemiluminescence was recorded using a Lumac® Biocounter (Lumac Systems Inc., Titusville, FL, USA) followed by activation of the neutrophils with the synthetic, chemotactic tripeptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM , final), and measurement of LECL. The final volume in each vial was 1 ml and the results are expressed in relative light units (rlu). The peak

values for FMLP-stimulated systems were reached at 40-50 sec after addition of the stimulant and have been corrected for background (i.e. subtraction of the value for the corresponding unstimulated systems).

2.2.6 *Elastase release*

Neutrophil degranulation was measured according to the extent of release of the primary granule enzyme, elastase. Neutrophils were incubated at a concentration of 2×10^6 cells/ml in HBSS with and without active pneumolysin (0.0167 - 41.75 ng/ml) for 10 min at 37°C. FMLP (0.1 μ M, final) in combination with a submaximal concentration of cytochalasin B (CB, 1 μ M, final), which potentiates the activation of neutrophil degranulation by FMLP, was then added to the cells which were incubated for 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400 x g for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase by a micromodification of a standard colourimetric procedure (Beatty *et al.*, 1982). Briefly, 125 μ l of supernatant was added to the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (3 mM in dimethyl sulphoxide) in 0.05 M Tris-HCl (pH 8.0) and elastase activity was monitored spectrophotometrically at a wavelength of 405 nm.

2.2.7 *CR3 expression*

The levels of spontaneous and FMLP/CB (0.1 μ M: 1 μ M)-activated expression of the β_2 -integrin, CR3, on neutrophils were measured after 15 min of treatment of the cells with active pneumolysin (0.0167 - 41.75 ng/ml) at 37°C. The cells (2×10^6) in 1 ml HBSS (final volume) were treated with 5 μ l of anti-CD11b-RPE monoclonal antibody or an isotopic control antibody (Dako A/S, Gostrup, Denmark) for 10 min. Analysis of CR3 expression was performed using a Coulter Epics XL-MLC flow cytometer (Coulter Corp. Miami, FL, USA) equipped with an air-cooled argon laser at 488 nm.

2.2.8 *Phospholipase A₂ activity*

This was measured using a radiometric thin layer chromatography (TLC) procedure (Krajewska & Anderson, 1993). Neutrophils (1×10^7 /ml) were

coincubated with 5 $\mu\text{Ci/ml}$ radiolabelled arachidonic acid (5,6,8,9,11,12,14,15- $^3\text{H(N)}$, 185 Ci/mmol) for 15 min at 37°C in Ca^{2+} -free HBSS containing 5 μM indomethacin, to allow incorporation of ^3H -arachidonic acid into membrane phospholipids. The cells were then washed twice and resuspended to $1 \times 10^7/\text{ml}$ in Ca^{2+} -replete HBSS. The cells ($2.5 \times 10^6/\text{ml}$) were then preincubated for 10 min at 37°C followed by the addition of active pneumolysin (0.0167 - 41.75 ng/ml). The final volume was 2 ml. After 3 min of incubation at 37°C the reactions were terminated and ^3H -arachidonic acid extracted by the addition of 5 ml chloroform/methanol (2:1 vol/vol) and thorough mixing. The lower organic phase was removed and evaporated to dryness under a nitrogen stream. The lipids were reconstituted in 40 μl chloroform/methanol and spotted onto silica gel precoated TLC plates (Merck, Darmstadt, Germany) together with 2 μM unlabelled arachidonic acid standard to facilitate detection. The plates were developed in chloroform/acetone (96:4 vol/vol) and then exposed to iodine vapors. The arachidonic acid spots were localized, excised and assayed for radioactivity.

2.2.9 Spectrofluorimetric measurement of cytosolic Ca^{2+}

Fura-2/AM (Calbiochem Corp., La Jolla, CA, USA) was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments (Grynkiewicz *et al.*, 1985). Neutrophils ($1 \times 10^7/\text{ml}$) were preloaded with fura-2 (2 μM) for 30 min at 37°C in PBS, washed twice and resuspended in indicator-free HBSS. The fura-2-loaded cells ($2 \times 10^6/\text{ml}$) were then preincubated for 10 min at 37°C after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable base-line was obtained, active or mutant pneumolysin (0.0167 - 41.75 ng/ml and 1.62 - 8077 ng/ml respectively) or an equal volume of HBSS (control system) was added to the cells and fluorescence intensity monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils. The effects of treatment of the cells with 5 mM EGTA, as well as those of U-73122 (10 μM), a phospholipase C (PLC) inhibitor (Tatrai *et al.*, 1994), on pneumolysin-mediated alterations in cytosolic Ca^{2+} levels were also investigated. EGTA was

added to the cells 60 sec prior to pneumolysin, while U-73122 was present with the cells throughout the 10 min preincubation period.

2.2.10 Membrane potential

The potential sensitive fluorescent dye, 3,3-dipentylloxycarbocyanine (di-O-C₅(3)) was used to measure changes in membrane potential in neutrophils (Seligmann & Gallin, 1980). The cells (1×10^6 /ml) were preincubated for 10 min at 37°C in HBSS containing 80 nM (final) di-O-C₅(3) after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 nm and 510 nm respectively. The neutrophils were then treated with active pneumolysin (0.0167 - 41.75 ng/ml) or an equivalent volume of HBSS (control system) and the subsequent alterations in fluorescence intensity monitored over a 5-10 min period.

2.2.11 Ca²⁺-ATPase activity

Plasma membrane Ca²⁺-ATPase activity was measured according to the rate of net efflux of ⁴⁵Ca²⁺ from neutrophils in a system which was uncomplicated by concomitant influx of the cation. The cells (1×10^7 /ml) were loaded with ⁴⁵Ca²⁺ (2 μCi/ml, specific activity 28.82 mCi/mg) for 30 min at 37°C in Ca²⁺-free HBSS. The neutrophils were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca²⁺-replete HBSS and held on ice until used, which was within 10 min of completion of loading with ⁴⁵Ca²⁺. The ⁴⁵Ca²⁺-loaded neutrophils (2×10^6 /ml) were then preincubated for 10 min at 37°C in Ca²⁺-replete HBSS followed by addition of active pneumolysin (8.37 and 41.75 ng/ml) and measurement of net efflux of ⁴⁵Ca²⁺ over 30 min at 37°C.

Reactions were stopped by the addition of 10 ml ice-cold Ca²⁺-replete HBSS to the tubes which were transferred immediately to an ice-bath. The cells were then pelleted by centrifugation at 400 x g for 5 min followed by washing with 15 ml ice-cold Ca²⁺-replete HBSS and the pellets finally dissolved in 0.5 ml of 0.5% triton X-100/0.1 M sodium hydroxide (NaOH) and the radioactivity assessed in a liquid scintillation spectrometer. The results are presented as the amount of cell-

associated radiolabelled cation (pmoles $^{45}\text{Ca}^{2+}/10^7$ cells). A comparison of this procedure with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope has been described by Anderson and Goolam Mahomed (1997).

2.2.12 Assay of transmembrane fluxes of potassium

Radiolabelled rubidium ($^{86}\text{Rb}^+$) was used as tracer for measuring K^+ uptake and efflux (Prasad *et al.*, 1987). For uptake studies, neutrophils ($1 \times 10^6/\text{ml}$) were suspended in isotonic Tris buffer (122 mM NaCl, 4 mM KCl, 1 mM MgSO_4 , 1 mM KH_2PO_4 , 20 mM Tris, 5 mM glucose, pH 7.4) and preincubated for 30 min at 37°C followed by serial addition of 2 μCi $^{86}\text{Rb}^+$ (Rubidium-86 chloride, specific activity 3.56 mCi/mg) and pneumolysin (8.37 and 41.75 ng/ml) to each tube (final volume 2 ml). After 30 min incubation at 37°C the reactions were terminated by the addition of ice-cold Tris buffer. The cells were then washed twice and the pellets finally dissolved in 0.5 ml 0.5% triton X-100/0.1 M NaOH and the radioactivity assayed in a liquid scintillation spectrometer. Sodium, potassium-ATPase (Na^+ , K^+ -ATPase) activity was taken as the difference in $^{86}\text{Rb}^+$ uptake in the presence and absence of 50 μM ouabain. The contribution of PLA_2 to alterations in the uptake of $^{86}\text{Rb}^+$ by pneumolysin-treated neutrophils was investigated by the inclusion of α -tocopherol (25 $\mu\text{g}/\text{ml}$ final, vitamin E, F.Hoffman-La Roche, Basel, Switzerland), which neutralizes lysophosphatidyl choline (Kagan, 1989), a potent inhibitor of Na^+ , K^+ -ATPase (Oishi *et al.*, 1990).

For efflux experiments, neutrophils were preloaded with $^{86}\text{Rb}^+$ by incubating the cells ($1 \times 10^7/\text{ml}$) with $^{86}\text{Rb}^+$ (5 $\mu\text{Ci}/\text{ml}$) for 30 min at 37°C in isotonic Tris Buffer. The cells were then washed twice with and resuspended in ice-cold Tris buffer at $2 \times 10^6/\text{ml}$ and the rate of efflux of the cation in control and active pneumolysin (8.37 and 41.75 ng/ml)-treated cells measured over a 10 min incubation period at 37°C .

The effects of active pneumolysin (41.75 and 83.70 ng/ml) on the activity of purified Na^+ , K^+ -ATPase (from porcine cerebral cortex) were measured using a spectrophotometric procedure described by Schwartz *et al.* (1969) based on the

ouabain (50 μ M)-inhibitable conversion of added ATP to ADP by the ATPase. ADP was assayed indirectly according to the reaction sequence: phosphoenolpyruvate + ADP \longrightarrow pyruvate + NADH \longrightarrow lactate + NAD⁺ catalysed by added pyruvate kinase and LDH, respectively. The conversion of NADH to NAD⁺ was monitored spectrophotometrically at 340 nm. Each system contained 20 milliunits/ml Na⁺, K⁺-ATPase, 2.5 mM phosphoenolpyruvate, 0.17 mM NADH, 0.25 mM ATP, 3 units/ml of pyruvate kinase and LDH, with and without pneumolysin and/or ouabain in a final volume of 3 ml assay buffer (25 mM Tris HCl, 5mM MgCl₂, 10 mM KCl, 100 mM KCl). All components (with the exception of ATP, pyruvate kinase and LDH) were preincubated for 5 min at 37°C after which ATP and the enzymes were added simultaneously and the reactions monitored over a 15 min time-course during which the rate was linear. Results are expressed as nmoles NAD⁺ generated/min.

2.2.13 Statistical analysis

The results of each series of experiments are expressed as either the mean \pm standard error of the mean (SEM) or the median values with 25-/75-percentiles. Levels of statistical significance were calculated by the Mann-Whitney U-test (two-tail) when two groups were compared or by analysis of variance with subsequent Tukey-Kramer multiple comparisons test for multiple groups. The computer-based software systems Instat II and Minitab were used for analyses. Significance levels were taken at a *P* value of <0.05.

2.3 Results

2.3.1 Cytotoxic effects of pneumolysin

The effects of pneumolysin on neutrophil ATP levels and on the release of cytosolic LDH using cells suspended in Ca²⁺-replete HBSS, as well as the corresponding comparative data for cells suspended in Ca²⁺-free HBSS are shown in Table 2.1. With cells suspended in Ca²⁺-replete HBSS a decrease in intracellular ATP in the setting of an increase in the release of LDH was observed at the highest concentration (41.75 ng/ml) of the toxin, but not at lower concentrations. However, the cytotoxic potential of pneumolysin was enhanced when the cells were suspended in Ca²⁺-free HBSS, with reduced ATP levels and

increased release of LDH observed at concentrations of 8.37 ng/ml and 1.67 ng/ml, respectively.

2.3.2 Superoxide production

The effects of active pneumolysin on superoxide production by FMLP-activated neutrophils are shown in Figure 2.1. Pneumolysin at concentrations of ≥ 1.67 ng/ml significantly ($P < 0.05$ by comparison with the pneumolysin-free control system) sensitized neutrophils for increased production of superoxide following activation with FMLP. Maximal enhancement was observed at ≥ 4.18 ng/ml pneumolysin, with no significant differences in the magnitude of enhancement at concentrations of 4.18 - 41.75 ng/ml. The absolute peak values for resting and FMLP-activated neutrophils were 285 (250/326) and 1398 (1282/1552) (background subtracted) rlu/ 10^6 cells respectively. The time-course study of superoxide production by control and pneumolysin (4.18 ng/ml)-treated neutrophils are shown in Figure 2.2. Pneumolysin enhancement of superoxide production by FMLP-activated neutrophils was associated with both an increase in, and prolongation of the peak response. Pneumolysin *per se* (in the absence of FMLP) did not affect superoxide production by neutrophils, the values for control cells and for those treated with 8.37 and 41.75 ng/ml of the toxin being 285 (250/326), 331 (330/335) and 316 (290/358) rlu, respectively.

Pretreatment of neutrophils with the mutant toxin at concentrations of up to 8077 ng/ml did not affect either spontaneous or FMLP-activated production of superoxide by neutrophils. The values for spontaneous production of superoxide by control and mutant-pneumolysin (8077 ng/ml)-treated systems were 1724 (1509/1859) and 1486 (1373/1658) rlu, while the corresponding values for FMLP-activated systems were 3525 (3264/3966) and 3763 (3390/4669) rlu (background subtracted).

The possible involvement of Ca^{2+} in pneumolysin-mediated enhancement of superoxide production was investigated using Ca^{2+} -free HBSS. These results are shown in Table 2.2. Relative to the responses observed in Ca^{2+} -replete HBSS,

suspension of neutrophils in Ca^{2+} -free medium resulted in a modest reduction in superoxide production by control neutrophils, possibly through loss of Ca^{2+} from stores (Anderson & Goolam Mahomed, 1997), while pneumolysin failed to stimulate, but rather inhibited superoxide production. The responses of unstimulated neutrophils suspended in Ca^{2+} -replete HBSS in the absence or presence of pneumolysin were 1049 (1030/1246) and 1272 (1229/1311) rlu respectively, while the corresponding responses of cells in Ca^{2+} -free HBSS were 723 (655/726) and 904 (581/940) rlu. Similar results were observed when the cells were suspended in Ca^{2+} -replete HBSS containing 5 mM EGTA.

2.3.3 Degranulation

The effects of active pneumolysin on the release of elastase by FMLP/CB-activated neutrophils are shown in Figure 2.1. At concentrations of ≥ 4.18 ng/ml the toxin significantly ($P < 0.05$) potentiated the release of elastase with maximal enhancement observed at 41.75 ng/ml pneumolysin. As in the case of superoxide production, however, the toxin *per se* in the absence of FMLP/CB did not affect the release of elastase from neutrophils. The amounts of enzyme released by control cells and those treated with 8.37 and 41.75 ng/ml of the toxin were 48 (46/54), 46 (41/57) and 58 (50/72), milliunits enzyme/ 10^7 cells, respectively, while the FMLP-activated neutrophils released 215 (132/229) milliunits enzyme/ 10^7 cells.

2.3.4 CR3 expression

The effects of active pneumolysin *per se* on CR3 expression on neutrophils, as well as on FMLP/CB-activated expression of this β_2 -integrin are shown in Figure 2.3. At concentrations of 1.67 ng/ml and upwards, pneumolysin caused a dose-related upregulation of CR3 expression on FMLP/CB-stimulated and -unstimulated cells, achieving statistical significance ($P < 0.05$) at concentrations of 8.37 and 41.75 ng/ml.

2.3.5 Phospholipase A₂ activity

The effects of active pneumolysin on neutrophil PLA₂ activity are shown in Figure 2.4. At concentrations of ≥ 8.37 ng/ml pneumolysin significantly ($P < 0.05$) increased PLA₂ activity in these cells.

2.3.6 Ca²⁺ fluxes

The effects of pneumolysin on cytosolic Ca²⁺ in neutrophils are shown in Figure 2.5 which depicts the fura-2 fluorescence responses (from 2 different experiments using cells from different donors) of control cells and cells treated with 1.67, 8.37 and 41.75 ng/ml pneumolysin. Exposure of the cells to 8.37 and 41.75 ng/ml of the toxin was accompanied by a dose-related increase (after a 1-2 min lag in the case of 8.37 ng/ml toxin) in fluorescence intensity. These experiments were repeated on 9 different occasions on which almost identical results were obtained. The median values with 25-/75-percentiles for cytosolic Ca²⁺ concentrations for control cells and those treated with 8.37 and 41.75 ng/ml of the toxin were 65 (57/90), 231 (178/237) ($P < 0.05$) and 387 (336/468) ($P < 0.05$), respectively. The effects of the mutant pneumolysin (161.54 - 8077 ng/ml) on the fura-2 fluorescence responses of neutrophils are shown in Figure 2.6 and demonstrate that the mutant toxin did not affect cellular Ca²⁺ levels.

The effects of EGTA and U-73122 on the fura-2 fluorescence responses of neutrophils treated with pneumolysin (8.37 and 41.75 ng/ml) are shown in Figure 2.7 (data from 3 experiments using cells from different donors). EGTA prevented the increase in cytosolic Ca²⁺ following exposure of the cells to pneumolysin, while fluorescence intensity was slightly increased in cells treated with U-73122. These experiments were repeated on 4 different occasions on which similar results were obtained. These observations indicate that the pneumolysin-mediated increase in the concentration of cytosolic Ca²⁺ in neutrophils, results from influx of extracellular Ca²⁺ as opposed to PLC-mediated release of the cation from intracellular stores. The decline in fluorescence intensity observed in neutrophils treated with 41.75 ng/ml pneumolysin in the presence of EGTA may be due to penetration of EGTA into the cells resulting from toxin-mediated alterations in membrane integrity.

Treatment of neutrophils with U-73122 (10 μ M) completely abolished the FMLP-mediated transient increase in neutrophil cytosolic Ca^{2+} levels, confirming the efficacy of this agent as an inhibitor of PLC-mediated mobilization of Ca^{2+} from intracellular stores (Figure 2.8).

2.3.7 Membrane potential

The effects of active pneumolysin, at concentrations of 1.67, 8.37 and 41.75 ng/ml, on neutrophil membrane potential, are shown in Figure 2.9. At concentrations of 8.37 and 41.75 ng/ml, pneumolysin caused depolarization of neutrophils (after a lag period of 1-2 min in the case of 8.37 ng/ml of the toxin) with no repolarization observed during the time-course of the experiment. These experiments were repeated using cells from 7 different donors with almost identical results obtained on each occasion.

2.3.8 Ca^{2+} -ATPase activity

At concentrations of 8.37 and 41.75 ng/ml, pneumolysin did not inhibit, but rather slightly potentiated the efflux of $^{45}\text{Ca}^{2+}$ from neutrophils which had been preloaded with the cation. Following incubation for 30 min at 37°C the amounts of Ca^{2+} released from pneumolysin-untreated control cells and from cells treated with 8.37 and 41.75 ng/ml of the toxin were 50 (42/58), 74 (73/77) and 75 (70/75) pmoles $\text{Ca}^{2+}/10^7$ cells, respectively (results of 4 different experiments).

2.3.9 Na^+ , K^+ -ATPase activity

Treatment of neutrophils with the toxin resulted in a striking, dose-related decrease in the influx of $^{86}\text{Rb}^+$ in the setting of a dramatic increase in net efflux of the cation. With respect to influx, during which the cells were exposed to the toxin for 30 min at 37°C in medium containing $^{86}\text{Rb}^+$, the amounts of cell-associated $^{86}\text{Rb}^+$ for control neutrophils and for cells treated with 8.37 and 41.75 ng/ml pneumolysin were 55710 (54450/57950), 4760 (4641/4870) and 4657 (4547/4709) counts per minute (cpm) ($P < 0.05$ for comparison of both concentrations of the toxin with the control system). In the case of efflux experiments, in which the cells were pre-loaded with $^{86}\text{Rb}^+$ and resuspended in

medium which was free of the radiolabelled cation, followed by treatment of the cells with the toxin for 10 min at 37°C, the corresponding values were 9399 (8638/9958), 278 (277/367) and 269 (260/276) cpm ($P < 0.05$ for comparison of both concentrations of the toxin with the control system).

Two additional series of experiments were undertaken to investigate the possible inhibition of Na^+ , K^+ -ATPase by pneumolysin, either indirectly as a result of activation of PLA_2 , or by direct inhibition of the cation transporter, as a mechanism of toxin-mediated efflux of K^+ . In the first of these, pretreatment of neutrophils with vitamin E (25 $\mu\text{g}/\text{ml}$, final) only partially attenuated the pneumolysin-mediated reduction in the net influx of the cation. The absolute values for ouabain-inhibitable influx of $^{86}\text{Rb}^+$ into control neutrophils without and with vitamin E, and into cells treated with 8.37 ng/ml pneumolysin in the absence and presence of vitamin E were 32380 (31260/33400), 32000 (30780/32800), 10690 (10370/10850) and 15020 (14860/15250) cpm respectively (data from 3 experiments). In the second series of experiments it was observed that pneumolysin at concentrations of up to 83.7 ng/ml did not inhibit the activity of purified Na^+ , K^+ -ATPase, the absolute values for the control and pneumolysin (41.75 and 83.7 ng/ml)-treated systems being 16 (16/19), 22 (22/23) and 24 (23/24) nmoles NAD^+/min , respectively.

Table 2.1: Effects of pneumolysin (0.167 – 41.75 ng/ml) on neutrophil ATP levels and release of LDH in HBSS with and without Ca^{2+} .



Treatment	ATP levels (nanomoles/ 10^7 cells) in HBSS		LDH release (% of total) in HBSS	
	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}
Neutrophils only (control)	49.2 (42.0/54.3)	50.1 (47.8/51.5)	6.2 (6.1/6.2)	7.4 (7.3/7.4)
Neutrophils + 0.167 ng/ml pneumolysin	46.0 (44.9/47.0)	45.2 (44.8/45.4)	6.2 (6.1/6.2)	7.8 (7.7/7.8)
Neutrophils + 1.67 ng/ml pneumolysin	42.3 (39.7/47.5)	45.8 (44.5/48.3)	6.2 (6.1/6.2)	11.8 (11.6/12.1) ^a
Neutrophils + 4.18 ng/ml pneumolysin	43.7 (43.4/44.2)	39.0 (37.1/42.3)	6.8 (6.6/7.1)	19.2 (19.1/19.2) ^{a,b}
Neutrophils + 8.37 ng/ml pneumolysin	46.7 (45.3/47.5)	31.4 (30.0/32.8) ^{a,b}	8.4 (8.2/8.7)	21.7 (21.6/21.7) ^{a,b}
Neutrophils + 41.75 ng/ml pneumolysin	35.2 (32.8/37.1) ^a	10.1 (9.7/10.2) ^{a,b}	12.8 (12.4/13.2) ^a	38.5 (37.8/39.3) ^{a,b}

Data from 2-5 experiments are presented as the median values with 25-75-percentiles.

^a $P < 0.05$ in comparison with the relevant pneumolysin-free control system.

^b $P < 0.05$ for comparison between corresponding values in Ca^{2+} -replete and Ca^{2+} -free HBSS.

Table 2.2: Effects of pneumolysin on superoxide production by neutrophils suspended in HBSS with and without Ca²⁺.

Treatment	Superoxide production by neutrophils	
	With Ca ²⁺	Without Ca ²⁺
FMLP (1 µM) only	6745 (6320/7265)	4997 (4828/5057)
FMLP + pneumolysin (4.18 ng/ml)	10620 (10550/10930) ^a	1862 (1805/1938) ^a

Data from 6 experiments are presented as the median values with 25-/75-percentiles (background subtracted) for lucigenin-enhanced chemiluminescence responses of neutrophils.

^a $P < 0.05$ for comparison with the relevant pneumolysin-free control systems.

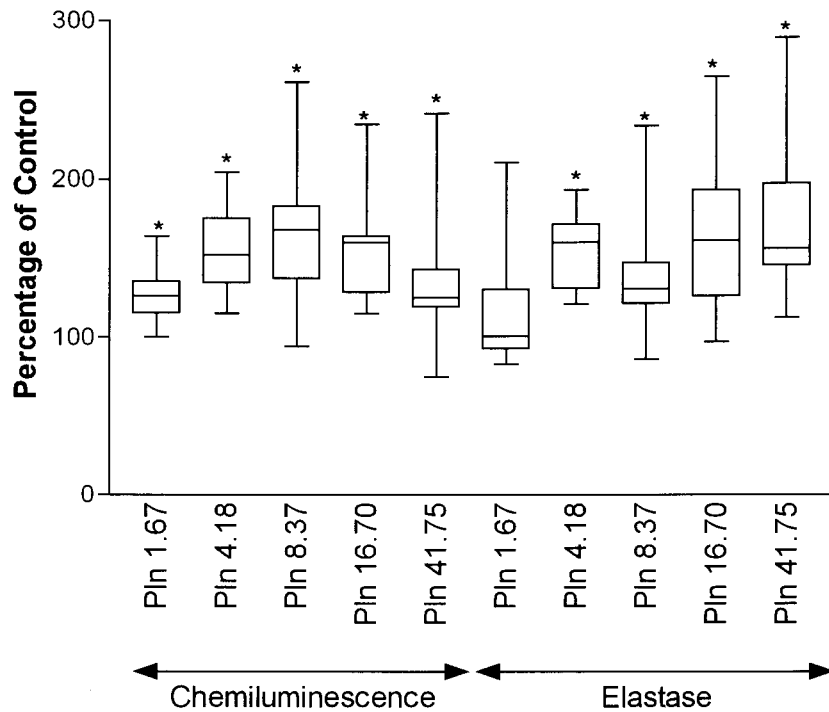


Figure 2.1: The effects of pneumolysin (Pln, 1.67 - 41.75 ng/ml) on superoxide production by FMLP (1 μ M)-activated neutrophils and on elastase release from FMLP/CB (0.1 μ M/1 μ M)-activated neutrophils. The results of a total of 27 and 24 different experiments for superoxide production and elastase release respectively, are presented as the median percentage of the pneumolysin-free control systems with 25-/75-percentiles.

* $P < 0.05$ for comparison with the pneumolysin-free control systems.

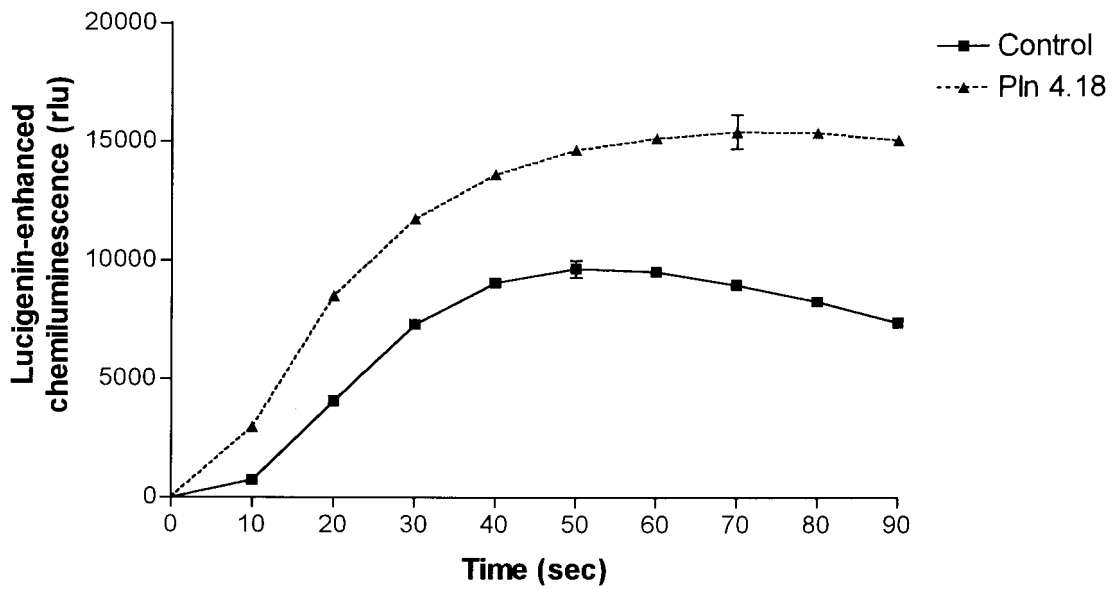


Figure 2.2: Time-course of superoxide production by control and pneumolysin (4.18 ng/ml)-treated, FMLP-activated neutrophils. The mean value of a single representative experiment (triplicate determinations) expressed as relative light units/ 10^6 cells, with standard error bars for the peak responses, are shown.

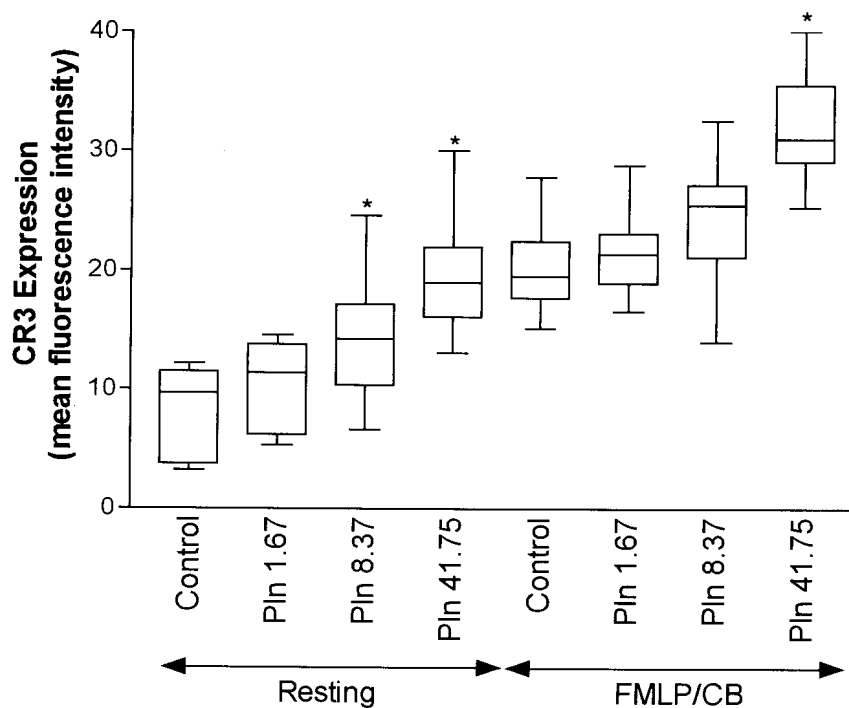


Figure 2.3: The effects of pneumolysin (1.67 - 41.75 ng/ml) on spontaneous and FMLP/CB (0.1 μ M/1 μ M)-activated CR3 expression on neutrophils. Data from 9 experiments are presented as median values for the mean fluorescence intensity with 25-/75-percentiles.

**P* < 0.05 for comparison with the pneumolysin-free control systems.

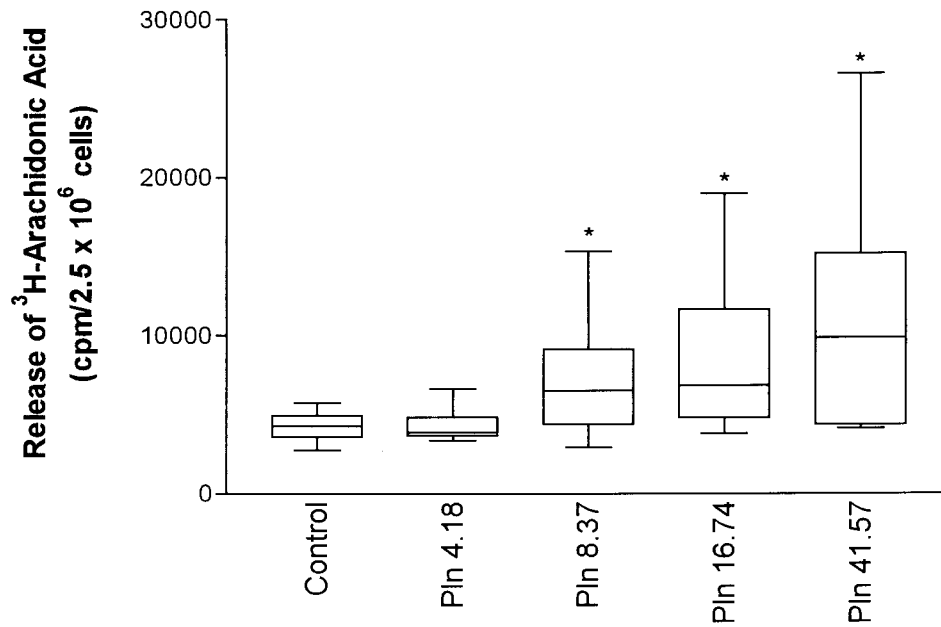


Figure 2.4: The effects of pneumolysin (Pln, 4.18 - 41.75 ng/ml) on neutrophil phospholipase A₂ activity. The results of a total of 16 experiments are presented as the median values with 25-75 percentiles (in counts per minute) of ³H-arachidonic acid released from labelled phospholipids.

**P* < 0.05 for comparison with the pneumolysin-free control systems.

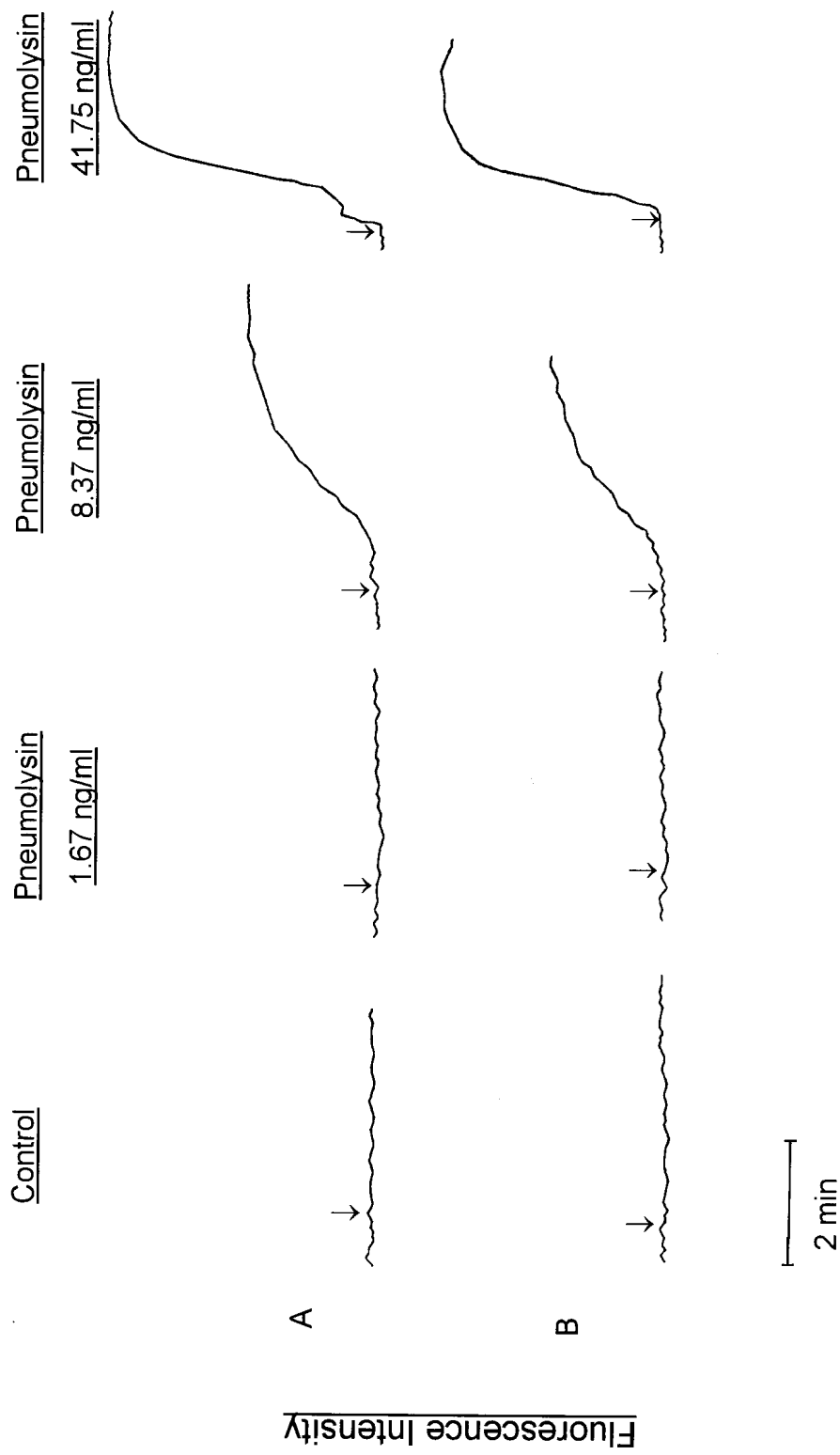


Figure 2.5: Fura-2 fluorescence responses of control and pneumolysin (1.67, 8.37 and 41.75 ng/ml)-treated neutrophils. Pneumolysin was added as indicated (↓) when a stable baseline was obtained (± 1 min). Data from 2 typical experiments (A and B) using different donors out of a total of 9 different experiments are shown.

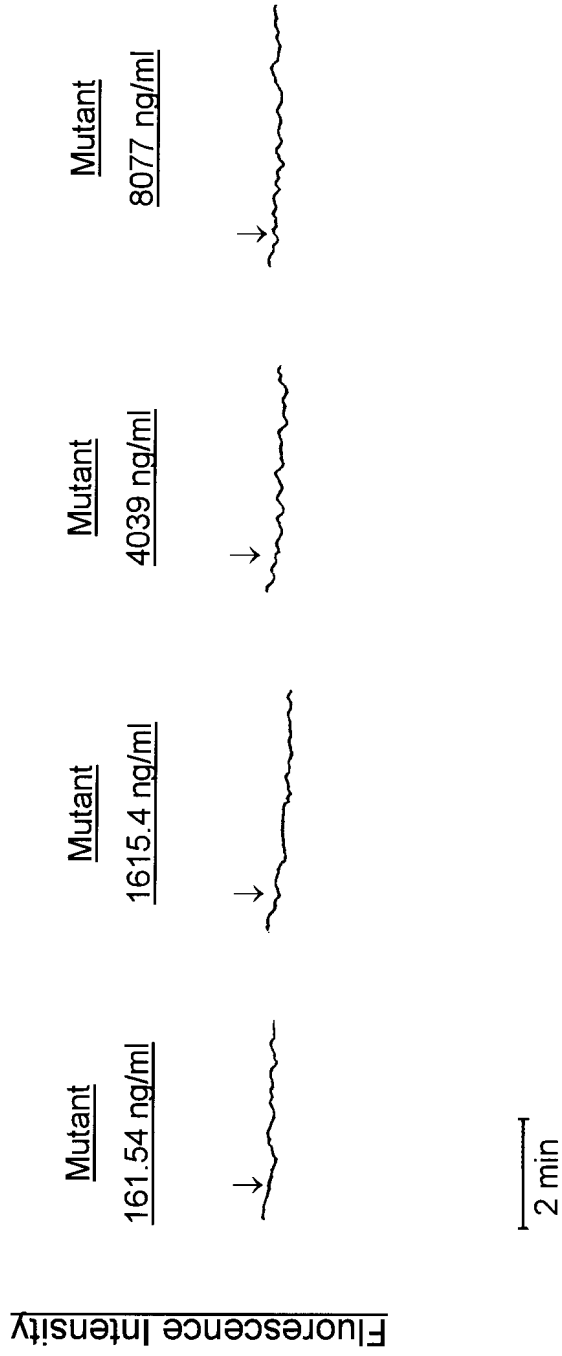
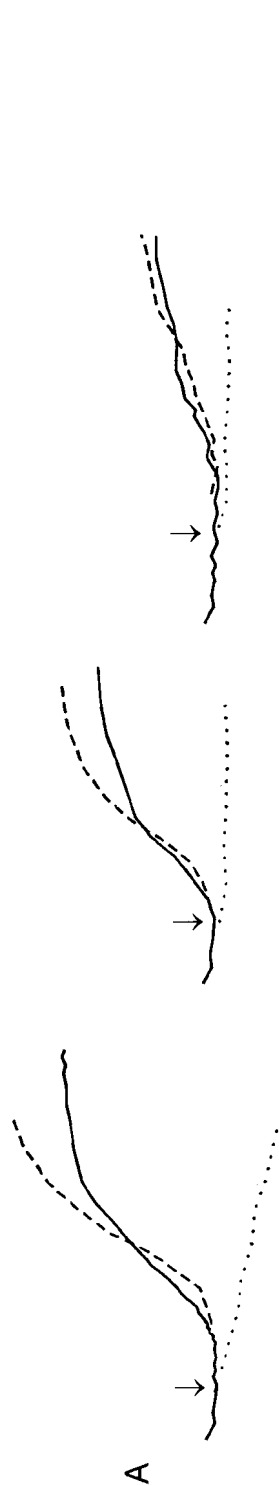
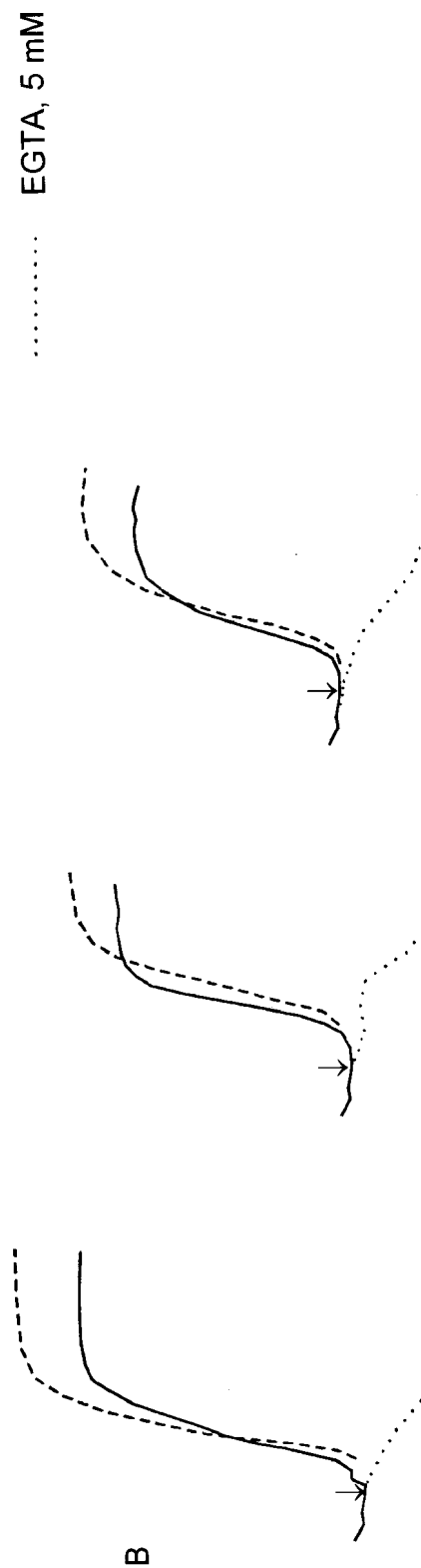


Figure 2.6: The effects of mutant pneumolysin (161.54 - 8077 ng/ml) on the fura-2 fluorescence response of neutrophils. The mutant toxin was added as indicated (\downarrow) when a stable baseline was obtained (± 1 min).

Pneumolysin 8.37 ng/ml



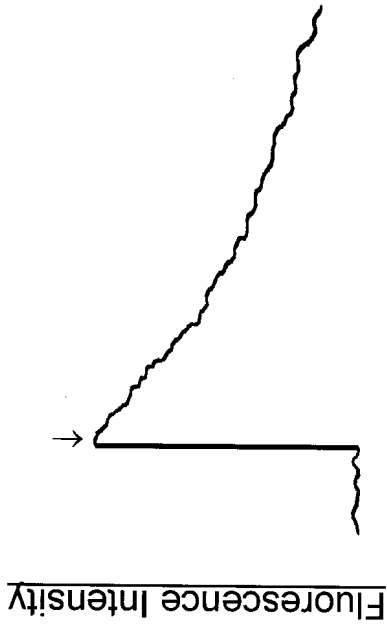
Pneumolysin 41.75 ng/ml



Fluorescence Intensity

Figure 2.7: The effects of EGTA (5 mM) and U-73122 (10 μ M) on the fura-2 fluorescence responses of neutrophils treated with 8.37 ng/ml (A) and 41.75 ng/ml (B) pneumolysin. Pneumolysin was added as indicated (\downarrow) when a stable baseline was obtained (\pm 1 min). Data are from 3 typical experiments using cells from different donors.

Without U-73122



With U-73122



2 min

Figure 2.8: The fura-2 fluorescence responses of FMLP (1 μM)-activated neutrophils in the absence and presence of U-73122 (10 μM). FMLP was added as indicated (\downarrow) when a stable baseline was obtained (± 1 min).

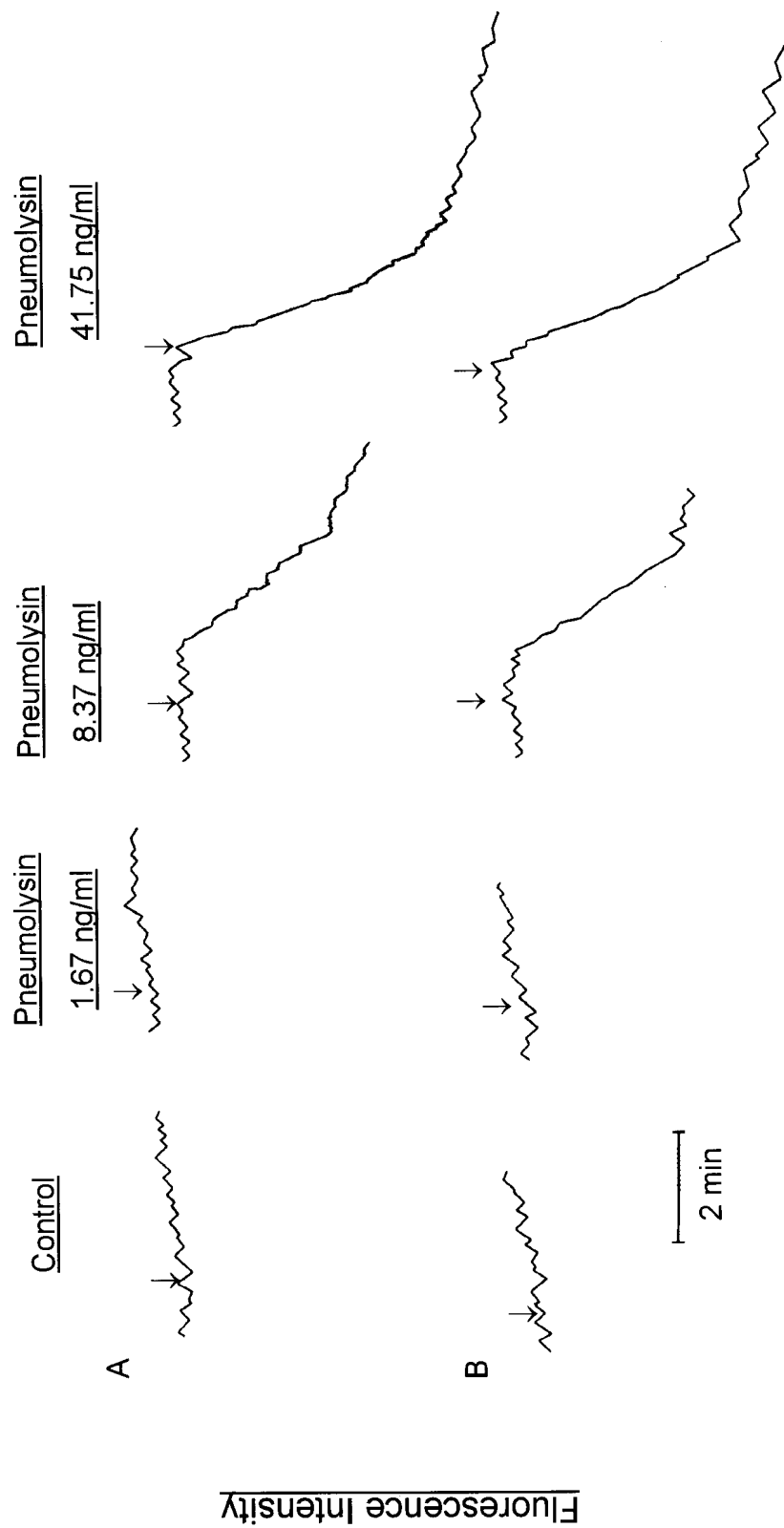


Figure 2.9: The effects of pneumolysin (1.67, 8.37 and 41.75 ng/ml) on neutrophil membrane potential. Pneumolysin was added as indicated (\downarrow) when a stable baseline was obtained (\pm 1 min). Data from 2 typical experiments (A and B) using cells from different donors out of a total of 7 different experiments are shown.

2.4 Discussion

In this study, treatment of human neutrophils with pneumolysin resulted in enhanced expression of CR3 and increased activity of PLA₂, as well as sensitization of the cells for increased production of superoxide and release of the primary granule enzyme, elastase, on subsequent exposure to FMLP. These proinflammatory actions were observed at concentrations of the toxin (≥ 1.67 ng/ml) which had either no, or minimal effects on cellular energy metabolism and viability. The relevance of these concentrations of pneumolysin to the *in vivo* setting during pneumococcal disease is uncertain because it is extremely difficult to detect free toxin in either the blood or the alveolar space, as it is rapidly tissue bound. However, in culture media, *Streptococcus pneumoniae* was found to produce pneumolysin at a concentration of up to 8.8 μ g/ml in 24 hours (Feldman, 1990). The maximal concentration of pneumolysin used in the laboratory research presented in this thesis, was 41.75 ng/ml, which had no, or minimal effects on cellular energy metabolism and viability over a 15 minute time course. Although the effects of pneumolysin on degranulation and PLA₂ activity are similar to those described in some earlier reports (Johnson *et al.*, 1981; Rubins *et al.*, 1994), others have reported that exposure of neutrophils to the toxin, at concentrations equivalent to or lower than those used in the current study, results in inhibition of stimulus-activated superoxide production (Paton & Ferrante, 1983; Nandoskar *et al.*, 1986; Saunders *et al.*, 1989). The reasons for these differences between the earlier studies (Paton & Ferrante, 1983; Nandoskar *et al.*, 1986; Saunders *et al.*, 1989) and the present study are unclear, but may relate to minor variations in experimental procedures, including the Ca²⁺ concentration of the cell-suspending medium, as well as the nature of the stimulus used to activate the cells. For example, phorbol esters, which, unlike FMLP, are membrane receptor- and Ca²⁺-independent activators of neutrophils (Naccache *et al.*, 1985), were used in several previous studies (Paton & Ferrante, 1983; Nandoskar *et al.*, 1986; Saunders *et al.*, 1989) with inhibition of superoxide production being attributed in one of these to the pore-forming actions of the toxin (Saunders *et al.*, 1989). It is possible that phorbol esters, which unlike FMLP, cause prolonged activation of the neutrophil respiratory burst, may potentiate the cytotoxic activity of

pneumolysin. I am unaware of other reports on pneumolysin-mediated upregulation of CR3 expression on resting and stimulated neutrophils. This is of potential importance in the recruitment and activation of neutrophils and other types of inflammatory cells during infection with *S. pneumoniae*.

Transient increases in cytosolic Ca^{2+} precede and are a prerequisite for activation of many of the proinflammatory activities of neutrophils, including adhesion to vascular endothelium, production of reactive oxidants, degranulation, and activation of cytosolic PLA_2 (Takenawa *et al.*, 1983; Lew *et al.*, 1986; Thelen *et al.*, 1993; Pettit & Hallett, 1996). In the current study, treatment of neutrophils with proinflammatory concentrations of pneumolysin resulted in a dose-related increase in cytosolic Ca^{2+} , apparently as a result of influx of extracellular cation, as opposed to its mobilization from intracellular stores. Evidence for the relationship between pneumolysin-mediated Ca^{2+} influx and alterations in the proinflammatory activities of neutrophils was strengthened by the observations that the sensitizing effects of the toxin on superoxide production by activated neutrophils were abolished when the cells were suspended in Ca^{2+} -free medium and by the observation that treatment of neutrophils with a mutant toxin, which was attenuated with respect to cytolytic activity, did not induce Ca^{2+} influx or sensitize the cells for increased production of superoxide.

Influx of Ca^{2+} into pneumolysin-treated neutrophils is probably attributable to the well-documented pore-forming actions of the toxin (Mitchell & Andrew, 1997). Several lines of evidence support this contention. First, the activity of the plasma membrane Ca^{2+} efflux pump of neutrophils was not inhibited by pneumolysin, excluding interference with discharge of the cation as a possible mechanism of increased cytosolic Ca^{2+} in these cells. Second, as mentioned above, Ca^{2+} influx into pneumolysin-treated neutrophils was dependent on the membrane modifying actions of the toxin, because mutant pneumolysin was ineffective. Additional evidence was derived from experiments in which cells suspended in Ca^{2+} -free medium were exposed to pneumolysin. This resulted in potentiation of the cytotoxic action of the toxin which is in agreement with previous studies in which divalent cations, particularly Ca^{2+} and zinc (Zn^{2+}), were found to counteract

cellular damage inflicted by pneumolysin and other pore-forming toxins (Harshman & Sugg, 1985; Korchev *et al.*, 1992; Korchev *et al.*, 1998). The protective effects of the divalent cations were attributed to voltage-dependent closure of channels rather than to a blocking mechanism (Korchev *et al.*, 1992; Korchev *et al.*, 1998). These observations, which may explain the inhibition of superoxide production by pneumolysin-treated, FMLP-activated neutrophils in Ca^{2+} -free HBSS, suggest that extracellular Ca^{2+} concentrations at sites of pneumococcal infection may determine the action of pneumolysin i.e. proinflammatory or cytolytic. In either case the outcome would be detrimental to surrounding host tissues.

At the same concentrations that caused influx of Ca^{2+} and sensitization of the proinflammatory activities of the cells, pneumolysin caused efflux of K^+ (detected by $^{86}\text{Rb}^+$) and membrane depolarization. These effects of pneumolysin on intracellular K^+ could not be attributed to direct inhibition of Na^+ , K^+ -ATPase, because the activity of this cation transporter was unaffected by the toxin in a cell-free system. Moreover, treatment of neutrophils with vitamin E, which neutralizes lysophospholipids (Kagan, 1989), only partially attenuated the decrease in intracellular K^+ following exposure of the cells to pneumolysin, suggesting that PLA_2 -mediated inhibition of Na^+ , K^+ -ATPase (Oishi *et al.*, 1990) may represent a minor, secondary, indirect mechanism of toxin-induced fluxes of K^+ . This contention is supported by data from additional experiments, shown in Appendix 2 (page 108), which showed that treatment of neutrophils with vitamin E partially attenuated pneumolysin-mediated depolarization in these cells. These data are also compatible with a pore-forming mechanism of pneumolysin-mediated alterations in fluxes of K^+ and Na^+ .

In addition to the proinflammatory interactions with human neutrophils described in this study, pneumolysin has been reported to initiate the production and release of IL-1, IL-6, and TNF- α by human and murine macrophages (Houldsworth *et al.*, 1994; Braun *et al.*, 1999) and to activate iNOS in these cells (Braun *et al.*, 1999). Because induction of NO synthase and cytokine release are dependent on Ca^{2+} -mediated activation of cytosolic nuclear transcription factors (Dolmetsch *et al.*,

1997), it is conceivable that influx of Ca^{2+} may underpin pneumolysin-mediated enhancement of the proinflammatory activities of both neutrophils and macrophages. Taken together, the findings of these and other studies (Kadioglu *et al.*, 2000) support the involvement of pneumolysin in the pathogenesis of inflammation-mediated tissue damage during pneumococcal infection.

Although, circulating neutrophils were used in my laboratory research, recruited neutrophils in the lung are likely to be sensitized (primed) and may be more responsive to pneumolysin. However, this is difficult to prove because alveolar neutrophils are not attainable in sufficient numbers to undertake the experiments on which the thesis is based. In the rabbit model, it has been shown that bacteremic pneumococcal infection is associated with an accelerated release of neutrophils from the bone marrow, and that these neutrophils preferentially sequester in the lung (Sato *et al.*, 1998). The up-regulatory effects of pneumolysin on neutrophil β_2 -integrin expression combined with the complement-activating properties of the toxin, may also contribute to systemic inflammatory responses and neutrophil mobilization from the bone marrow.

Exaggerated inflammatory responses mediated by pneumolysin may favour microbial survival by promoting premature, auto-oxidative exhaustion of phagocytes (Baehner *et al.*, 1977) and oxidative dysfunction of B and T lymphocytes (El-Hag *et al.*, 1986). Alternatively, release of pneumolysin, which occurs during autolysis of bacteria (Mitchell & Andrew, 1997), may represent a diversionary strategy by which incoming inflammatory cells and proteins are directed towards disintegrating micro-organisms, as opposed to viable bacteria at different sites. In either case, pneumolysin would contribute to microbial persistence.

Chapter 3:

The effects of pneumolysin on prostaglandin E₂ and leukotriene B₄ production

3.1 Introduction

Although pneumolysin has been shown to increase the activity of PLA₂ in both neutrophils (Chapter 2) and pulmonary endothelial cells (Rubins *et al.*, 1994), the effects of the toxin on the production of arachidonic acid-derived mediators of inflammation have not been described. In this study, I have investigated the effects of recombinant pneumolysin on the production of PGE₂ and LTB₄ by human neutrophils *in vitro*.

3.2 Materials and Methods

3.2.1 *Pneumolysin*

Recombinant, active pneumolysin, at final concentrations of 8.37 and 41.75 ng/ml, was used as described in 2.2.1.

3.2.2 *Neutrophils*

Purified human neutrophils were prepared as described in 2.2.3.

3.2.3 *Prostaglandin E₂ and leukotriene B₄*

Neutrophils (2×10^6 cells/ml) in HBSS were preincubated at 37°C for 10 min followed by the addition of pneumolysin (8.37 and 41.75 ng/ml, final) or an equal volume (100 µl) of HBSS to control systems. The final volume in each tube was 1 ml. For systems in which the cells were activated with FMLP (1 µM, final) the cells were preincubated for 5 min at 37°C, after which pneumolysin was added, followed 5 min later by FMLP. After incubation the reactions were terminated by the addition of 1 ml of ice-cold HBSS to each tube and the tubes transferred to an ice-bath. The tubes were then centrifuged to pellet the cells and PGE₂ and LTB₄ were assayed for in the supernatants by competitive binding radioimmunoassay procedures (Du Pont NEN Research Products, Boston, Mass, USA). The results are expressed as nanograms PGE₂ or LTB₄/10⁷ cells.

3.2.4 *Time-course studies*

Preliminary time-course experiments were performed using cells from a single donor and incubation times of 1, 3, 5 and 10 min after the addition of pneumolysin (8.37 and 41.75 ng/ml) only, or FMLP with and without the toxin. PGE₂ and LTB₄ levels were then measured in the supernatants of these cells.

3.2.5 *The role of extracellular calcium in the production of PGE₂ and LTB₄*

In an additional series of experiments using cells from 3 different donors, the extracellular calcium-chelating agent, EGTA (5 mM, final), was added to the cells 5 sec prior to pneumolysin or FMLP. PGE₂ and LTB₄ levels were then measured in the supernatants.

3.2.6 *Statistical analysis*

The results are expressed as the median values with 25-/75-percentiles. The levels of statistical significance were calculated by the Mann-Whitney U-test (two-tail) by the computer-based software system, Instat II. Significant levels were taken at a *P* value <0.05.

3.3 **Results**

3.3.1 *Time-course study*

PGE₂ and LTB₄ production by neutrophils, exposed to pneumolysin for 1 to 10 min, with and without FMLP, are shown in Figure 3.1. An increase in the concentrations of PGE₂ and LTB₄ in supernatants of neutrophils treated with pneumolysin alone, FMLP alone, and in particular the combination of the toxin and chemoattractant, was observed within 1 min of activation of the cells, and for most systems peak concentrations were detected at around 5 min of incubation. This incubation time was therefore used for subsequent experiments.

3.3.2 *Prostaglandin E₂ and leukotriene B₄*

The effects of pneumolysin on the production of PGE₂ and LTB₄ by FMLP-treated and -untreated neutrophils from 10 different donors are shown in Figures 3.2 and

3.3, respectively. Exposure of neutrophils to pneumolysin in the absence of FMLP increased the production of both PGE₂ and LTB₄, which, in the case of PGE₂, was statistically significant at both concentrations of the toxin ($P < 0.05$), but only at the highest concentration for LTB₄ ($P < 0.05$). FMLP *per se* significantly ($P < 0.05$) increased the production of both PGE₂ and LTB₄ by neutrophils, an effect which was striking and significantly ($P < 0.05$) potentiated by pretreatment of the cells with pneumolysin at both 8.37 and 41.75 ng/ml.

3.3.3 Role of extracellular calcium

The effects of removal of extracellular Ca²⁺ by treatment of the cell-suspending medium with EGTA (5 mM) on the production of PGE₂ and LTB₄ by pneumolysin-treated neutrophils, with and without FMLP, are shown in Figures 3.4 and 3.5, respectively. EGTA completely attenuated the pneumolysin-mediated enhancement of LTB₄ production by FMLP-treated and -untreated neutrophils, but did not affect the production of these bioactive lipids in control systems, in the presence and absence of FMLP and in the absence of pneumolysin. Similar results were obtained for PGE₂ production. In the presence of EGTA, the pneumolysin-treated systems, both with and without FMLP, did not differ significantly from the corresponding pneumolysin-free control systems in PGE₂ and LTB₄ production.

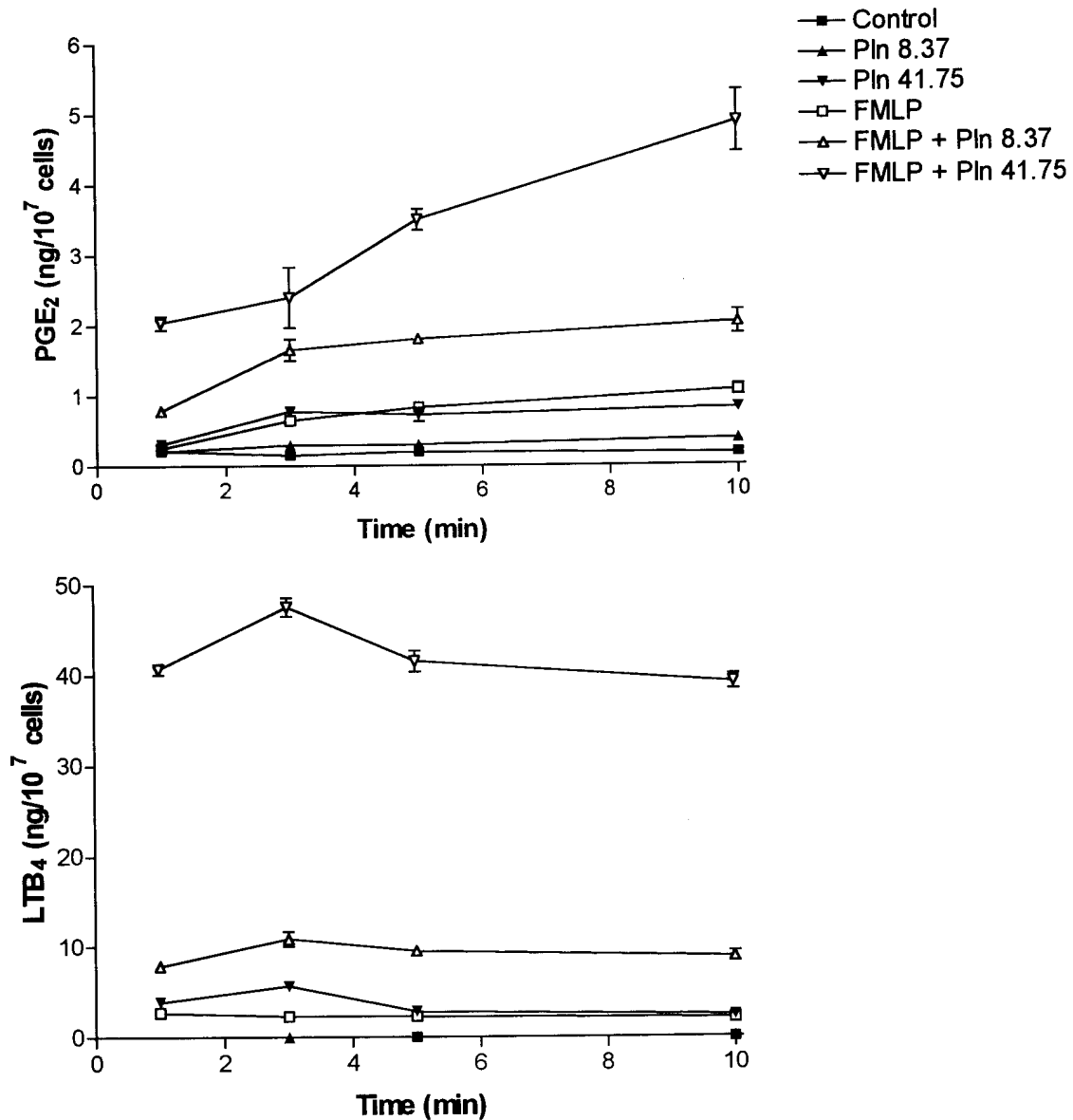


Figure 3.1: PGE₂ and LTB₄ production by neutrophils treated with pneumolysin (Pln, 8.37 and 41.75 ng/ml) for 1, 3, 5 and 10 min. Data from a single experiment (duplicate determinations) are shown, with the amounts of LTB₄ and PGE₂ detected in the supernatants expressed as ng/10⁷ cells.

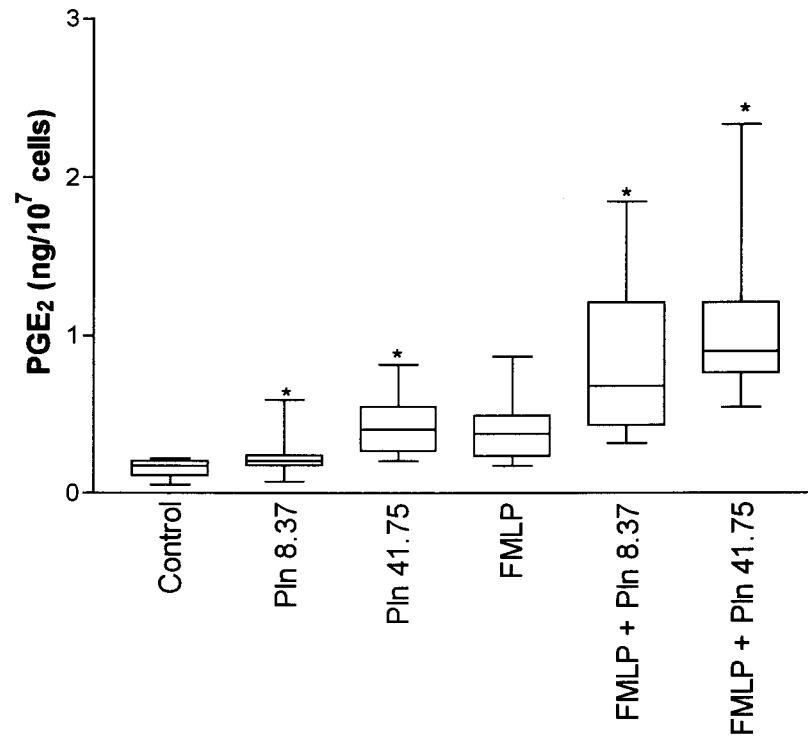


Figure 3.2: Effects of pneumolysin (Pln, 8.37 and 41.75 ng/ml) on the production of PGE₂ by unstimulated and FMLP (1 μ M)-activated neutrophils. The results of 9 experiments using cells from different donors are expressed as the median values with 25-/75-percentiles.

* $P < 0.05$ for comparison with the appropriate pneumolysin-free control systems.

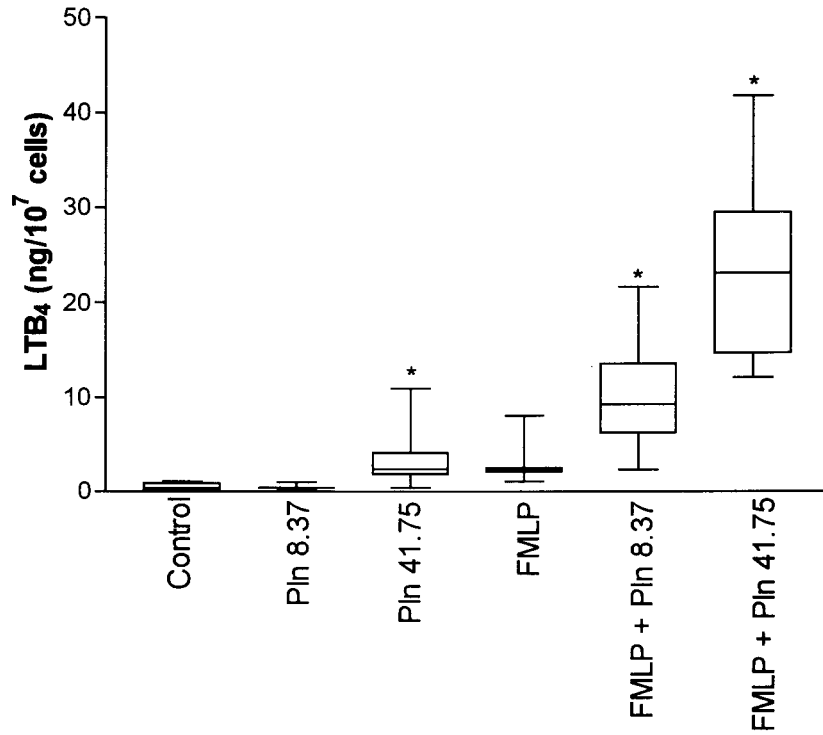


Figure 3.3: Effects of pneumolysin (Pln, 8.37 and 41.75 ng/ml) on the production of LTB₄ by unstimulated and FMLP (1 μ M)-activated neutrophils. The results of 10 experiments using cells from different donors are expressed as the median values with 25-/75-percentiles.

* $P < 0.05$ for comparison with the appropriate pneumolysin-free control systems.

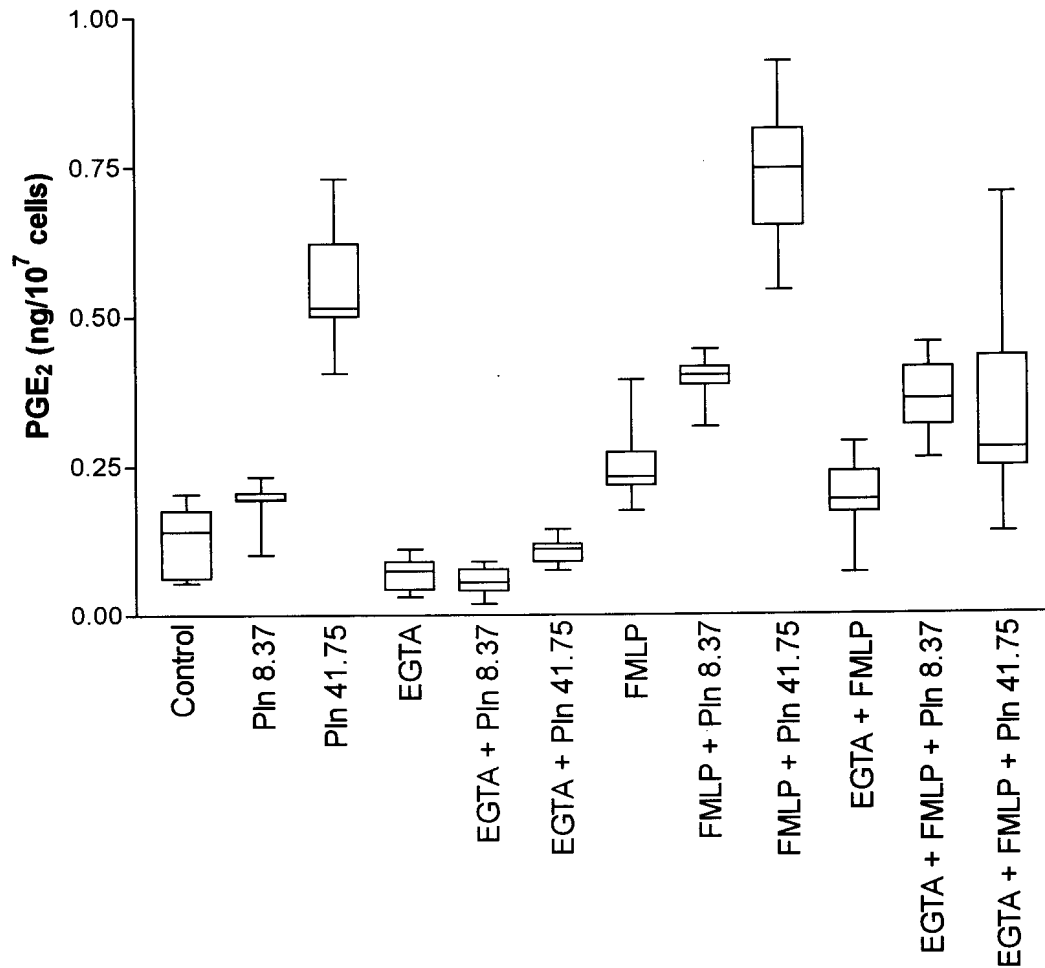


Figure 3.4: Effects of EGTA (5 mM) on pneumolysin (PIn, 8.37 and 41.75 ng/ml)-mediated enhancement of production of PGE₂ by unstimulated and FMLP (1 μM)-activated neutrophils. The results of 2 experiments, with 4 replicates in each, using cells from different donors are expressed as the median values with 25-75-percentiles.

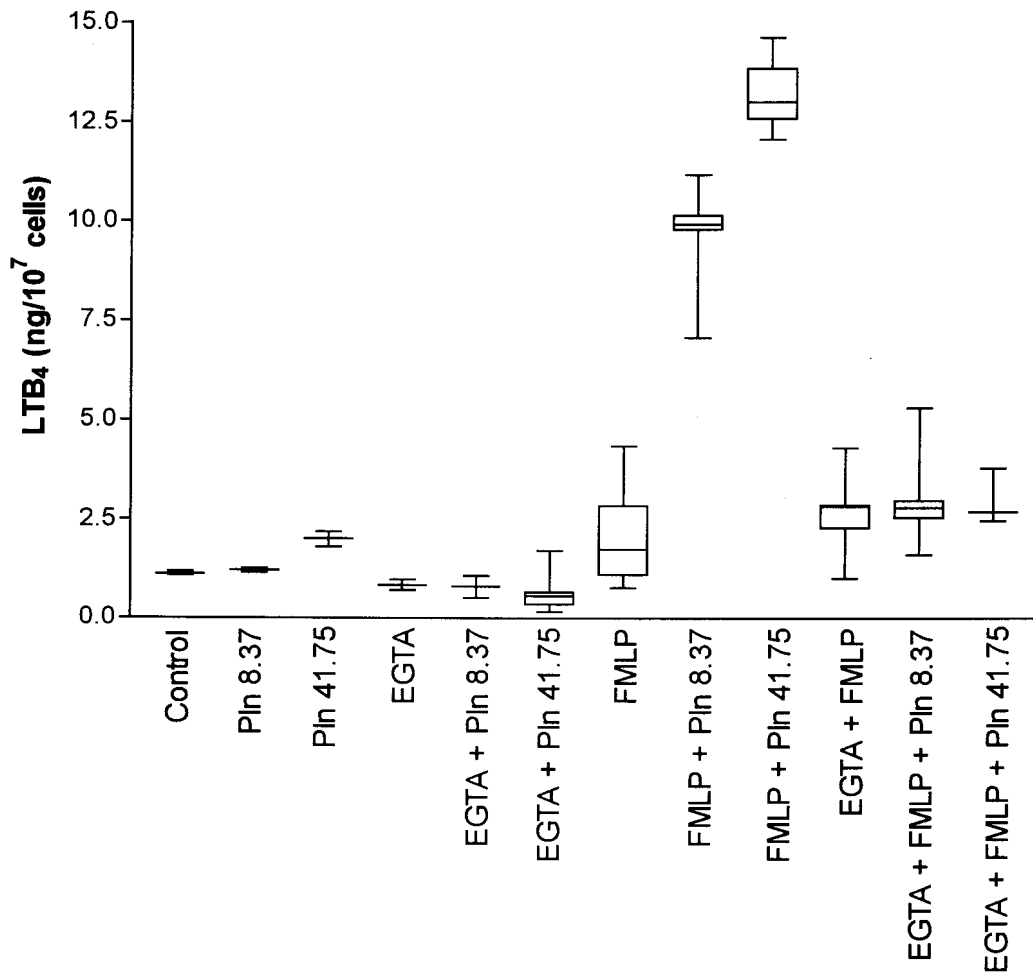


Figure 3.5: Effects of EGTA (5 mM) on pneumolysin (PIn, 8.37 and 41.75 ng/ml)-mediated enhancement of production of LTB₄ by unstimulated and FMLP (1 μ M)-activated neutrophils. The results of 3 experiments, with triplicate determinations, using cells from different donors are expressed as the median values with 25-/75-percentiles.

3.4 Discussion

Results presented in the previous chapter demonstrated that pneumolysin, at concentrations which had either no or minimal effects on cellular energy metabolism and viability, augmented the activity of PLA₂ in neutrophils. In the current study, most likely as a consequence of its enhancing effects on PLA₂, pneumolysin was found to cause a dose-related increase in the production of both PGE₂ and LTB₄ by human neutrophils. Although this was evident with the toxin *per se*, it was most striking with the combination of pneumolysin and FMLP, which is most likely to mimic the situation at sites of inflammation.

Pneumolysin-mediated augmentation of the proinflammatory activities of human neutrophils is achieved by a pore-forming mechanism resulting in influx of extracellular Ca²⁺, as shown in Chapter 2. In the current study, inclusion of the Ca²⁺-chelating agent, EGTA, in the cell-suspending medium completely attenuated the pneumolysin-mediated enhancement of PGE₂ and LTB₄ production by neutrophils, probably by preventing Ca²⁺-dependent activation of PLA₂ (Rubins *et al.*, 1994; Chapter 2).

Receptor-mediated pro-adhesive, chemotactic, secretory and pro-oxidative interactions of LTB₄ with phagocytes, particularly neutrophils, have been well documented (Cleasson & Dahlén, 1999) and potentiation of these proinflammatory activities by pneumolysin may contribute to hyperacute inflammation during pneumococcal infection (Kadioglu *et al.*, 2000). Pneumolysin-mediated augmentation of the production of PGE₂ by neutrophils may also contribute to dysregulated inflammatory responses by increasing vascular permeability, thereby facilitating influx of proinflammatory polypeptides, as well as by delaying neutrophil apoptosis (Rossi *et al.*, 1995).

In addition to augmentation of the production of proinflammatory cytokines, reactive oxidants, including NO, proteolytic enzymes, and adhesion molecules (Houldsworth *et al.*, 1994; Braun *et al.*, 1999; Chapter 2), data from this study demonstrate that pneumolysin also increases the production of the

proinflammatory lipids PGE₂ and LTB₄ by human neutrophils. Acting in concert these various proinflammatory agents may promote hyperacute inflammation during infection with *S. pneumoniae*. The relationship between the proinflammatory activities of pneumolysin and microbial persistence is, however, more complex, with several possible scenarios. Exaggerated inflammatory responses may favour accelerated eradication of the microbial pathogen. Alternatively, hyperactivation of phagocytes may result in premature auto-oxidative inactivation of their protective functions (Baehner *et al.*, 1977), as well as inhibition of the proliferative responses of neighbouring lymphocytes (El-Hag *et al.*, 1986), favouring microbial persistence.

Chapter 4:

The effect of pneumolysin on the synthesis and release of interleukin-8

4.1 Introduction

Pneumolysin possesses cytotoxic and proinflammatory properties (Houldsworth *et al.*, 1994; Braun *et al.*, 1999; Chapters 2 and 3), both of which are thought to contribute to the pathogenesis of invasive disease caused by *S. pneumoniae* (Feldman *et al.*, 1991; Musher *et al.*, 2001a). In murine models of experimental infection of the airways, pneumolysin-producing strains of the pneumococcus cause exaggerated pulmonary inflammatory responses that are characterized by an excessive influx of neutrophils into the lungs (Kadioglu *et al.*, 2000). To my knowledge pneumolysin is not a chemoattractant, however, the above observation suggests that pneumolysin may affect production of IL-8, a chemokine actively synthesized and released by neutrophils, which in turn amplifies recruitment of neutrophils from the blood to the lungs, but also causes autocrine activation of these cells (Cassatella, 1999; van Eeden & Terashima, 2000; Witko-Sarsat *et al.*, 2000).

In this study I have investigated the effects of recombinant pneumolysin, at concentrations, previously found to sensitize or activate those neutrophil proinflammatory functions that do not require biosynthetic activity, on the synthesis and release of IL-8 by these cells.

4.2 Materials and Methods

4.2.1 Preparation of pneumolysin

Recombinant active pneumolysin was expressed in *E. coli* and purified from cell extracts as described in 2.2.1. Final concentrations of 8.37 and 41.75 ng/ml, were used in this study.

4.2.2 Neutrophils

Purified neutrophils were prepared, as described in 2.2.3. To obtain ultra-pure neutrophil preparations (>98% purity), the isolated cell suspensions were depleted

of contaminating cells by flow cytometry using a Beckman Coulter Altra Cell Sorter equipped with a water-cooled Coherent Enterprise laser. A pure monocyte preparation was obtained, by using magnetic beads coated with a mix of anti-CD 2, 7, 16 (a and b), 19 and 56 monoclonal antibodies (Dynal® ASA, Oslo, Norway). These ultra-pure neutrophil and monocyte suspensions were used in a limited series of confirmatory experiments to identify the relative contributions of neutrophils and monocytes to IL-8 production.

4.2.3 IL-8 and TNF- α production by neutrophils

Neutrophils (2×10^6 /ml) were preincubated for 10 min with and without cycloheximide, a protein synthesis inhibitor, (10 μ g/ml) in HBSS supplemented with 1 mg/ml bovine serum albumin prior to the addition of pneumolysin (8.37 and 41.75 ng/ml, final), or the synthetic chemotactic tripeptide FMLP (1 μ M, final) individually and in combination. When used in combination, pneumolysin was added to the cells 1 min prior to FMLP. Toxin- and/or FMLP-free control systems received an equal volume of HBSS. The final volume in each tube was 2 ml. Total (intra- and extracellular) and extracellular IL-8 and TNF- α were measured using antibody capture enzyme-linked immunosorbent assay (ELISA) procedures (Roche Diagnostics Corp, Indianapolis, USA) after 0, 0.25, 1, 2, 4 and 6 hours of incubation at 37°C following the addition of pneumolysin and/or FMLP to the cells. Cell-associated IL-8 and TNF- α were measured in the lysates of neutrophils which had been treated with 0.01% lysophosphatidylcholine (LPC) followed by centrifugation at 300 x g for 5 min to remove cellular debris, while extracellular cytokines were measured in cell-free supernatants following the removal of the cells by centrifugation.

These experiments were also performed using cells suspended in nominally Ca²⁺-free HBSS containing the Ca²⁺-chelating agent, EGTA (100 μ M, final) to ensure total depletion of the cation. The results of these investigations are expressed as picograms cytokine/10⁶ neutrophils. Under these conditions, no influx of Ca²⁺ into pneumolysin (41.75 ng/ml)-treated neutrophils, could be detected (Chapter 2).

4.2.4 Quantitation of IL-8 specific mRNA

This was measured using the Quantikine® (R & D Systems, Minneapolis, USA) mRNA colourimetric microplate assay. Following exposure of neutrophils (2×10^6 /ml) to pneumolysin (8.37 and 41.75 ng/ml, final) for 6 hours at 37°C total RNA was extracted and purified using the SV total RNA isolation system (Promega Corp., Madison, USA) and adjusted spectrophotometrically to 40 µg/ml for each sample. The RNA samples (150 µl) were then hybridized with IL-8 gene-specific biotin-labelled capture oligonucleotide probes (50 µl) and digoxigenin-labelled detection probes in a microplate and incubated at 65°C for 60 min. The hybridization solution (150 µl) was then transferred to a streptavidin-coated microplate on which the RNA-probe is captured, and incubated on a shaker for 60 min at room temperature. This was followed by washing (x4) and addition of 200 µl of anti-digoxigenin conjugate to each well followed by incubation on a shaker for 60 min at room temperature after which the plates were washed (x6) followed by addition of substrate solution (50 µl). After a further incubation for 60 min at room temperature, 50 µl of amplifier solution was added to each well and the reactions stopped after a 30 min incubation at room temperature and optical densities measured at 490 nm on a plate spectrophotometer.

4.2.5 Intracellular cytokine detection

Following exposure to pneumolysin (8.37 and 41.75 ng/ml) and/or FMLP for 0, 2, 4 and 6 hours at 37°C, neutrophils were pelleted by centrifugation and surface stained with CD14- or CD16-FITC, washed and resuspended to 5×10^5 cells/ml in 0.5 ml cold 4% paraformaldehyde fixative and incubated at room temperature for 10 min. Following fixation, the cells were washed once in PBS and resuspended in 2 ml HBSS containing 0.1% saponin and 0.05% sodium azide, then concentrated by centrifugation and resuspended in 200 µl of the same buffer. Intracytoplasmic IL-8 was then marked by the addition of 10 µl of phycoerythrin-conjugated monoclonal antibody to recombinant human IL-8 (mouse IgG₁, R & D Systems, Minneapolis, USA) for 10 min, while an isotypic antibody was used as a negative control. Analysis of IL-8 was performed at 575 nm, while CD14/CD16 was monitored at 525 nm using an Altra Cell Sorter (Beckman Coulter) equipped with a water-cooled Coherent Enterprise laser.

4.2.6 Statistical analysis

The results of each series of experiments are expressed as median with 25-/75-percentiles. Levels of statistical significance were calculated by the Mann-Whitney U-test (2-tail). The level of significance was set at $P < 0.05$.

4.3 Results

4.3.1 Effects of pneumolysin on IL-8 and TNF- α production by neutrophils

Maximal production of IL-8, detected extracellularly, in both control systems and systems containing pneumolysin, with and without FMLP, was observed during 6 hours of incubation at 37°C, with extremely low levels of the cytokine detected at the very early incubation times (0, 0.25 and 1 hours), shown in Figure 4.1. The effects of pneumolysin and FMLP alone and in combination on the extracellular release of IL-8 by neutrophils, as well as on the total amount of cell-associated cytokine (extracellular and intracellular) after the 6 hour incubation period in the presence and absence of cycloheximide, as well as in Ca²⁺-replete and Ca²⁺-free HBSS are shown in Figures 4.2 and 4.3 respectively. While the amount of extracellular and total IL-8 increased over time in systems containing untreated (control) neutrophils, the concentrations of the chemokine (both extracellular and total) were significantly higher ($P < 0.05$) in systems containing neutrophils treated with pneumolysin alone, while FMLP alone caused a significant increase ($P < 0.05$) in extracellular, but not total IL-8. The most striking increases, however, were observed in neutrophils treated with the combination of pneumolysin and FMLP, particularly with the higher concentration of the toxin in the case of extracellular cytokine.

Inclusion of cycloheximide or depletion of Ca²⁺ from the medium abrogated the production of IL-8 by both untreated and pneumolysin/FMLP-treated neutrophils. Neutrophils exposed to 41.75 ng/ml pneumolysin, with or without FMLP, in Ca²⁺-free HBSS produced less IL-8 (both extracellular and total) than the corresponding control systems or the system containing 8.37 ng/ml of the toxin. This probably results from the enhanced cytotoxic activity of pneumolysin in Ca²⁺-free cell-suspending medium (Chapter 2).

TNF- α was undetectable in the supernatants and lysates of control neutrophils and cells treated with pneumolysin and/or FMLP over the 6 hour time-course of the experiment.

4.3.2 Pneumolysin-mediated alterations in IL-8 specific mRNA

The effects of pneumolysin on the synthesis of IL-8, in the presence and absence of FMLP are shown in Figure 4.4. The results are expressed as the amount of IL-8 specific mRNA in amol/ml. A significant ($P < 0.05$) increase in neutrophil mRNA was observed in cells treated with pneumolysin at 41.75 ng/ml, and in cells treated with the combination of FMLP and pneumolysin (41.75 ng/ml). The values for the control, pneumolysin-free systems, without and with FMLP, were not significantly different. Almost identical results were obtained in a limited series of experiments using ultra-pure neutrophils prepared by flow cytometry, while the cytokine was detectable at very low levels, in the corresponding neutrophil-depleted, monocyte-enriched cell suspensions (Figure 4.5).

4.3.3 Intracellular localization of IL-8

Using flow cytometry, IL-8 was localized intracytoplasmically in permeabilized neutrophils, being detected after 2, 4 and 6 hours of incubation (Figure 4.6). The results are expressed as mean fluorescence intensity, indicating the fluorescent channel number.

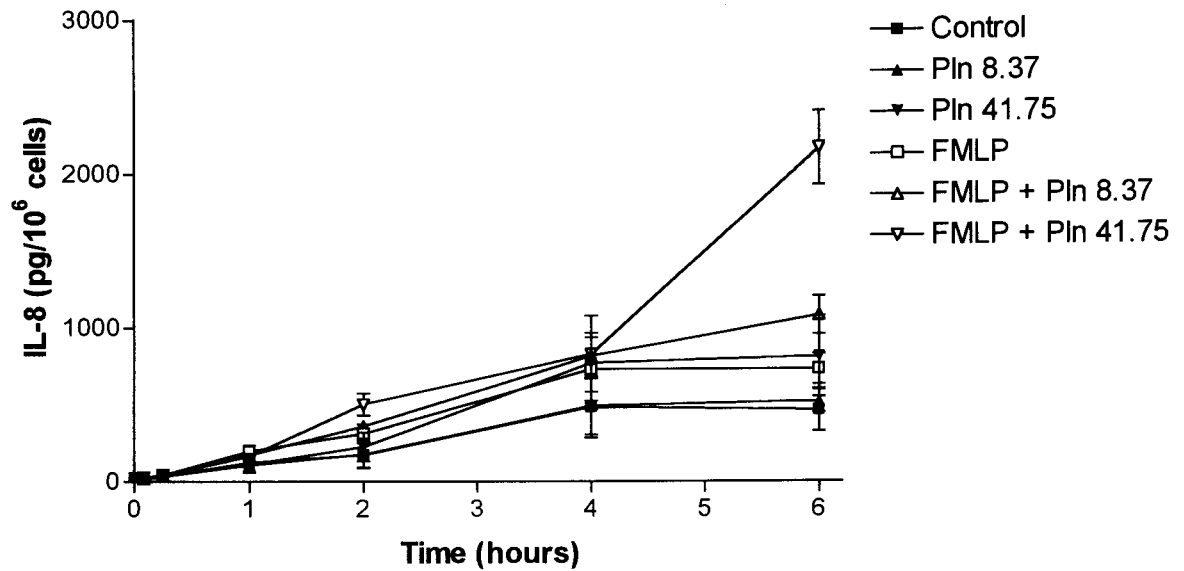


Figure 4.1: The effects of pneumolysin (Pln, 8.37 and 41.75 ng/ml) on the release of IL-8 by neutrophils following exposure to the toxin for 0, 0.25, 1, 2, 4 and 6 hours. The results represent the mean values (\pm SEM) of a single experiment (in duplicate).

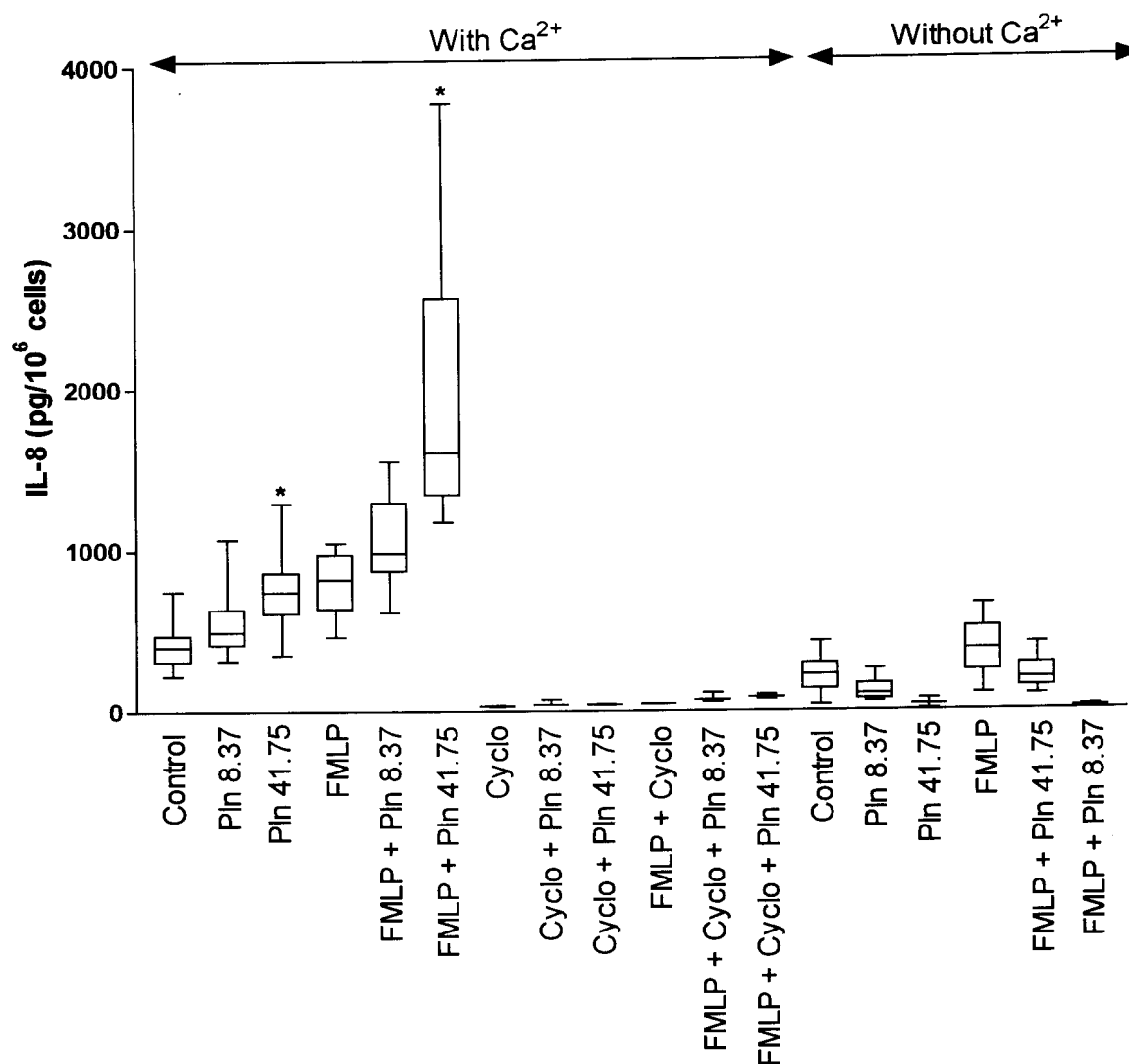


Figure 4.2: Box and whisker plots of IL-8 in the supernatants of unstimulated and FMLP (1 μ M)-activated neutrophils in the presence and absence of pneumolysin (Pln, 8.37 and 41.75 ng/ml). The results shown are the median values with 25-/75-percentiles observed following a fixed incubation period of 6 hours at 37°C for neutrophils suspended in HBSS with and without Ca²⁺ (7 and 2 experiments with triplicate determinations in each, respectively) in the presence and absence of cycloheximide (Cyclo, 10 μ g/ml).

* $P < 0.05$ compared with the corresponding pneumolysin-free control systems.

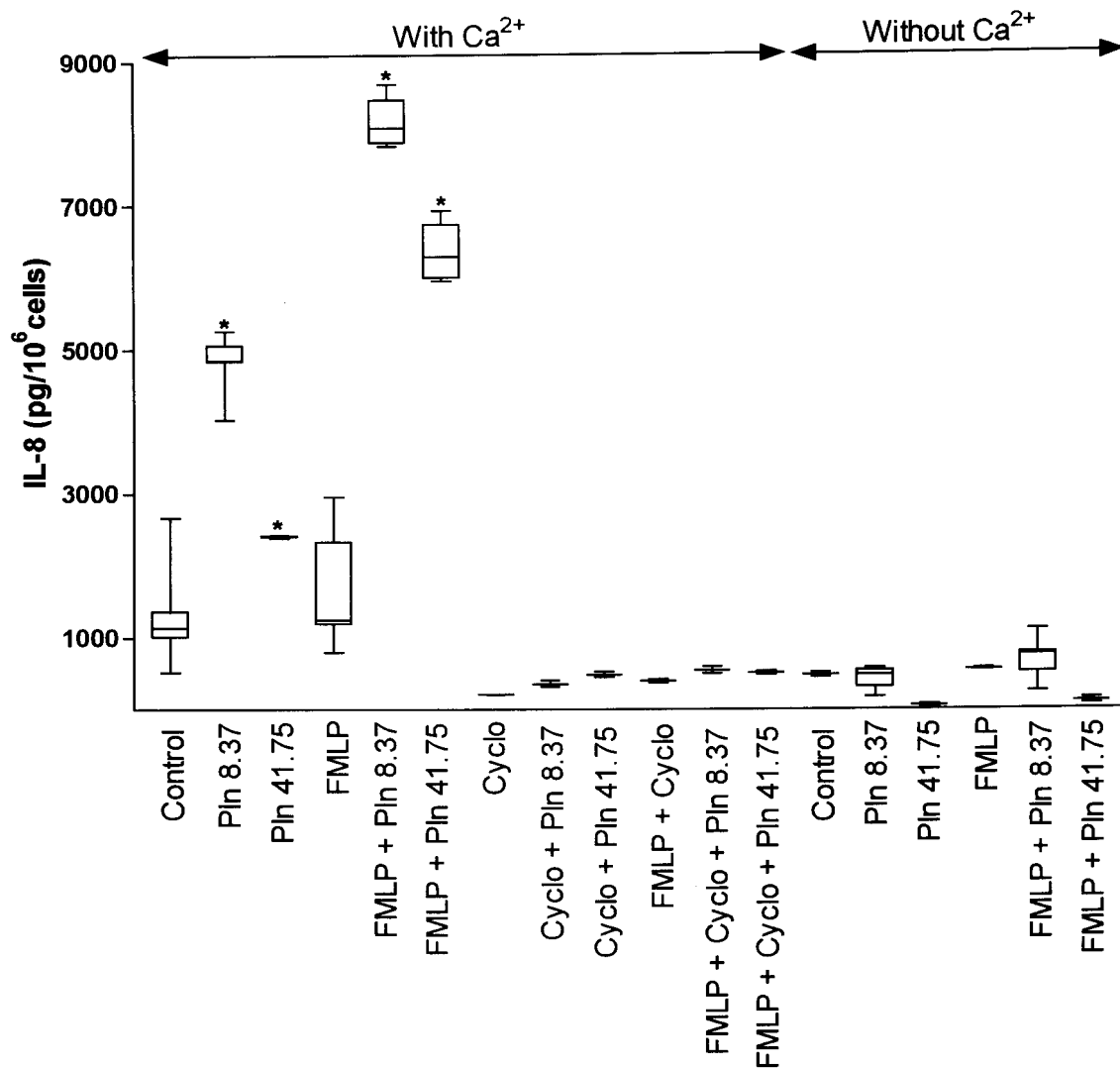


Figure 4.3: Box and whisker plots of the total (extracellular and intracellular) amount of IL-8 produced by unstimulated and FMLP (1 μ M)-activated pneumolysin (Pln, 8.37 and 41.75 ng/ml)-treated neutrophils over a 6 hour time-course at 37°C. The results are the median values with 25-/75-percentiles observed for neutrophils suspended in HBSS with and without Ca²⁺ (7 and 2 experiments with triplicate determinations in each, respectively) in the presence and absence of cycloheximide (Cyclo, 10 μ g/ml).

* $P < 0.05$ compared with the corresponding pneumolysin-free control systems.

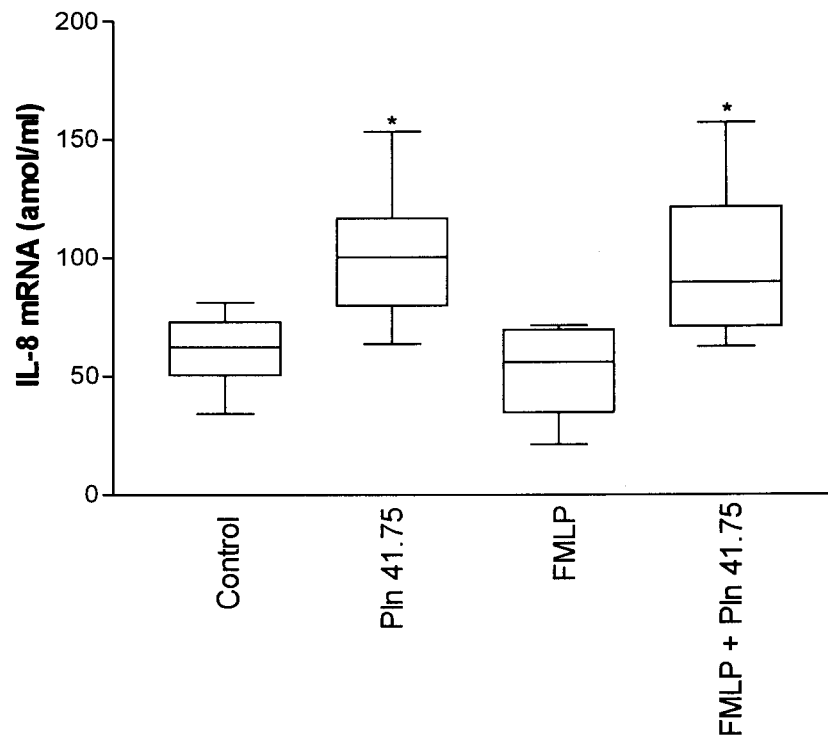


Figure 4.4: The effects of pneumolysin (Pln, 41.75 ng/ml) on the synthesis of IL-8 specific mRNA by neutrophils, in the presence and absence of FMLP (1 μ M). The results are expressed as median values with 25-/75-percentiles, for a total of 12 experimental determinations.

* $P < 0.05$ when compared to the corresponding pneumolysin-free control system.

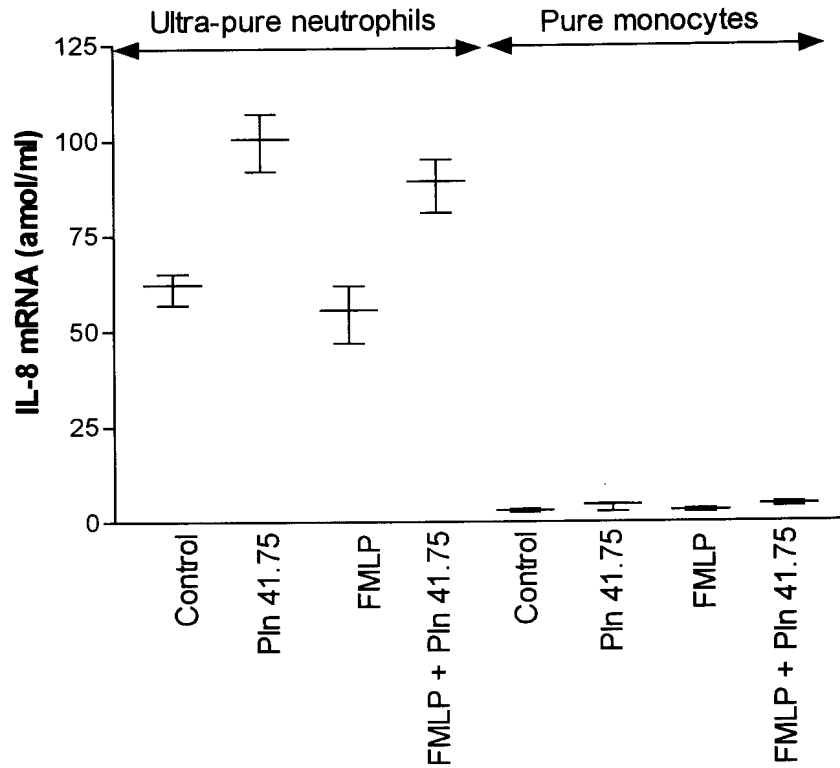


Figure 4.5: The effects of pneumolysin (Pln, 41.75 ng/ml), in the presence and absence of FMLP (1 μ M), on the IL-8 specific mRNA synthesis by ultra-pure neutrophils and pure monocytes. The results represents the median values with 25-/75-percentiles of a single experiment, with triplicate determinations.

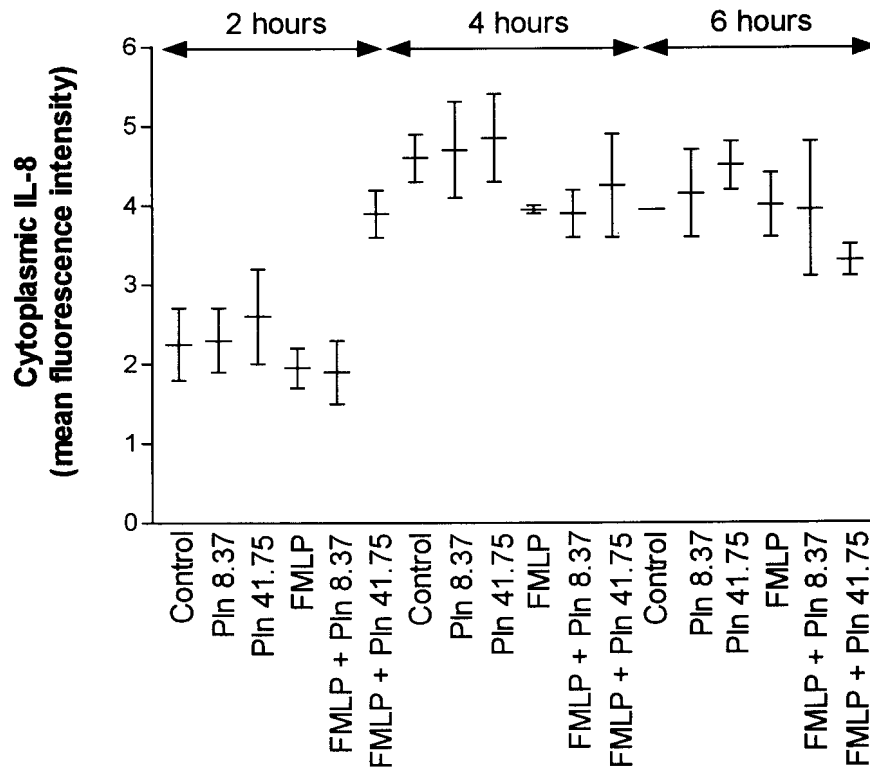


Figure 4.6: The effects of pneumolysin (Pln, 8.37 and 41.75 ng/ml), in the presence and absence of FMLP (1 μ M), on neutrophil intracytoplasmic IL-8 levels. The results represent the median values with 25-/75-percentiles of 2 experiments (duplicate determinations), using cells from different donors.

4.4 Discussion

In this study, I have investigated the effects of pneumolysin, at concentrations that potentiate several abruptly activatable, proinflammatory activities of human neutrophils, on the production of IL-8 by these cells. Exposure of neutrophils to the toxin alone was accompanied by a significant increase in both the synthesis and release of the chemokine, whereas FMLP alone increased the release of IL-8 without significantly affecting its synthesis. However, the most striking increases were observed when the chemoattractant and the toxin were used in combination. Interestingly, extracellular concentrations of IL-8 were higher in neutrophils treated with pneumolysin at 41.75 ng/ml in comparison with cells treated with 8.37 ng/ml, whereas the opposite was observed with respect to intracellular IL-8. These observations may be attributable to pneumolysin-mediated alterations in plasma membrane integrity at higher concentrations of the toxin over the 6 hour time-course of the experiments, as described in Chapter 2, resulting in leakage of intracellular IL-8.

I am unaware of any studies that may have documented the exact concentration of pneumolysin in the airways during pneumococcal infection, probably because of the high tissue affinity of the toxin. However, pneumolysin concentrations of up to 9 µg/ml, which are considerably higher than those used in the present study, have been detected in broth cultures of the pneumococcus (Feldman *et al.*, 1990).

Pneumolysin-mediated increases in neutrophil IL-8 appeared to be the consequence of *de novo* synthesis of the cytokine, since these were associated with the appearance of IL-8 specific mRNA. The contribution that contaminating monocytes make to IL-8 synthesis during exposure of the cells to pneumolysin, with and without FMLP, was negligible.

Activation of synthesis of IL-8 by neutrophils treated with pneumolysin, with and without FMLP, was completely attenuated by depletion of Ca²⁺ from the cell-suspending medium. This dependence of IL-8 synthesis on the influx of extracellular Ca²⁺ is in agreement with previous reports, in which, over a time-course similar to that which this study used for pneumolysin, the calcium ionophore, A23187, and the endo-membrane Ca²⁺-ATPase inhibitor, thapsigargin,

were found to activate IL-8 synthesis by neutrophils by a mechanism dependent on influx of extracellular Ca^{2+} (Kuhns *et al.*, 1998). Although the molecular/biochemical mechanism by which pneumolysin causes activation of synthesis of IL-8 by neutrophils remains to be established, Ca^{2+} -dependent activation of the proinflammatory transcription factor, $\text{NF}\kappa\text{B}$, is likely to be involved (Dolmetsch *et al.*, 1997).

Although the involvement of pneumolysin in pneumococcal virulence and dissemination is well documented (Feldman *et al.*, 1991; Musher *et al.*, 2001a), the possible role that toxin-mediated dysregulation of the synthesis of IL-8 by neutrophils and other cell types (Madsen *et al.*, 2000) plays in the pathogenesis of pneumococcal disease remains to be established. Excessive production of this and other chemokines (Rijneveld *et al.*, 2002), if it occurs *in vivo*, may contribute to exaggerated inflammatory responses, such as those caused by pneumolysin-producing strains of the pneumococcus in a murine model of experimental lung infection (Kadioglu *et al.*, 2000); this, in turn, may favour microbial invasiveness as a consequence of neutrophil-mediated damage to respiratory epithelium and, by promoting disorientation and premature deactivation of neutrophils, may also enable the pneumococcus to subvert host defenses.

Chapter 5:

The effect of pneumolysin on the oxidative inactivation of α -1-proteinase inhibitor

5.1 Introduction

As described in Chapter 1, infections with *S. pneumoniae* continue to be associated with considerable morbidity and mortality worldwide. There are a number of well-described factors that increase an individual's risk of acquiring pneumococcal infection, some of which are also associated with more severe disease. Recently, cigarette smoking has been documented to be a strong, independent risk factor for invasive pneumococcal infection (Nuorti *et al.*, 2000). A dose-response relationship was noted between cigarette smoking and the development of pneumococcal disease. Although likely to be multi-factorial, the mechanisms by which cigarette smoking predisposes to this infection, remain uncertain, and further research in this area would enhance our understanding of the pathogenesis of pneumococcal disease (Nuorti *et al.*, 2000).

Interestingly, replacement therapy in individuals with hereditary deficiency of α -1-proteinase inhibitor (API) results in a marked reduction in the frequency and severity of pulmonary infections (Lieberman, 2000). Moreover, acquired, localized dysfunction of API has been reported to occur in the lungs of cigarette smokers, primarily as a consequence of oxidative inactivation of the inhibitor by smoke-derived oxidants, as well as by oxidants generated by smoke-activated phagocytes (Gadek *et al.*, 1979). Taken together, these observations suggest that API is a potential link between cigarette smoking and pneumococcal disease.

In this context it is noteworthy that pneumolysin, a pro-oxidative toxin which augments the production of reactive oxidants by activated phagocytes, is thought to be involved in the pathogenesis of invasive pneumococcal disease (Feldman *et al.*, 1991; Musher *et al.*, 2001b). In the current study, I have investigated the effects of pneumolysin alone and in combination with human neutrophils on the elastase inhibitory capacity (EIC) of API.

5.2 Materials and Methods

5.2.1 Preparation of pneumolysin

Recombinant active pneumolysin was expressed in *E. coli* and purified from cell extracts as described in 2.2.1. Final pneumolysin concentrations of 8.37 and 41.75 ng/ml were used.

5.2.2 Neutrophils

Purified neutrophils were prepared as described in 2.2.3.

5.2.3 Elastase inhibitory capacity of alpha-1-protease inhibitor

Purified API (0.25 mg/ml, final) was preincubated with neutrophils (5×10^6 /ml) in HBSS for 5 min at 37°C prior to the addition of pneumolysin (8.37 and 41.75 ng/ml, final) or an equal volume of HBSS to control systems and a further 5 min incubation at 37°C. This was followed by addition of the synthetic chemoattractant, FMLP (1 μ M, final), or an equal volume of HBSS to control systems. The final volume in each tube was 250 μ l and the tubes were then incubated for 15 min at 37°C after which the neutrophils were pelleted by centrifugation and the supernatants harvested and assayed for EIC using a micro-modification of a spectrophotometric procedure (Ras *et al.*, 1992). Briefly, 50 μ l of the supernatant was added to 250 μ l of 0.2 M Tris-HCl (pH 8.0) containing 21 milliunits (final) of porcine elastase and the mixtures incubated for 15 min at 37°C. Following incubation, 50 μ l of the mixture was added to 150 μ l of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanalide (0.6 mM in Tris-HCl) in the wells of microtiter plates and elastase activity monitored spectrophotometrically at a wavelength of 405 nm over a 30 min time-course at 37°C. The results are expressed as the magnitude (%) of inactivation of the EIC of API.

Rubins and colleagues (1992) reported that API inhibits the haemolytic and cytotoxic activities of purified pneumolysin. In a follow-up article, Rubins and Freiberg (1994) indicated that nanomolar concentrations of contaminating

cholesterol, found in some commercially available API preparations, inhibited the cytotoxic activities, previously attributed to API, of pneumolysin. To control for possible interference by contaminants present in the API preparation with the biological activity of pneumolysin, I have measured the effects of the proteinase inhibitor on the haemolytic activity of the toxin, as well as its effects on mediating influx of Ca^{2+} into neutrophils, as described in Appendix 1 and 2.3.6, respectively.

In an additional series of experiments, the effects of wortmannin (selective inhibitor of phosphatidylinositol-3-kinase, 1 μM , final) which inhibits activation of NADPH oxidase, and methionine (reactive oxidant scavenger, 100 μM , final), on neutrophil-mediated inactivation of API were investigated (Tsan & Chen, 1980; Li *et al.*, 1994). These agents were added to the cells 5 min prior to pneumolysin (8.37 ng/ml).

The effects of depletion of extracellular Ca^{2+} were investigated by using cells suspended in nominally Ca^{2+} -free HBSS containing the Ca^{2+} -chelating agent, EGTA (100 μM , final), to ensure total depletion of the cation. Under these conditions, I was unable to detect influx of Ca^{2+} into pneumolysin-treated neutrophils.

5.2.4 *Superoxide generation*

The effects of pneumolysin (8.37 ng/ml), with and without FMLP (1 μM), in the presence and absence of wortmannin and methionine on superoxide production by neutrophils, were measured using a LECL procedure, as described in 2.2.5. The incubation conditions were as described above. LECL was monitored over a 5 min time-course at 37°C using a Lumac Biocounter (Lumac Systems) following the addition of pneumolysin, in the presence and absence of FMLP, to the cells. The final volume in each vial was 1 ml HBSS supplemented with 0.1% bovine serum albumin, containing 0.2 mM lucigenin and 1×10^6 neutrophils. The results are expressed in relative light units (rlu).

5.2.5 Statistical analysis

Levels of statistical significance were calculated by the Mann-Whitney U-test (2-tail). The level of significance was set at $P < 0.05$. The results of each series of experiments are expressed as the median values with 25-/75-percentiles.

5.3 Results

5.3.1 Pneumolysin and API

In a preliminary series of experiments, it was established that at this concentration the API preparation used (0.25 mg/ml) did not interfere with either the haemolytic activity of pneumolysin (41.75 ng/ml) or with the toxin-mediated influx of Ca^{2+} into neutrophils. These results are shown in Figures 5.1 and 5.2 respectively.

As shown in Figure 5.3 the EIC of API was unaffected by pneumolysin alone (at 8.37 and 41.75 ng/ml), neutrophils alone, or the combination of pneumolysin with neutrophils. However, addition of FMLP (1 μ M) to neutrophils was accompanied by significant ($P < 0.05$) inhibition of the EIC of API which was substantially augmented in a dose-related manner by pre-treatment of the cells with pneumolysin ($P < 0.05$ for comparison between FMLP only and FMLP in combination with pneumolysin at both concentrations tested, as well as for comparison between the two concentrations of pneumolysin). The inactivating effects of FMLP-activated neutrophils in both the presence and absence of pneumolysin, on the EIC of API were completely attenuated by depletion of Ca^{2+} from the cell-suspending medium.

The effects of treatment of neutrophils with wortmannin and methionine on the inactivation of the EIC of API following activation of the cells with FMLP in the presence and absence of pneumolysin (8.37 and 41.75 ng/ml) are shown in Figure 5.4. These agents significantly ($P < 0.05$) attenuated the functional inactivation of API by FMLP-activated neutrophils both with and without pneumolysin.

5.3.2 Superoxide generation

As described in Chapter 2, pneumolysin *per se* does not affect the generation of superoxide by resting neutrophils, but significantly ($P < 0.05$) augmented the production of the reactive oxidant by FMLP-activated cells. The median values

(background values for unstimulated cells subtracted) with 25-/75-percentiles for FMLP-activated systems in the absence and presence of pneumolysin (8.37 ng/ml) were 15890 (14430/18140) and 31110 (30080/32180) rlu, respectively. Superoxide production by these systems was significantly ($P < 0.05$) decreased by inclusion of wortmannin, the respective values being 493 (483/501) and 4141 (2417/5143), while being unaffected by methionine, the values being 14180 (13270/15380) and 32180 (31110/34930) for systems without and with pneumolysin (8.37 ng/ml). Although these pro-oxidative interactions of pneumolysin are identical to those described in Chapter 2, the LECL values are somewhat higher. This is attributable to the inclusion of bovine serum albumin in the cell-suspending medium.

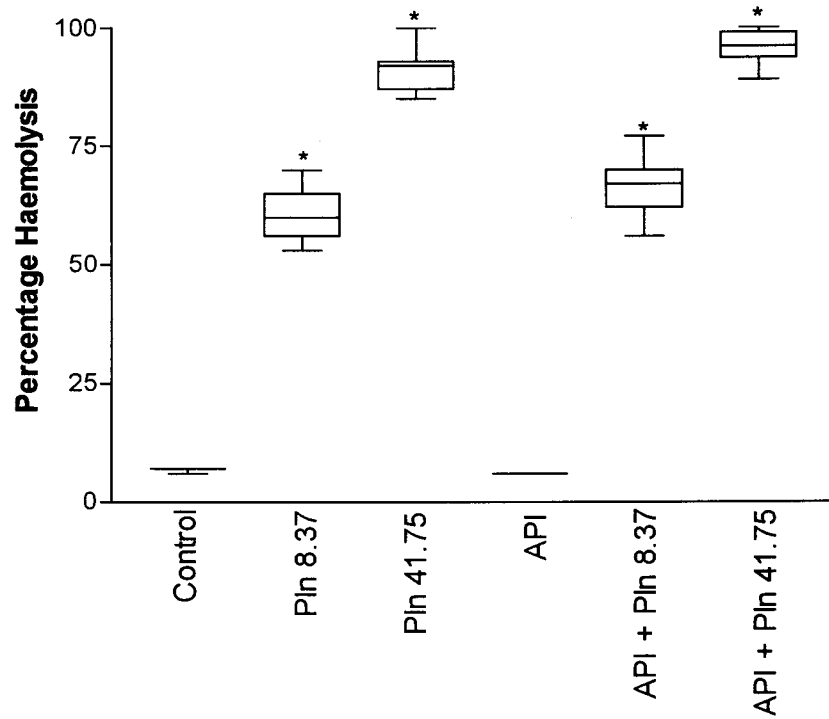


Figure 5.1: The effects of pneumolysin (Pln, 8.37 and 41.75 ng/ml) in the presence and absence of API (0.25 mg/ml), on erythrocyte haemolysis. The results for a total of 12 experimental determinations are presented as the median values with 25-/75-percentiles, and expressed as percentage haemolysis. * $P < 0.05$ in comparison with the relevant pneumolysin-free control system.

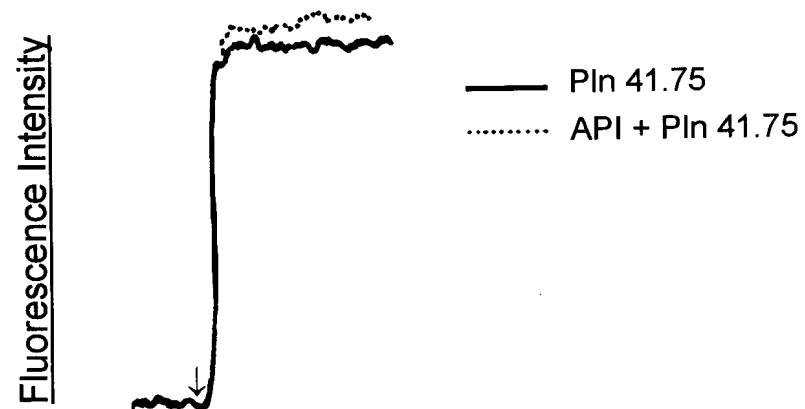


Figure 5.2: The fura-2 fluorescence responses of pneumolysin-treated neutrophils in the presence and absence of 0.25 mg/ml API. Pneumolysin (41.75 ng/ml, final) was added as indicated (↓), after a stable baseline was obtained (± 1 min). This is a representative trace from 2 experiments.

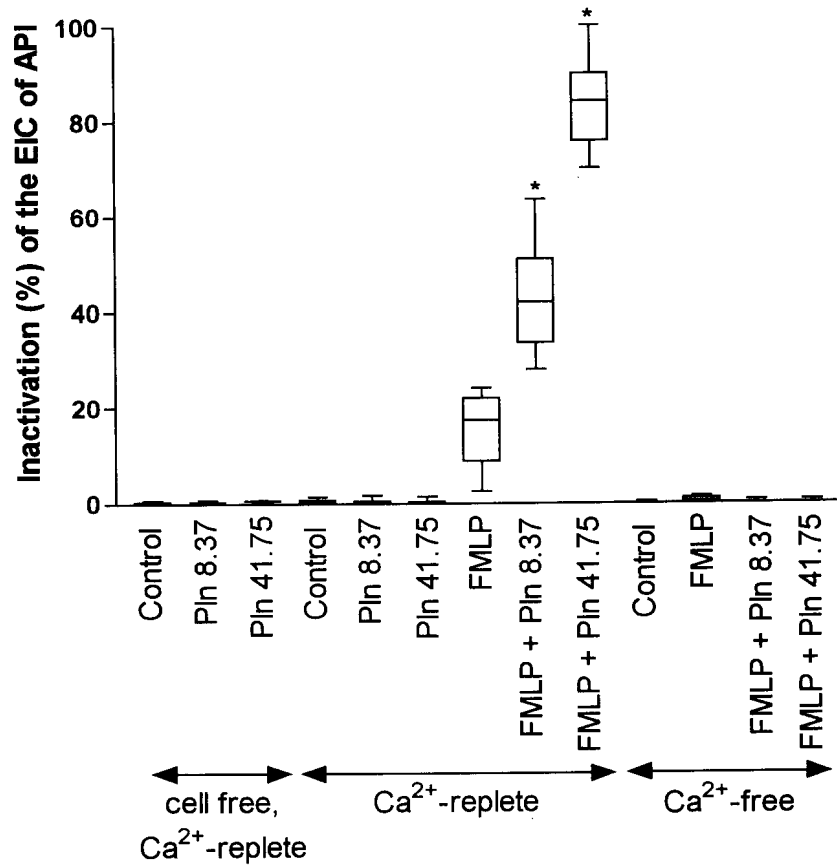


Figure 5.3: Box and whisker plots of the inactivation of the EIC of API exposed to pneumolysin (PIn, 8.37 and 41.75 ng/ml) only (cell free), or to pneumolysin-treated neutrophils, with and without FMLP (1 μ M), in Ca²⁺-replete and Ca²⁺-free medium (data from 5 different experiments with 5 replicates in each).

* $P < 0.05$ for comparison of the values for FMLP-activated neutrophils in the absence of pneumolysin with the corresponding systems in the presence of the toxin.

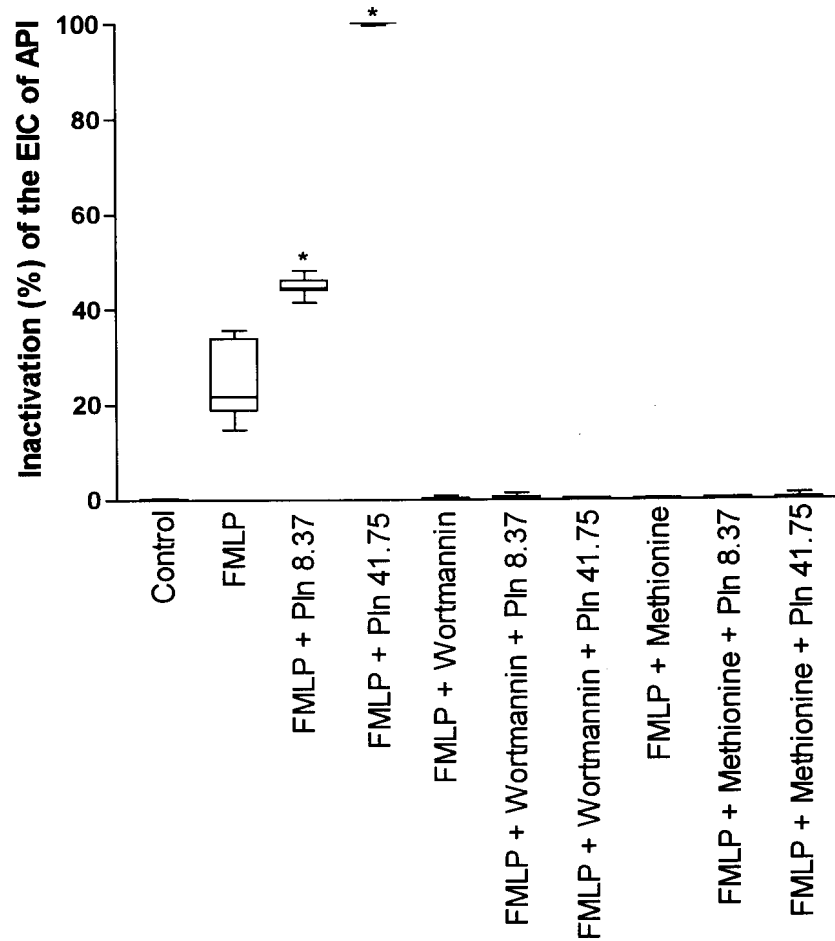


Figure 5.4: Box and whisker plots of the inactivation of the EIC of API exposed to pneumolysin (Pln, 8.37 and 41.75 ng/ml)-treated and –untreated neutrophils in the presence and absence of wortmannin (1 μ M), or methionine (100 μ M). The data shown are from 2 different experiments with 5 replicates in each.

* P <0.05 for comparison of the values for FMLP-activated neutrophils in the absence of pneumolysin with the corresponding values in the presence of the toxin.

5.4 Discussion

In this study, pneumolysin, at concentrations which are not cytotoxic for neutrophils over the time-course studied, has been found to sensitize human neutrophils for exaggerated inactivation of API on exposure of the cells to the chemoattractant, FMLP. These pro-proteolytic interactions of pneumolysin with neutrophils appear to be secondary to the pro-oxidative effects of the toxin on the cells since they were associated with augmentation of FMLP-mediated activation of neutrophil membrane-associated oxidative metabolism and attenuated by wortmannin, an inhibitor of activation of NADPH oxidase, as well as by methionine, a scavenger of hypochlorous acid which protects the critical oxidation-sensitive methionine residue at position 356 in the elastase inhibitory site of API (Tsan & Chen, 1980).

The pro-oxidative interactions of pneumolysin with neutrophils are strictly Ca^{2+} -dependent being secondary to the pore-forming actions of the toxin, which promote influx of extracellular cation and activation or sensitization of Ca^{2+} -dependent proinflammatory functions of the cells, as described in Chapter 2. This relationship is also underscored in the current study since depletion of Ca^{2+} from the cell-suspending medium completely abrogated pneumolysin-mediated enhancement of inactivation of API by FMLP-activated neutrophils.

Although the involvement, if any, of pneumolysin-mediated augmentation of inactivation of API by chemoattractant-activated neutrophils in the pathogenesis of pneumococcal disease remains to be established, this mechanism could, theoretically, either restrict or favour microbial dissemination. In the case of the former, neutrophil-derived granule proteases such as elastase and cathepsin G, both of which are inactivated by API, have been reported to be potential mediators of neutrophil antimicrobial activity (Belaaouaj *et al.*, 1998; Reeves *et al.*, 2002). On the other hand, interference with API may increase the vulnerability of respiratory epithelium to elastase (Rennard *et al.*, 1991; Lewis *et al.*, 1995), facilitating spread of the pneumococcus. The latter scenario may be the most probable, since the pneumococcus also produces copious amounts of H_2O_2 (Duane *et al.*, 1993), which may contribute further to oxidative inactivation of API.



Pneumolysin therefore possesses, albeit indirectly, pro-proteolytic properties. Although the etiologic significance is unknown, it is possible that these interactions between pneumolysin and pulmonary phagocytes, if they occur in the airways, may contribute to the development of invasive pneumococcal disease, and may be of particular significance in cigarette smokers.

Chapter 6:

Concluding Comments

The laboratory research described in this thesis was designed to investigate the effects of pneumolysin on the proinflammatory activities of human neutrophils *in vitro*.

My findings can be summarized as follows:

Pneumolysin, most notably in combination with FMLP, potentiated the following neutrophil activities:

- O_2^- production
- Release of elastase from neutrophil primary granules
- Expression of the β_2 -integrin, CR3
- PLA₂ activity, resulting in the increased production of the arachidonic acid mediators of inflammation, PGE₂ and LTB₄
- IL-8 synthesis and release
- Oxidative inactivation of API

These proinflammatory interactions of pneumolysin with neutrophils, which have not to my knowledge been described previously, are strictly dependent on the toxin-mediated influx of extracellular Ca^{2+} , with no apparent involvement of mobilization of the cation from intracellular stores.

These observations suggest that pneumolysin-directed antiinflammatory pharmacologic strategies may have therapeutic potential in acute pneumococcal disease. Possible approaches which merit investigation include identification of pharmacological agents which:

- Antagonise the physical interactions of pneumolysin with neutrophils and other types of immune and inflammatory cells
- Potentiate the clearance of Ca^{2+} from the cytosol of activated neutrophils.

Potential targets include:

- i) the calmodulin-upregulated plasma membrane Ca^{2+} -ATPase, and/or the Na^+ , Ca^{2+} -exchanger operating in the forward mode (Ca^{2+} out, Na^+ in), both of which promote efflux of cytosolic Ca^{2+}
- ii) 3',5' cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA)-upregulated endomembrane Ca^{2+} -ATPase which sequesters/resequesters Ca^{2+} into intracellular storage organelles.

I am unaware of pharmacological agents which fall into the first category, or indeed of any which potentiate the activity of the plasma membrane Ca^{2+} -ATPase or the Na^+ , Ca^{2+} -exchanger. However, the endomembrane Ca^{2+} -ATPase is amenable to upregulation by pharmacological agents which selectively elevate cAMP in immune and inflammatory cells, resulting in accelerated clearance of cytosolic Ca^{2+} and suppression of proinflammatory activity. In the case of neutrophils, these include:

- i) Selective inhibitors of the cAMP-hydrolysing enzyme type 4 phosphodiesterase (PDE4), with subtype PDE4B2 being the predominant type present in neutrophils (Wang *et al.*, 1999)
- ii) Selective agonists of adenylate cyclase-coupled subtype $\text{A}_{2\text{A}}$ adenosine receptors.

Treatment of human neutrophils with selective PDE4 inhibitors or subtype $\text{A}_{2\text{A}}$ receptor agonists *in vitro* results in increased intracellular concentrations of cAMP, upregulation of the endomembrane Ca^{2+} -ATPase, accelerated clearance of cytosolic Ca^{2+} , and down-regulation of the proinflammatory activities of these cells (Anderson *et al.*, 1998; Anderson *et al.*, 2000).

Rolipram is the prototype PDE4 inhibitor. Although, the effects of rolipram on the course of experimental pneumococcal disease do not appear to have been studied (Toward & Broadley, 2002). Using a model of LPS-induced inflammatory lung disease in guinea pigs, they observed that administration of rolipram attenuated the infiltration of inflammatory cells into the airways, and reversed LPS-induced bronchoconstriction, underscoring the antiinflammatory potential of this agent.

Sullivan *et al.* (1999) investigated the effects of an $\text{A}_{2\text{A}}$ receptor agonist, 2-cyclohexylmethylidenehydrazinoadenosine (WRC-0470), on harmful inflammatory

responses both *in vitro* and *in vivo*. WRC-0470 alone, and synergistically in combination with rolipram, increased neutrophil intracellular cAMP concentrations and reduced cytokine-enhanced neutrophil adherence, superoxide release and degranulation *in vitro*. Using a rat model of acute experimental bacterial meningitis, induced by intracisternal administration of *E. coli* LPS, these investigators also observed that administration of WRC-0470, both with and without rolipram, inhibited pleocytosis and reduced the LPS-induced increase in blood-brain barrier permeability, compatible with decreased neutrophil-mediated tissue damage.

In addition to the aforementioned pharmacological strategies, the use of antimicrobial agents which possess secondary antimicrobial properties is an alternative approach which merits consideration. In this context macrolide, azalide and ketolide antimicrobial agents have been reported to suppress the proinflammatory activities of human neutrophils (Mokgobu *et al.*, 1999) and to inhibit the synthesis of IL-8, a major neutrophil chemoattractant (Elssner & Vogelmeier, 2001), by bronchial epithelial cells and neutrophils (Feldman & Anderson, 1999; Tsuchihashi *et al.*, 2002). Moreover, cefoperazone a cephalosporin antimicrobial agent, has been reported to protect API against oxidative inactivation by stimulated neutrophils (Dallegrì *et al.*, 1999), but in this case additional data on less toxic agents belonging to the same group of pharmacological agents is needed.

In conclusion, while conceding that these various pneumolysin/neutrophil-directed antiinflammatory chemotherapeutic strategies are somewhat speculative, and must clearly be evaluated in animal models of experimental pneumococcal infection, they do, however, represent a logical and potentially clinically relevant extension of the laboratory research described in this thesis.

Appendix 1

Evaluation of the haemolytic activities of the preparation of recombinant pneumolysin used for all the studies presented in this dissertation.

Materials and methods:

Erythrocytes were prepared from clotted venous blood from healthy, adult volunteers. The blood was centrifuged at 490 x g for 15 min at room temperature. The bottom phase collected, washed with PBS three times and resuspended to a 5% erythrocyte solution.

The erythrocytes (0.5%, final) were incubated in HBSS for 30 min at 37°C before the active pneumolysin (0.837 - 83.70 ng/ml) was added (final volume of 1 ml). A 100% haemolysis control, in which erythrocytes were treated with the detergent lysophosphatidylcholine (LPC) at 100 µg/ml (final), was included. After 5 min intact erythrocytes were removed by centrifugation and the supernatants assayed spectrophotometrically at 405 nm for haemoglobin content. The results are expressed as a percentage of the 100% haemolysis control (Anderson *et al.*, 1996).

Results

The haemolytic activity of pneumolysin (0.837 - 83.70 ng/ml) is shown in Figure A1. The toxin caused dose-related haemolysis which reached 100% at 41.75 ng/ml.

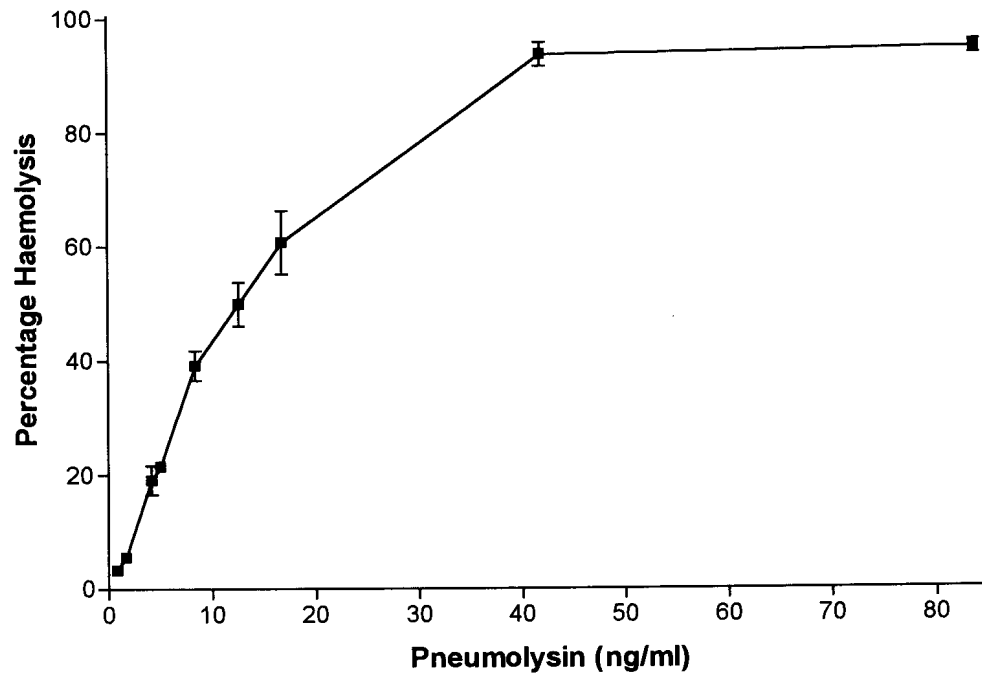


Figure A1: Pneumolysin (0.837 - 83.70 ng/ml)-mediated haemolysis of human erythrocytes. The results of a total of 8 experiments are presented as the mean percentage haemolysis \pm SEMs.

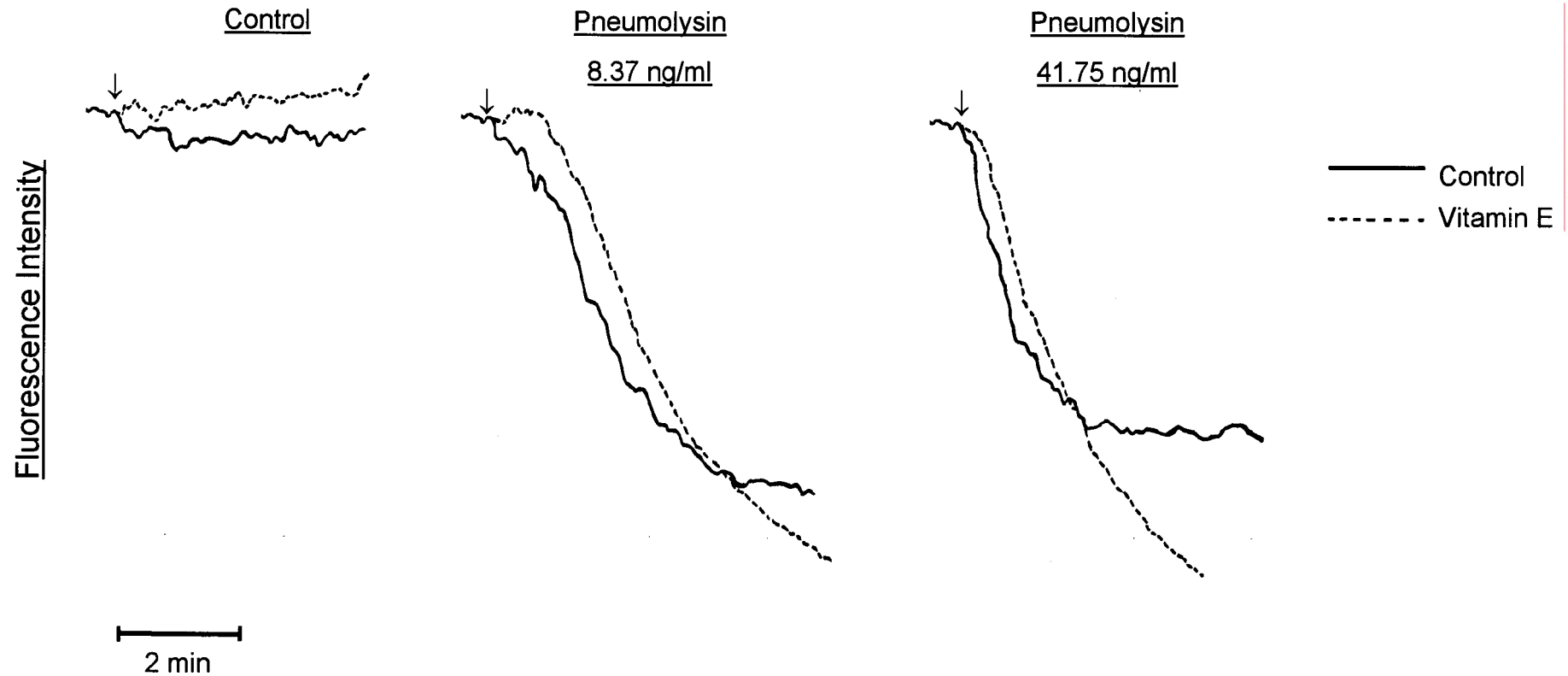
Appendix 2

Figure A2: The effects of pneumolysin (8.37 and 41.75 ng/ml) on human neutrophil membrane potential in the presence and absence of vitamin E (25 μ g/ml). Pneumolysin was added as indicated (\downarrow) when a stable baseline was obtained (\pm 1 min).

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