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THE ROLE OF PNEUMOLYSIN IN PRO-INFLAMMATORY MEDIATOR PRODUCTION

A thesis submitted to the University of Glasgow for the degree of Ph.D.

by

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ABSTRACT

Pneumolysin is a pore forming toxin produced by *Streptococcus pneumoniae* and is necessary for full virulence during pneumococcal infection. Pneumolysin monomers oligomerise in cell membranes and form pores which can lead to cell lysis or, at low concentrations, lead to production of pro-inflammatory mediators. We attempted to define the role of oligomerisation in pneumolysin-induced pro-inflammatory mediator production. Site directed mutagenesis was used to alter amino acid residues within the pneumolysin structure thought to be essential for oligomerisation.

An assay was developed to detect oligomerisation by measuring changes in fluorescence in the environment of an extrinsic fluor, ANS. Contrary to expectations, the data presented here show that mutating these residues does not abolish oligomerisation of the toxin. We therefore have not been able to resolve the role of oligomerisation in mediator production. Evidence that the lytic activity of the toxin is involved is shown by use of a mutant toxin in which the lytic activity is abolished to less than 0.1%. Lytic concentrations of the toxin also stimulate much greater production of both nitric oxide and tumour necrosis factor from macrophages than sublytic concentrations.

Experiments using the whole organism confirm the role of pneumolysin in mediator production by *Streptococcus pneumoniae*. These data have also revealed other interesting interactions between the pneumococcus and host cells that involve pneumolysin.

Data from microarray experiments reported here confirm increased expression of several pro-inflammatory mediators previously reported to be induced by pneumolysin. Other mediators expected to show enhanced gene regulation were not confirmed. This highlights the complexity of these methods and the need for further research into these mechanisms. Data from the microarray and experiments using laser scanning cytometry confirm the ability of pneumolysin to induce apoptosis in macrophages.

We have confirmed the importance of pneumolysin in the development of an inflammatory response and shown how it may be pivotal in the outcome of the host response to pneumococcal infection.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

°C	Degrees celcius
µl	Microlitre
µm	Micrometre
µM	Micromolar
-/-	Deficient
A	Absorbance
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis Inducing Factor
ANS	8-Anilino-1-naphthalene-sulphonic acid
AOM	Acute Otitis Media
APS	Ammonium Persulphate
AUC	Analytical Ultracentrifugation
BAB	Blood Agar Base
Bad	BCL2-Associated Death Promoter
Bak	BCL2-Antagonist/Killer
BALF	Bronchus Associated Lymphoid Fluid
BALT	Bronchus Associated Lymphoid Tissue
Bax	BCL2-Associated X Protein
BBB	Blood-Brain Barrier
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CBP	Choline Binding Protein
Cfu	Colony forming unit
CNS	Central Nervous System
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CV	Column Volumes
D39	*S. pneumoniae* Strain D39
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DTNB	Dithio(bis)nitrobenzoate
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic Acid

ELISA	Enzyme-Linked Immunosorbent Assay
EST	Expressed Sequence Tag
EtOH	Ethanol
EU	Endotoxin Units
F12k	Modified Ham's F12 (Medium)
FA	Formaldehyde Agarose
F-antigen	Forssman Antigen
FBS	Foetal Bovine Serum
Fc	Crystallisable fragment (of immunoglobulin)
g	Gram
G(M)-CSF	Granulocyte-(Macrophage) colony stimulating factor
GBS	Group B Streptococcus
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid])
HIC	Hydrophobic Interaction Chromatography
His-Tag	Histidine Affinity Tag
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HU	Haemolytic Units
IκB	Inhibitory κB
ICE	Interleukin Converting Enzyme
IEC	Ion-Exchange Chromatography
IFNγ	Interferon gamma
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
IPTG	Isopropyl-β-D-Thiogalactopyranoside
IRAK	IL-1 Receptor-Associated Kinase
kb	Kilobase
kDa	Kilodalton
LAL	Limulus Amebocyte Lysate
LB broth	Luria-Bertani broth
LDH	Lactate Dehydrogenase

LPS	Lipopolysaccharide
LSC	Laser Scanning Cytometry
LT	Leukotriene
LTA	Lipotechoic Acids
M	Molar
mAb	Monoclonal Antibody
MC	Metal Chelate (Chromatography)
MCP	Macrophage Chemotactic Protein
MIP	Macrophage Inflammatory Protein e.g. MIP-1α
MMP	Matrix Metalloproteinase
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
NFκB	Nuclear factor kappa B
NIK	NFκB-Inducing Kinase
NK-cells	Natural Killer Cells
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
Nramp	Natural Resistance Macrophage Protein
OD	Optical Density
ORF	Open Reading Frame
PAF	Platelet Activating Factor
PARP	Poly(ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
PC	Phosphorylcholine
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PI	Propidium Iodide
PLN-A	D39 pneumococci containing inactivated pneumolysin gene
Ply	Pneumolysin
Ply-TNB	Thionitrobenzoate derivatised pneumolysin
PMB	Polymyxin B
pNA	p-nitroaniline
Pnc	Pneumococci
Psa	Pneumococcal Surface Adhesin

Psp	Pneumococcal Surface Protein
RANTES	Regulated upon activation normal T-cell expressed and secreted
RNA	Ribonucleic Acid
ROS/ROI	Reactive Oxygen Species/ Intermediates
rpm	Revolutions per minute
S. pneumoniae	*Streptococcus pneumoniae*
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
STM	Signature Tagged Mutagenesis
TA	Techoic Acids
TAT	Thiol-Activated Toxin
TGF-β	Transforming Growth Factor-β
TLR	Toll-like Receptor
TNF	Tumour necrosis factor
TPR	Tetratrico Peptide Repeat
TRAFs	TNF Receptor Associated Factor
U	Units
V	Voltage
WT	Wild type
x g	Acceleration in the Earth's gravitational field
X-Gal	O-Nitrophenyl-β-D-Galactopyranoside
z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone

1. INTRODUCTION

1.1 Public Health Impact of *Streptococcus pneumoniae*

Streptococcus pneumoniae, also known as the pneumococcus, is a Gram-positive bacterium with significant health implications. It was formerly known as *Diplococcus pneumoniae* and was first isolated in 1881 by Sternberg (1881) and Pasteur (1881) independently. Normally the pneumococcus has a commensal relationship with humans, its main host. It is likely that everybody is colonised by this bacteria in the nasopharynx at some time in their life, with no ill effects. Several capsular types can be carried at one time and colonisation can occur at birth. The pneumococcus was first implicated in disease in 1883 when the organism was associated with lobar pneumonia (Friedlander 1883). Pneumococcal disease leads to a wide range of important human pathologies, from common upper respiratory tract infections to severe invasive manifestations such as pneumonia, meningitis and septicaemia. Disease occurs when the respiratory tract, central nervous system (CNS) or blood is invaded and infection established. Infection is more likely to result following the recent acquisition of a different strain than from a strain already being carried. This bacterium is a major public health problem all over the world and is an important concern for the WHO (World Health Organisation). A 10% reduction in pneumococcal deaths worldwide would save more lives than the complete eradication of *Haemophilus influenzae* and *Salmonella typhi* (Cadoz 1998). Antimicrobial drugs have reduced the overall mortality from pneumococcal disease but can be ineffective unless given early. Furthermore, several strains are exhibiting increased drug resistance.

Acute respiratory infections kill an estimated 2.6 million children under the age of five annually. The pneumococcus causes over one million of these deaths, most of which occur in developing countries, where it is probably the most important pathogen during early infancy. In Europe and the United States, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia, estimated to affect one in one thousand adults each year (WHO 1999).

National surveillance of invasive pneumococcal infections in England and Wales is based on the reporting of pneumococcal isolates from normally sterile sites. In 2000 and 2001, *S. pneumoniae* was the 4th most reported bacterial infection in England and Wales (PHLS Communicable Disease Surveillance Centre 2001a, 2001b). The incidence of invasive pneumococcal infections is highest among young children and elderly people, and the majority of infections occur during winter months (Aszkenasy *et al.* 1995).

1.2 Structure of *Streptococcus pneumoniae*

Streptococci are Gram-positive, catalase negative and facultative anaerobic bacteria. They are further classified by haemolysis when grown on blood agar, biochemical tests and by group-specific carbohydrate antigens in the cell wall known as Lancefield groups. Colonies of strains containing streptolysin-O display a large zone of complete red cell lysis called β-haemolysis. Some other streptococci including pneumococci, exhibit α-haemolysis on blood agar, demonstrated by formation of a green/brown zone around colonies due to discolouration and loss of potassium from the red blood cells. Lancefield groups are designated by letters A through O. β-haemolytic streptococci found in humans are usually group A (Brock and Madigan 1991). Group B streptococci are also commonly isolated from humans and other animals. *Streptococcus pneumoniae* does not possess Lancefield antigens.

The organism is an oval or spherical coccus of 0.5-1.25μm in diameter and is surrounded by a polysaccharide capsule. Two phenotypes of the pneumococcus have been identified, opaque and transparent, named after their colony appearance on transparent medium. This variation appears in all strains and there is spontaneous variation between colony phenotypes known as phase variation. Animal models show the transparent phenotype to be more efficient at colonisation of the nasopharynx whereas the opaque phenotype is more virulent in a model of invasive infection. These characteristics are associated with the altered regulation of certain elements in the pneumococcus. The opaque variant produces more capsular polysaccharide, especially under anaerobic conditions, which could inhibit adherence but prevent clearance. The transparent phenotype produces more H_2O_2, which may act to kill other commensal organisms present in the respiratory tract and also acts as a toxin (Weiser 1998).

1.3 Clinical Disease caused by *Streptococcus pneumoniae*

In recent years, there has been an increase in the understanding of interactions between the pneumococci and its host, both in terms of how virulence factors of the organism contribute to pathogenesis and how the host's response to infection can be harmful as well as protective. However there is still much more to learn. One in three children asymptomatically carry pneumococci in the nasopharynx at any given time, but little is understood about the transition from commensal to pathogen. An important aspect of adherence is that the interaction is benign unless the human cells become activated. Therefore the ability to invade human cells may be more of a result of changes in the host

cell surface perhaps for example by a concurrent viral infection, rather then by the bacteria itself (Tuomanen and Masure 2000).

1.3.1 Meningitis

The mortality and morbidity associated with bacterial meningitis have remained significant despite advances in antimicrobial therapy and supportive care. The main causes are *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Incidence of disease due to *H. influenzae* has been reduced since the introduction of a successful conjugate vaccine. Acute bacterial meningitis remains a major problem with 10% of survivors suffering from neurological sequelae such as learning deficits, mental retardation, sensory-motor deficits and most commonly, hearing loss (Grimwood *et al.* 1995). Meningitis caused by *S. pneumoniae* is rapidly fatal if not treated early and effectively. The exact pathophysiological mechanisms of pneumococcal meningitis are unclear but both host and bacterial factors play a role.

In order to cause meningitis, bacteria need to cross the blood-brain barrier (BBB). In an *in vitro* BBB model, pneumococci cause rounding and detachment of brain microvascular endothelial cells and decrease the transendothelial electrical resistance, indicating an opening of tight junctions (Zysk *et al.* 2001). Intra-cerebral inoculation of pneumococci also causes an opening of tight junctions between brain endothelial cells (Quagliarello *et al.* 1986). No differences were detected between endothelial damage caused by *S. pneumoniae* isolated from meningitis patients or from patients with bacteraemic pneumonia. This led to the hypothesis that pneumococcal meningitis develops as a consequence of an impairment of host defences rather than being related to special properties of pneumococcal strains (Zysk *et al.* 2001).

Pneumococcal multiplication in the CNS induces production of pro-inflammatory cytokines such as tumour necrosis factor (TNF), nitric oxide (NO), chemokines such as interleukin (IL)-8 and the anti-inflammatory mediators IL-10 and transforming growth factor-β (TGF-β) (Freyer *et al.* 1996; van Furth *et al.* 1996). Fragments of the pneumococcal cell wall have been shown to stimulate the host inflammatory response (Tuomanen *et al.* 1985) and cause the release of TNF, IL-1 and IL-6 (Tuomanen *et al.* 1986). This leads to a complex network of additional mediators, which seem to be involved in meningeal inflammation (Täuber and Moser 1999). Cytokines are released from cells normally present in the CNS, such as

endothelial cells, microglial cells, and astrocytes. They up-regulate adhesion molecules leading to an influx of leukocytes across the BBB causing the characteristic inflammation of bacterial meningitis. Opening of tight junctions leads to an influx of serum components including chemotactic factors such as C5a which in turn causes neutrophils to appear in the cerebrospinal fluid (CSF) within 8-12 hours. Due to a lack of opsonins in this compartment, the neutrophils have no effect on the proliferation of the bacteria (Smith *et al.* 1973; Propp *et al.* 1977). Down-regulation of this inflammatory response decreases the neurological complications in clinical and experimental systems (Tuomanen *et al.* 1989; Saez-Llorens *et al.* 1990; Saukkonen *et al.* 1990).

Early in experimental pneumococcal meningitis, cerebral blood flow increases due to vasodilation mediated by NO (Koedel *et al.* 1995). NO continues to play a role as a regulator of cerebral blood flow far into the course of disease (Leib *et al.* 1998). It can have beneficial effects as inhibition of nitric oxide synthase (NOS) leads to a harmful increase in cerebral ischaemia. However NO can also be neurotoxic directly, or by combining with superoxide to form peroxynitrite (Kim *et al.* 1995).

The increased inflammatory reaction leads to multiple, well-defined alterations of intracranial physiology. These include brain oedema, an increase in intracranial pressure, a decrease in cerebral blood flow and an increased resistance to CSF resorption (Scheld *et al.* 1980; Täuber *et al.* 1985; Tureen *et al.* 1990; Tureen *et al.* 1992). The increase in intracranial pressure contributes critically to cerebral ischaemia (Tureen *et al.* 1992).

It has been demonstrated that *S. pneumoniae* is able to cause meningitis independent of bloodstream involvement upon different routes of infection. This may account for the difficulty in treating human infections caused by this organism (Marra and Brigham 2001). Several approaches that interfere selectively with the mechanisms of neuronal injury are effective in animal models, including the use of non-bacteriolytic, protein synthesis-inhibiting antibiotics, antioxidants and inhibitors of transcription factors, matrix metalloproteinases, and caspases (Nau and Bruck 2002). Lysis of bacteria by antibiotics can lead to an increased inflammatory response due to the release of pneumolysin toxin and bacterial cell wall products.

Densely ciliated ependymal cells cover the ventricular surface of brain and cerebral aqueducts separating the CSF (which is infected during meningitis) from the neuronal tissue. Ciliary beat may prevent margination of the bacteria to the brain surface. Pneumolysin may enable entry into the cerebrum by impairment of the BBB. Wild type pneumococci cause rounding and detachment of the brain microvascular endothelial cells whereas mutant D39 pneumococci containing an inactivated pneumolysin gene (PLN-A) had no effect (Zysk *et al.* 2001). Furthermore, pneumolysin caused damage comparable to that by live *S. pneumoniae.* Pneumolysin causes rapid ciliary stasis, sloughing of cilia and cytoplasmic extrusion (Mohammed *et al.* 1999). Following ependymal damage, regeneration of ciliated ependymal cells is rarely seen (Tureen 1995).

In a rabbit model of meningitis, PLN-A caused less damage to the dentate gyrus than wild type pneumococci. However, to reduce neuronal damage, hydrogen peroxide (H_2O_2) production by *S. pneumoniae* must also be inhibited. *S. pneumoniae* can damage the hippocampus directly through induction of neuronal apoptosis initiated by the pneumococcal toxins pneumolysin and H_2O_2 (Braun *et al.* 2002).

1.3.2 Pneumonia

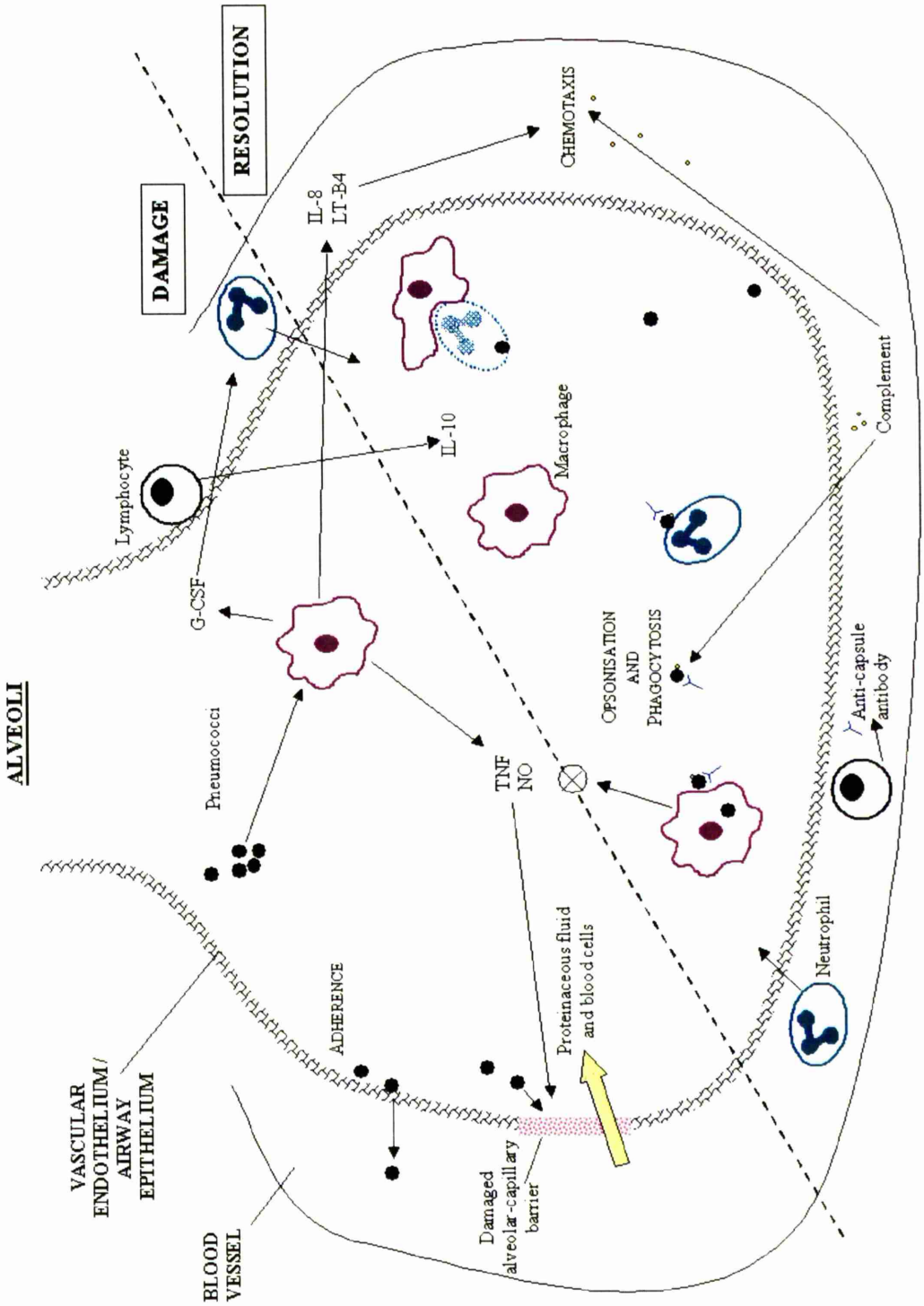
Pneumococci gain access to the lung by aspiration from the nasopharynx. Progression to pneumonia requires additional events, for example, a pre-existing viral infection. *In vitro* adherence of *S. pneumoniae* to respiratory tract epithelial cells is enhanced by the presence of the influenza virus (Hakansson *et al.* 1996). The pneumococcus itself can directly contribute to its spread to the lungs by causing impairment of the ciliary activity and pneumolysin-dependent disruption of the epithelial tight junctions, which are essential for normal mucus production (Rayner *et al.* 1995; Wilson *et al.* 1996). Pneumococcal pneumonia is associated with bacteraemia more frequently than other bacterial pneumonias (Fang *et al.* 1990).

In the classic description of pneumonia there are four stages: engorgement, red hepatisation, grey hepatisation and resolution. Disease starts when bacteria invade the alveoli of the lung. This leads to congestion of alveolar capillaries and an outpouring of serous fluid into the lung. This acts in favour of the bacteria as both a source of nutrients and a mode of transport to neighbouring alveoli (Harford and Hara 1950). The engorgement of capillaries with blood cells causes the appearance of red hepatisation, and neutrophils, a few macrophages and

many erythrocytes pass from the capillaries into the alveolar spaces (Loosli and Baker 1962). Within a few hours, this exudate continues to accumulate in the alveoli and the capillaries become compressed. The number of erythrocytes starts to decrease and the number of leukocytes rises (McKinsey and Bisno 1980). The intravascular deposition of fibrin leads to decreased perfusion and the characteristic grey hepatisation (Kline and Internitz 1915). Pneumococci evading the immune response spread to the lung tissues and blood, causing septicaemia. Most deaths occur within the first five days of illness (Sato *et al.* 1998), although splenectomised individuals can succumb in as little as 18-24 hours with no apparent symptoms (Musher 1992). The actual cause of death from pneumococcal pneumonia is unknown. Death can occur even after antibiotic treatment and bacterial clearance has started (Austrian and Gold 1964).

The host response to pneumococcal pneumonia (Figure 1.1) was investigated in mice after intranasal inoculation with 10^7 cfu (colony-forming units) of bacteria (Bergeron *et al.* 1998). During the first four hours of disease, ineffective phagocytosis by alveolar macrophages was seen and increased levels of pro-inflammatory mediators in the bronchus associated lymphoid fluid (BALF) and lung tissue were found. After four hours, cytokine levels continued to increase and were associated with bacterial growth and neutrophil recruitment to the lungs. A spill over of IL-1 into the serum was also observed. After 24 hours, the bacteria progressed to the blood stream and a down-regulation of cytokines in the BALF and lung tissue occurred. Despite an overall loss in body weight, there was an increase in lung weight due to alveolar injury and the development of oedema. Between 48 and 72 hours there was strong recruitment of monocytes and lymphocytes to the lungs and release of NO in BALF and lung tissue. Bacteraemia was associated with cytokine release in the blood. After this time, high NO levels were observed, as well as an increase in bacterial growth, lung weight and mortality.

Figure 1.1 – Pneumococcal Infection in the Lung



This figure illustrates some of the interactions between *Streptococcus pneumoniae* and host cells during pneumococcal pneumonia. Pneumococci multiply in the alveolar space. Bacteria and bacterial products bind to endothelia, epithelia and leukocytes and elicit cellular damage and cytokine release. Further influx of leukocytes occurs in response to released chemokines and complement activation. The outcome of the infection depends at least partly on the ability of the host to withstand the inflammation associated with bacterial death. Abbreviations: LT, leukotrienes; G-CSF, granulocyte-colony stimulating factor; TNF, tumour necrosis factor; NO, nitric oxide; IL, interleukin;

Effective pulmonary host defence against respiratory pathogens is thought to be mediated via phagocytosis by alveolar macrophages and recruited neutrophils (Gordon *et al.* 2000). If the bacteria overcome these defences and gain entry to the blood, systemic protection is afforded by anticapsular antibodies (Macleod *et al.* 1945) that enable efficient engulfment of opsonised bacteria by phagocytes. The host response is orchestrated by rapid inflammation following infection that involves cytokines and other soluble mediators (see section 1.11.5). Neutrophils that have phagocytosed bacteria must then be cleared from the lungs, probably by macrophages, which in turn are removed from the lung by mucociliary transport. Fibroblasts and alveolar epithelial cells are then recruited to the lung, possibly by IL-1β and TNF to repair damage (Lesur *et al.* 1996). Despite massive inflammatory responses, patients who recover fully from pneumococcal pneumonia show no permanent damage to their lungs (Catterall 1999).

1.3.2.1 Role of Pneumolysin in Pneumonia

Pneumolysin causes severe lobar pneumonia when injected into the apical bronchus of rats (Feldman *et al.* 1991). The cytolytic and complement-activating properties of the toxin both contribute to pathogenesis of pneumococcal pneumonia at different stages of infection and by different mechanisms (Berry *et al.* 1995; Rubins *et al.* 1995; Rubins *et al.* 1996). Pneumolysin is also involved in cell recruitment during pneumococcal pneumonia as infection with pneumolysin-deficient bacteria results in delayed and reduced neutrophil influx to the lung (Kadioglu *et al.* 2000).

1.3.2.2 Predisposing Factors for Pneumococcal Pneumonia

People at the extremes of age are most likely to suffer from pneumococcal disease. The highest incidences are found in children aged between 18 and 24 months and in individuals over seventy (Austrian and Gold 1964).

Investigation of host genetic factors which may be responsible for susceptibility to infectious diseases revealed a locus on mouse chromosome one, termed *Bcg*, *Lsh* or *Ity*. This locus is important in controlling infections due to intracellular organisms such as *Mycobacterium* species, *Listeria monocytogenes* and *Salmonella typhimurium* (Skamene *et al.* 1979; Forget *et al.* 1981; Plant *et al.* 1982). *Ity* encodes a natural resistance macrophage protein 1 (Nramp1) which affects the macrophage's ability to destroy ingested pathogens early in the infectious process (Vidal *et al.* 1993).

Genetic factors play a role in determining the outcome to pneumococcal infection, but at present, the nature of these is unknown. Adoptees have a five-fold greater risk of dying from an infectious disease if a biological parent died from the same infection (with pneumonia accounting for 29% of infections), whereas the risk is not increased if an adoptive parent dies from infection (Sørenson *et al.* 1988). Models of pneumococcal pneumonia in inbred strains of mice that are genetically resistant or susceptible to infection have been established (Gingles *et al.* 2001). Inbred BALB/c mouse strains display resistance to infection, whereas CBA/Ca strains are susceptible and die within 36 hours of infection. CBA/Ca mice are unable to control pneumococcal viability within the lungs and bloodstream. They display a reduced inflammatory response within lung airways and an elevated inflammatory response within lung tissue (Kerr *et al.* 2002).

Incidence of pneumococcal pneumonia is very high in HIV-infected patients; making *S. pneumoniae* the most commonly isolated respiratory pathogen in HIV-positive individuals (Gilks *et al.* 1996; Moore *et al.* 1998). Prevalence of HIV has meant an increasing percentage of females and a younger age group are becoming infected with pneumococcal pneumonia. Furthermore, the risk of bacteraemic pneumococcal pneumonia is increased approximately 100-fold (Redd *et al.* 1990), with a mortality rate in AIDS patients of 57% whereas the rate in other populations is 22-39% (Pesola and Charles 1992).

1.3.3 Otitis Media

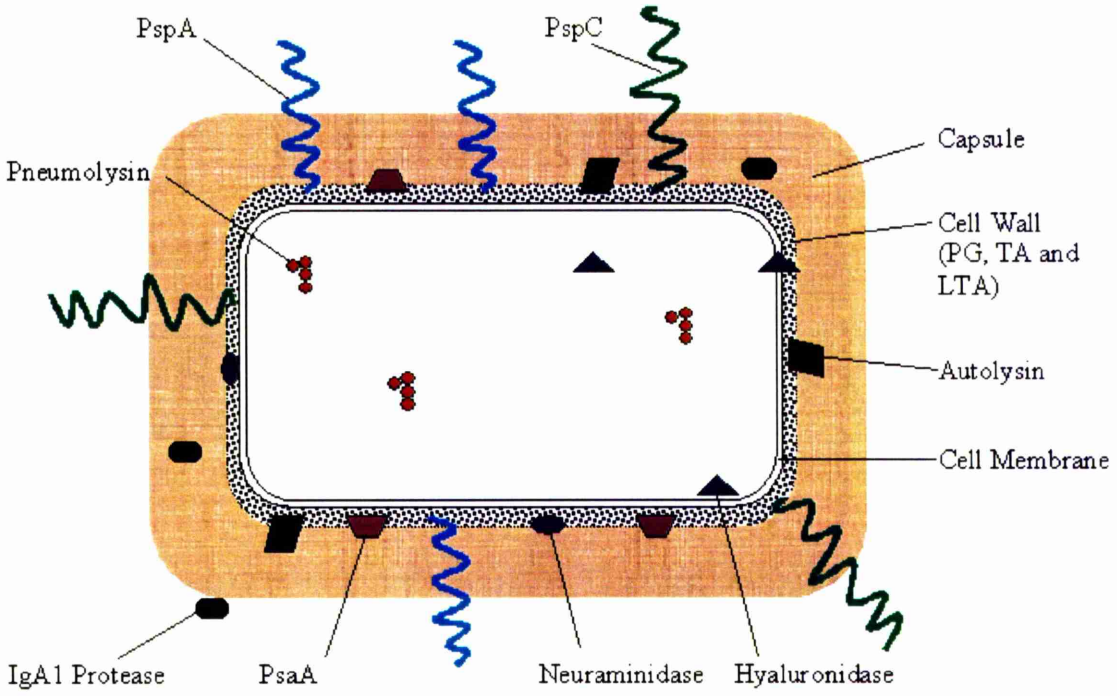
The pneumococcus is the leading cause of otitis media with virtually every child experiencing at least one episode by the age of five. To gain access to the middle ear, pneumococci must progress up the Eustachian tube. Once in the ear, bacterial replication leads to an influx of neutrophils driven by the release of IL-6, IL-1 and TNF (Carlsen *et al.* 1992). Bacterial lysis induced by antibiotics releases large amounts of bacterial cell wall fragments and pneumolysin. Pneumococcal otitis media is characterised by profound inflammation mainly induced by cell wall components (Tuomanen *et al.* 1985; Tuomanen *et al.* 1987; Ripley-Petzoldt *et al.* 1988; Carlsen *et al.* 1992). Animal models have shown that cell wall fragments are sufficient to induce the entire inflammatory cascade of otitis media (Ripley-Petzoldt *et al.* 1988; Bhatt *et al.* 1991; Carlsen *et al.* 1992; Bhatt *et al.* 1993; Bhatt *et al.* 1995). Pneumolysin is involved in damage to the hair cells of the cochlea (Winter *et al.* 1997) and the organ of Corti (Comis *et al.* 1993). Hearing loss in both otitis media and

meningitis is thought to be due to the toxic effects of pneumolysin and the side effects of inflammation (Tuomanen 2001).

1.4 Virulence Factors produced by *Streptococcus pneumoniae*

The pneumococcus produces several molecules, which may be important in pathogenesis (Figure 1.2).

Figure 1.2 – Virulence Factors produced by *Streptococcus pneumoniae*



This simplified picture shows some of the important virulence factors of *S. pneumoniae* and where their location is currently thought to be. The cell wall is comprised mainly of peptidoglycan (PG), techoic acids (TA) and lipotechoic acids (LTA). The TA and LTA contain choline, which acts as an anchor for many of the surface molecules. Abbreviations: Psp, pneumococcal surface protein; Psa, pneumococcal surface adhesin.

The extracellular polysaccharide capsule is a major virulence factor of *S. pneumoniae*. It forms the outermost layer of the pneumococcus; 90 different types of capsule are produced (Henrichsen 1995). The capsule provides a thick surface layer and completely conceals the inner structure of exponentially growing pneumococci (Sørensen *et al.* 1984). Differences in pneumococcal capsular types are the basis of serotype classification, originally differentiated by the capsule swelling (Quellung) reaction but now molecular techniques are employed for typing (Gillespie 1999). Expression of the capsular polysaccharide is essential for colonisation of the nasopharynx and virulence; encapsulated strains are at least 10^5 times more virulent than strains lacking the capsule (Avery and Dubos 1931; Watson and Musher

1990). In some serotypes the capsule can render the bacteria less susceptible to phagocytosis by host cells. Deposition of the complement component C3d on the capsule is thought to enhance anti-capsule antibody production by binding to B-cells via complement receptor 2 (CR2) (Griffioen *et al.* 1991; Griffioen *et al.* 1992). Production of anti-capsular antibody is highly protective host response (Smit *et al.* 1977; Snippe *et al.* 1983). However, serotypes that allow the deposition of C3 but prevent its degradation to C3b and C3d are poorly immunogenic but are more easily phagocytosed (Hostetter 1986). Type 3 mutants producing 20% of parental amounts of capsule could colonise as effectively as the parent but displayed a significant reduction in virulence via intravenous infection of a mouse model indicating a further role in virulence for the capsule (Magee and Yother 2001). The polysaccharide capsule is essential for *in vivo* survival and replication, but it is not toxic so other pneumococcal factors are required for full virulence.

Advances in technology should permit identification of sequences with homology to known genes, or genes involved in certain activities. Use of *S. pneumoniae* genome sequences published by The Institute for Genomic Research (TIGR) in 1997 (http://www.tigr.org/tdb) and 2001 (Tettelin *et al.* 2001) has enabled the identification of many virulence factors. In a recent large-scale study, the genome sequence was examined for open reading frames (ORFs) encoding proteins with secretion motifs or similarity to predicted virulence factors (Wizemann *et al.* 2001). Mice were immunised with 108 of the 130 ORFs identified and six were found to confer protection against pneumococcal challenge. The targetting of entire families of genes encoding proteins with recognisable structural features is also achievable. A conserved amino acid sequence LPXTGE is important in a mechanism of attachment of proteins to the pneumococcal cell surface. Although several members of this family had already been identified, use of the genome sequence revealed 15 of these motifs, including those in neuraminidase and hyaluronidase (Gosink *et al.* 2000).

Microarray techniques have allowed a number of genes involved in the regulation of pneumococcal competence to be identified (Rimini *et al.* 2000) and have been useful in starting to determine downstream expression of a two-component system (De Saizieu *et al.* 2000). The identification and construction of mutants of pneumococcal two-component signal transduction systems have identified one as essential for growth in two studies (Lange *et al.* 1999; Throup *et al.* 2000). Differences in the other two-component systems found by these two studies may demonstrate differences in the importance of these regulatory systems

to the pathogenesis of systemic versus respiratory infections. *In vivo* expression technology and signature tagged mutagenesis (STM) have enabled large-scale identification of virulence genes from the pneumococcus (Polissi *et al.* 1998; Lau *et al.* 2001). Some virulence factors were found to be specific for pneumonia (Polissi *et al.* 1998). Further characterisation of genes identified this way has allowed increased understanding of importance virulence factors such as NADH oxidase (Yu *et al.* 2001) that could lead to new ways of combating disease.

1.4.1 Surface Molecules

The cell wall consists of a triple layered peptidoglycan (PG) backbone that anchors the capsular polysaccharide, the cell wall polysaccharide and possibly also proteins. Peptidoglycan is especially abundant in Gram-positive bacteria where it makes up half of the mass of the cell wall. It can induce a variety of biological effects which can, like LPS in Gram-negative organisms, lead to sepsis. Chemicals such as cytokines produced by host cells after PG stimulation mediate these effects. A specific fragment of PG (*N*-acetyl-glucosamine-β-[1→4]-*N*-acetylmuramyl-1-alanine-d-isoglutamine) is responsible for the synergism with lipoteichoic acids (LTA) or IFNγ to induce NO formation in mouse macrophages (Kengatharan *et al.* 1998).

The cell wall polysaccharide structure is common to all serotypes (Alonso De Velasco *et al.* 1995). It is a complex teichoic acid (TA), containing phosphorylcholine (PC) residues attached to the peptidoglycan. LTA is inserted into the plasma membrane via its lipid moiety and also contains phosphorylcholine. LTA is powerful inhibitor of autolysin (Horne and Tomasz 1985), which is involved in cell lysis and cleaves PG during cell division. TA and LTA contribute strongly to host responses associated with acute inflammation. The structure of LTA (also known as the F-antigen) contributes to the ability of a particular bacterium to cause shock and multiple organ failure (Kengatharan *et al.* 1998). However, immunisation of mice with LTA does not protect against pneumococcal infection (Horne and Tomasz 1985).

Surface molecules such as membrane-linked proteases could be responsible for the increase in matrix metalloproteinase (MMP)-2 production seen from porcine endothelial cells stimulated by heat-killed *S. pneumoniae* (Michel *et al.* 2001). Over expression of MMPs can form part of an inflammatory reaction and could contribute to the development of shock.

A recently identified, 24-kDa surface protein called Spa (streptococcal protective antigen) (Dale *et al.* 1999), evokes protective antibodies and confers resistance to phagocytosis in human blood and virulence in mice. In group A streptococci, both Spa and M-protein are required for optimal virulence (McLellan *et al.* 2001). The M-protein is found on the surface of group A streptococci and is involved in the ability of the bacteria to resist opsonisation by complement and phagocytic killing by neutrophils (Lancefield 1962; Jacks-Weis *et al.* 1982).

A fibronectin-binding protein pavA is localised to the pneumococcal cell outer surface and has a role in cell binding to fibronectin (present on glycosidic receptors on epithelial cells). In *Staphylococcus aureus*, fibronectin proteins have been shown to be involved in adherence to human airway epithelium (Mongodin *et al.* 2002). Isogenic pavA mutants of pneumococci displayed 10^4-fold attenuation in virulence in a mouse sepsis model (Holmes *et al.* 2001).

Originally thought to be an adhesin, pneumococcal surface adhesin (Psa)A is now thought to indirectly alter adhesion of the pneumococci by acting on choline binding protein expression. The presence of the gene encoding PsaA has been confirmed in all 90 pneumococcal serotypes (Morrison *et al.* 2000). Immunisation with PsaA protected mice from challenge with virulent pneumococci (Talkington *et al.* 1996). It is a potential vaccine candidate as mentioned in section 1.5.1. The gene encoding PsaA is situated in a locus containing *psaB* and *psaC*. Knockout mutants of each of these genes, as well as the downstream homologue *psaD*, resulted in a similar phenotype with defective adhesion and reduced transformation efficiency. Mutagenesis of all these genes resulted in penicillin tolerance due to a failure to translate the autolysin gene (Novak *et al.* 1998). When an insertion-duplication mutant of D39 pneumococci was created without a functional PsaA gene it was completely avirulent and adherence of the bacteria to A549 lung epithelial cells was impaired (Paton *et al.* 1997). X-ray crystallography revealed a metal binding site in PsaA (Lawrence *et al.* 1998), strengthening the idea that this protein is a putative ATP-binding cassette-type binding protein involved in the uptake of Mn^{2+} and Zn^{2+}.

1.4.1.1 Choline Binding Proteins

An unusual feature of the pneumococcal cell wall structure is the presence of phosphorylcholine in the TA and LTA (Tomasz 1967). Pneumococcal choline-bearing cell wall structures connect the choline-binding proteins to the bacterial surface. Choline may

also interact with receptors on host cells and play a part in adherence and induction of host cell signalling, resulting in inflammation. The choline binding proteins (CBPs) are a family of surface proteins non-covalently bound to the phosphorylcholine moiety of the cell wall by a conserved choline-binding domain. Twelve CBPs have been identified using the pneumococcal genome sequence and are shown in Table 1.1. These proteins are involved in adherence to the nasopharynx and also have a role in causing sepsis (Gosink *et al.* 2000). Mutant pneumococci containing no CBPs displayed altered hydrophobic and electrostatic surface characteristics and were poorly adherent to human cells in comparison to wild type bacteria (Swiatlo *et al.* 2002).

Table 1.1 – Choline Binding Proteins of *Streptococcus pneumoniae*

CHOLINE-BINDING PROTEIN	ROLE IN VIRULENCE	REFERENCES
PspA	Role in sepsis. Decreases complement deposition. Binds to Lactoferrin	Talkington *et al.* 1992; Hammerschmidt *et al.* 1999; Neeleman *et al.* 1999; Tu *et al.* 1999
PspC (CbpA, SpsA)	Colonisation of nasopharynx. Uses polymeric Ig receptor	Rosenow *et al.* 1997; Zhang *et al.* 2000
LytA	Release of pneumolysin and cell wall degradation products	Berry *et al.* 1989a
LytB	Colonisation of nasopharynx	Gosink *et al.* 2000
LytC	Colonisation of nasopharynx	Gosink *et al.* 2000
PcpA	May play a role in adhesion	Sanchez-Beato *et al.* 1998
CbpD	Colonisation of nasopharynx	Gosink *et al.* 2000
CbpE	Reduced colonisation of nasopharynx. Decreased adherence to human cells	Gosink *et al.* 2000
CbpF	None	Gosink *et al.* 2000
CbpG	Reduced colonisation of nasopharynx. Reduced binding to human cells. Role in sepsis. Putative serine protease	Gosink *et al.* 2000
CbpI	None	Gosink *et al.* 2000
CbpJ	None	Gosink *et al.* 2000

Adapted from Mitchell and Kerr (2001).

Pneumococcal surface protein A (PspA) is found in most clinical isolates of pneumococci (Crain *et al.* 1990). It exhibits immunogenic properties and is required for full virulence of the pneumococcus (McDaniel *et al.* 1987). It is able to reduce the consumption of complement by pneumococci, leading to reduced complement mediated clearance and phagocytosis of the bacteria (Briles *et al.* 2000b). Despite displaying structural and antigenic variability between different pneumococcal strains, passive immunisation with antibodies against PspA or its N-terminal fragment protect mice against challenge with various serotypes (McDaniel *et al.* 1984; McDaniel *et al.* 1991; Talkington *et al.* 1991). PspC (also known as CbpA and SpsA) has a very similar structure to PspA; it is thought that protection against carriage, seen after immunisation with anti-PspA antibodies, may partly be due to antibody cross reaction with PspC, as PspA-negative pneumococci still cause significant colonisation (Briles *et al.* 2000b). PspC can bind to human secretory IgA, which may aid virulence by hindering bacterial clearance.

Most organisms contain one or more lytic enzymes. They are involved in a variety of functions such as cell wall synthesis, separation of daughter cells at the end of cell division and cell motility. *S. pneumoniae* contains a powerful autolysin coded by the gene *lytA*. Autolysin has been characterised as *N*-acetylmuramyl-$_L$-alanine amidase (Howard and Gooder 1974). LTA is a powerful inhibitor of this enzyme and probably has an *in vivo* role in controlling autolysin activity (Holtje and Tomasz 1975). Autolysin-negative mutants are less virulent than wild type pneumococci and immunisation with autolysin confers some protection against pneumococcal challenge in mice (Berry *et al.* 1989a). The effects of autolysin however, seem to be mediated by the release of pneumolysin (from the pneumococcal cytoplasm) and cell wall products (Lock *et al.* 1992). *In vivo*, autolysin activity can be triggered by lysozyme (Bruyn *et al.* 1992) inducing pneumococcal lysis and increased inflammation. Two other *lyt* genes have also been described, *lytB* and *lytC* (Garcia *et al.* 1999).

The pneumococcus appears to take advantage of the host inflammatory response by engaging the up-regulated platelet-activating factor (PAF) receptor in a choline-dependent fashion (Cundell *et al.* 1995). Coincident with the appearance of this receptor on host cells following inflammatory stimuli, pneumococci undergo enhanced adherence and internalisation. Resting eukaryotic cells containing N-acetylgalactosamine β-1-4-galactose or N-acetylgalactosamine β-1-3-galactose can tether pneumococci, but these cells do not

support translocation. Activated cells expressing the PAF receptor strongly support pneumococcal adherence and invasion of the transparent, but not opaque, pneumococcal phenotype. Surprisingly, binding to the PAF receptor by pneumococci fails to stimulate signal transduction (Cundell *et al.* 1995).

1.4.2 Neuraminidase

Neuraminidases are produced by all clinical isolates of *S. pneumoniae*. There are three identified neuraminidase enzymes coded by the genes *nanA*, *nanB* and *nanC* in the pneumococcus. The C-terminal end of the nanA enzyme contains the typical Gram-positive cell surface anchorage domain LPXTGE and has been shown to be a surface protein (Camara *et al.* 1994). The precise role of neuraminidases in virulence is not known. These enzymes cleave sialic acid residues from a wide variety of glycoproteins, glycolipids, and oligosaccharides on cell surfaces or in body fluids. This mechanism could damage host tissues and also reveal potential binding sites for pneumococcal adhesion (Krivan *et al.* 1988). Studies using a nanA-deficient strain of D39 have shown that *nanA* gene products have a significant impact on the changes of the carbohydrate moeties in epithelium and expose N-acetylglucosamine (GlcNAc) and D-galactose residues (Tong *et al.* 2001). The same group showed that this mutant was eliminated from the nasopharynx earlier than the wild type strain and was completely eliminated from the middle ear eleven days before the parent strain in a chinchilla model of otitis media (Tong *et al.* 2000).

1.4.3 Hyaluronidase

Hyaluronidase is an enzyme that degrades hyaluronic acid, a component of connective tissue. Virtually all invasive strains of *S. pneumoniae* produce hyaluronidase. This enzyme may play a role in translocation of organisms from the blood to the brain across the BBB as strains unable to produce hyaluronidase do not appear to invade the brains of infected mice (Kostyukova *et al.* 1995). Hyaluronidase also appears to play a role in either the invasion of the bacteria into the blood stream or in the survival of the organisms within the blood (Mitchell *et al.* 1997; Zwijnenburg *et al.* 2001). Further studies are evaluating the role of hyaluronidase in virulence.

1.4.4 Pneumolysin

Pneumolysin is a 53-kDa protein produced by all clinical isolates of *Streptococcus pneumoniae*. It is a member of a family of thiol-activated toxins (or Cholesterol-dependent

cytolysins) produced by a wide range of Gram-positive bacteria. The pneumolysin gene shows a very high level of conservation between serotypes.

Several publications have indicated that pneumolysin is an important virulence factor during pneumococcal disease. Significant anti-pneumolysin antibody titres (Jalonen *et al.* 1989) and circulating pneumolysin immune complexes (Leinonen *et al.* 1990) were detected in sera from the majority of patients with pneumococcal pneumonia and levels of anti-pneumolysin antibodies in pneumonia patients were significantly lower than in healthy controls (Kanclerski *et al.* 1988). This implies the toxin is produced *in vivo* and lower anti-pneumolysin antibody levels may predispose an individual to infection. Pneumolysin was detected by immunofluorescence staining of lungs from mice challenged intranasally with virulent pneumococci confirming its production *in vivo* (Canvin *et al.* 1995). The elimination of the bacteria *S. pneumoniae* from infected individuals often fails to prevent a fatal outcome, but administration of anti-pneumolysin antibodies prolongs survival in infected mice (Paton *et al.* 1983). Together these data suggest that pneumolysin is an important virulence factor and plays a part in the fatal outcome of infection.

Berry *et al.* (1989b) confirmed that pneumolysin is directly involved in the pathogenesis of pneumococcal infections. Insertion-duplication mutagenesis was used to construct a pneumolysin-negative derivative of type 2 *S. pneumoniae*, known as PLN-A. Intravenous challenge with a wild type pneumococcal strain (D39) caused overwhelming bacteraemia (10^8-10^9 organisms/ml blood) and death within 24h. When PLN-A pneumococci were used, a more chronic bacteraemia (10^5-10^6 organisms/ml) was established, that persisted for over a week without detrimental effect. Inactivation of the pneumolysin gene in mice increased the LD_{50} approximately 100-fold. The pneumolysin-negative pneumococcus was back-transformed with a purified DNA fragment carrying the pneumolysin gene and this restored full virulence.

The PLN-A mutant has helped to define the biological role of pneumolysin in several systems. Compared to wild type, pneumolysin-negative pneumococci show reduced ability to replicate and induce much less inflammation in the lung (Canvin *et al.* 1995), display delayed invasion into the bloodstream and are more susceptible to phagocytosis. *In vivo* experiments studying the growth kinetics of PLN-A compared to wild type, showed pneumolysin was required for the growth of bacteria to greater than 10^7 CFU/ml. In mice co-

infected with D39 and PLN-A, both strains grew exponentially until the death of the mice indicating that pneumolysin acts extracellularly. The D39 pneumococci were not able to grow exponentially in mice that had been pre-infected with at least 10^6 CFU/ml of PLN-A pneumococci suggesting that the PLN-A strain was able to induce host resistance (Benton *et al.* 1995).

In a purified form, pneumolysin is lethal to animals. *In vitro*, it lyses all types of eukaryotic cells tested. At sublytic concentrations pneumolysin affects the immune system by inhibiting some cellular activities and activating others; as will be discussed below. The toxin can also activate the classical pathway of the complement cascade in the absence of specific antibodies (Paton *et al.* 1984).

In vivo, pneumolysin is released by pneumococcal lysis and interferes with the inflammatory response, permitting continued growth and invasion of the bacteria. Rubins *et al.* (1995) used a technique to inoculate wild type and pneumolysin-negative pneumococci into the trachea that delivered 99% of the bacteria into the lungs. Their results revealed that pneumolysin facilitates intra-alveolar replication of pneumococci, penetration of bacteria from alveoli into the interstitium of the lung and dissemination of pneumococci into the bloodstream. Both the cytotoxic and the complement-activating activities of pneumolysin contribute independently to acute pulmonary injury and the high rates of bacteraemia seen in pneumococcal pneumonia. These findings are consistent with a model of ineffective complement activation by pneumolysin, which may deplete complement factors and divert opsonins away from bacterial cells.

1.4.4.1 Complement Activation by Pneumolysin

Treatment of human serum with purified pneumolysin results in activation of the complement via the classical pathway in the absence of specific antibodies. The effect of this may be to deplete complement levels and abrogate protection (Paton *et al.* 1984) and may lead to an attack on host tissues and promote inflammation (Mitchell and Andrew 1997). This activity is completely separate from the cellular activities of the toxin as mutations that severely abrogate cell binding and lytic activity have no effect on the complement activating activity. Also pre-treatment with cholesterol does not inhibit the activation of complement by pneumolysin (Paton *et al.* 1984).

Pneumolysin is thought to activate the complement pathway using a mechanism related to its ability to bind the Fc fragment of IgG. Complement activation and antibody binding are apparently related, as amino acid substitution at the aspartic acid residue at position 385 in domain four abolishes complement activation and reduces IgG binding by 70% (Mitchell *et al.* 1991). This residue lies in one of the regions of pneumolysin homologous to C-reactive protein (CRP). CRP is an acute phase protein, which activates the classical complement pathway in the absence of specific antibody (Kaplan and Volankis 1974). However the reason why pneumolysin activates the complement cascade may not be due to its homology to CRP, but due to the similarity of domain four with the Fc fragment of IgG. Although there is no sequence homology, both molecules adopt a β-sandwich structure, which could bind the first component of complement (Rossjohn *et al.* 1998).

1.4.4.2 Release of Pneumolysin from *Streptococcus pneumoniae*

Unlike the other thiol-activated toxins, pneumolysin lacks a hydrophobic N-terminal signal peptide (Walker *et al.* 1987), consistent with its cytoplasmic location in *S. pneumoniae* (Johnson 1977). It is not secreted by the bacteria but thought to be released upon lysis of the cell under the influence of autolysin. Autolysin can be triggered by human lysozyme (Bruyn *et al.* 1992), thereby using a human defence system to induce pneumococcal lysis, release pneumolysin and enhance inflammation. One publication has shown that pneumolysin can be released in significant amounts from certain strains during log-phase growth when bacterial cell lysis is not believed to occur (Balachandran *et al.* 2001). This group found that extracellular release of pneumolysin from the pneumococcal strain WU2 was not dependent on autolysin action.

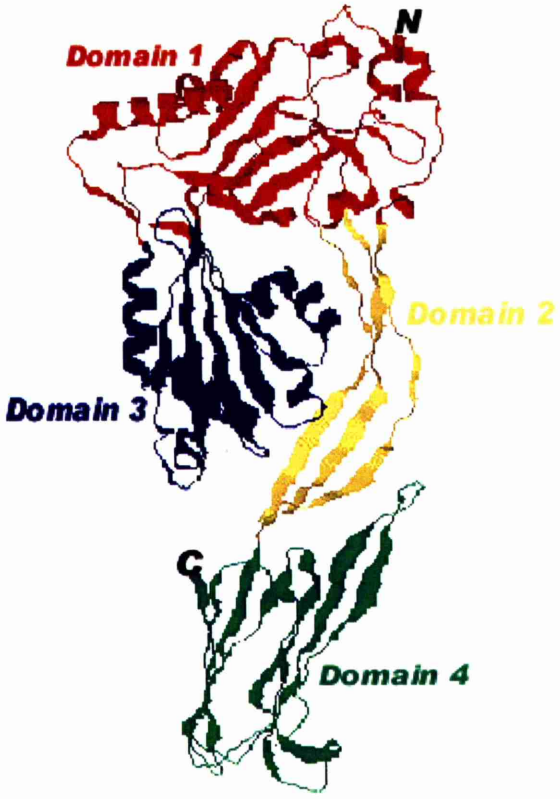
ClpC belongs to a family of heat shock proteins that act as molecular chaperones thought to play a role in the expression of several proteins, including pneumolysin. A ClpC-deficient mutant failed to express pneumolysin and several CBPs (Charpentier *et al.* 2000). However in a later study (Chastanet *et al.* 2001) ClpC was shown to act as a negative regulator, preventing competence gene expression under inappropriate conditions and did not play a major role in competence development, autolysis, pneumolysin production, or growth at high temperature of *S. pneumoniae* as previously thought. The regulation of pneumolysin has not yet been resolved.

Although inactivation of the pneumolysin gene significantly reduces the capacity of pneumococci to kill their host, it does not completely abolish it, indicating that other pneumococcal products are also involved. Pneumococcal virulence factors have different contributions in different forms of pneumococcal disease and at different stages of infection.

1.4.4.3 Pneumolysin Structure

Pneumolysin is a 470 amino acid polypeptide. It has a molecular weight of 52772, calculated from the deduced amino acid sequence (Walker *et al.* 1987). A preliminary crystallisation study described formation of pneumolysin crystals that only occurred in the presence of cholesterol (Kelly and Jedrzejas 2000), but a detailed crystal structure of pneumolysin has not yet been solved. A homology model has been created (Rossjohn *et al.* 1998) based on the crystal structure of perfringolysin O (Rossjohn *et al.* 1997) and is shown in Figure 1.3. The polypeptide chain runs back and forth several times through domains 1 to 3, whereas domain 4 is formed contiguously from its C-terminal part.

Figure 1.3 – Homology Model of Pneumolysin



This ribbon picture of the homology model of pneumolysin was created using RasMol and is based on the crystal structure of Perfringolysin O (Rossjohn *et al.* 1997). The four domains of pneumolysin are shown. The N- and C-terminal are marked by ***N*** and ***C*** respectively.

1.5 Pneumococcal Vaccines

Whole cell preparations were used as early vaccines (Wright *et al.* 1914), but after the introduction of antibiotics, research into vaccine development declined. In 1983 a commercial 23-valent polysaccharide vaccine was introduced (Pneumovax II; Pnu-Immune 23) that protects against almost 90% of infections caused by *S. pneumoniae* in the USA. The vaccine covers the 23 most common disease-causing serotypes; therefore protection is serotype specific.

Protection by the capsular polysaccharide vaccine depends on a T-cell independent antibody response. This type of immune response is very poor in children younger than two. The vaccine does not provoke a good response in other groups of people at high risk from pneumococcal disease such as alcoholics and the elderly. It is also ineffective in people who are HIV-positive unless given early (Feikin *et al.* 2001). Coupling the polysaccharide to a protein could promote switching of a thymus-independent to thymus-dependent response, making a vaccine more effective in these people.

1.5.1 Conjugate vaccines

A heptavalent conjugate pneumococcal vaccine (Prevnar, Wyeth Lederle) consists of seven serotypes of capsular polysaccharide conjugated to a carrier protein, the non-toxic diphtheria-toxin analogue CRM197. US trials found this vaccine to be highly effective in preventing invasive disease and pneumonia and otitis media in young children (Black *et al.* 2001). HIV-positive patients receiving this conjugate vaccine had higher antibody concentrations and opsonophagocytic titres after a second vaccine dose (of either conjugate or polysaccharide vaccine) than a placebo group (Feikin *et al.* 2001). These results offer hope that the vaccine could be effective in the potential patients that need it most.

A study assessing the serotypes of five hundred clinical isolates predicted that the seven-valent conjugate vaccine could potentially protect against 84% of acute otitis media (AOM) cases in the US (Joloba *et al.* 2001). However a study in Finland showed this vaccine to be effective in reducing only 6% of pneumococcal AOM and to actually increase AOM caused by serotypes not present in the vaccine (Eskola *et al.* 2001). Nevertheless, as AOM is so common (seven million cases in the US per annum) a 6% reduction in disease could benefit many children.

Potential coverage by nine- and eleven-valent vaccines could substantially decrease invasive pneumococcal disease in southern Israel (Fraser *et al.* 2001). A trial using PncCRM9 had a major impact on morbidity and antibiotic use in children between 12-35 months attending day care centres in Israel (Dagan *et al.* 2001b). This group also found that reduction of respiratory problems, including those not traditionally considered of pneumococcal origin and the ensuing lowered antibiotic use in day-care centre attendees by pneumococcal conjugate vaccination suggest a broader benefit from the vaccine than only preventing invasive disease (Dagan *et al.* 2001a). Trials using an eleven-valent pneumococcal conjugate vaccine (Aventis Pasteur) are currently underway.

In analogy with the Hib (*Haemophilus influenzae B*) vaccines, pneumococcal conjugate vaccines have been shown not only to protect against invasive disease, but also to suppress nasopharyngeal carriage of the pathogen (Obaro, 2002). Therefore, these vaccines could prevent non-invasive pneumococcal disease and reduce bacterial transmission in the community. Such a herd effect would add considerable value to the conjugate vaccines.

One of the biggest fears concerning the pneumococcal conjugate vaccine is that the frequency of disease will remain the same as a result of increases in infections with the pneumococcal serotypes not included in the vaccine. This phenomenon is known as serotype replacement. A similar situation could arise if another species of bacteria filled the niche currently occupied by *S. pneumoniae* as a commensal organism because a vaccine would also prevent colonisation of the nasopharynx by vaccine serotypes. Data from the Finnish trial (Eskola *et al.* 2001) suggested that the vaccinated group had more episodes of AOM (not necessarily caused by the pneumococcus) than the control group. However after several years follow up, no indication of serotype replacement has been observed in invasive disease among the participants of a Californian trial (Black *et al.* 2000).

The phenomenon of serotype replacement could potentially be overcome if a protective protein antigen in the vaccine was common to all serotypes of the pneumococcus. Three pneumococcal proteins are potential candidates for a vaccine; they are pneumococcal surface adhesin (Psa) A, pneumococcal surface protein (Psp) A and a pneumolysin toxoid. A vaccine combining these proteins could provide better protection than immunisation with any of the antigens alone (Briles *et al.* 2000a; Ogunniyi *et al.* 2001) probably because the different proteins have roles in different stages and types of pneumococcal disease. IgA antibodies to

all three proteins are naturally produced and levels increase with age and exposure to pneumococci (Simell *et al.* 2001). Anti-pneumolysin IgG levels have been linked with protection against invasive disease by pneumococci (Musher *et al.* 2001). High concentrations of antibodies to PsaA were associated with lower risk of the pneumococci carriage progression to pneumococcal AOM in children aged 9-24 months (Rapola *et al.* 2001b). These are all thymus-dependent antigens, so would induce antibody responses and immunological memory, even in young children. Indeed infants have been shown to be capable of producing a specific antibody response to PsaA and pneumolysin during AOM (Rapola *et al.* 2001a). Currently, the serotype selection in conjugate vaccines is more restricted than in polysaccharide vaccines. Conjugate vaccines are also more expensive to produce, restricting their use in poorer countries.

It is important to consider the (often controversial) consequences of widespread use of a new type of vaccine. Novak *et al.* (1998) raised the possibility that the loss of function of PsaA by vaccine-induced antibodies may promote penicillin tolerance.

1.6 Thiol-activated Toxins

In 1972, Mayer proposed that the C5-C9 complement proteins assembled into pore-forming protein complexes in lipid bilayers. With approaches that had been used in complement work, *Staphylococcus aureus* α-toxin became the first bacterial toxin recognised to form pores in the membranes of mammalian cells (Fussle *et al.* 1981). This was quickly followed by the discovery of several other pore-forming toxins such as aerolysin (Howard and Buckley 1982) and streptolysin-O (Bhakdi *et al.* 1985). Now it is thought that the majority of pathogenic bacteria produce pore-forming toxins. Unlike those from Gram-negative bacteria, pore-forming toxins from Gram-positive bacteria are generally produced and secreted as water-soluble, single chain monomers and do not require further processing to become active.

Within the large group of pore-forming toxins is a family of thiol-activated toxins (TATs), named because they lose activity on oxidation but regain activity following addition of reducing agents. Gram-positive bacteria mainly of the genera *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus* produce TATs. Currently, 23 have been identified (Palmer 2001), some examples are shown in Table 1.2. They share many properties including similar molecular weight, immunological cross-reaction and reversible loss of activity on oxidation and

reduction. These toxins show significant amino acid sequence homology, which may account for their similarity. A cysteine in a tryptophan (Trp)-rich conserved sequence, in all but two of the toxins, (ECTGLAWEWWR) near the C-terminus provides the thiol group that is required for the activity of many TATs.

Table 1.2 – Examples of Thiol-Activated Toxins

BACTERIA	THIOL-ACTIVATED TOXIN
Bacillus cereus	Cereolysin-O
Clostridium botulinum	Botulinolysin
Clostridium perfringens	Perfringolysin-O
Clostridium tetani	Tetanolysin
Listeria monocytogenes	Listeriolysin-O
Streptococcus intermedius	Intermedilysin
Streptococcus pneumoniae	Pneumolysin
Streptococcus pyogenes	Streptolysin-O

These toxins are thought to cause damage by interfering with the eukaryotic cell membranes. Most toxins probably interact with specific membrane acceptor molecules but only a small number have been definitively identified. It is thought that the binding site for pneumolysin, perfringolysin-O and streptolysin-O is cholesterol (Smyth and Duncan 1978; Ohno-Iwashita *et al.* 1988), which appears to be essential for the cytolytic activity of pneumolysin, but definite proof that this is the membrane receptor is lacking. Cholesterol could interact with the sulfhydryl group in the toxin and position the polypeptide into the membrane so the hydrophilic domains can adopt a transmembrane orientation resulting in channel formation (De Kruiff 1990). Once the toxin has bound to the membrane it is thought to oligomerise to form transmembrane pores that lead to cell lysis (Duncan and Schlegel 1975). The precise mechanism of cell binding is not understood. Pneumolysin can bind to cholesterol in solution but inaccessibility of cholesterol in cell membranes (Yeagle 1987) may prevent this happening. Alternatively, binding of toxin to cells may involve a receptor other than cholesterol and interaction with cholesterol may aid functional oligomer formation (Jacobs *et al.* 1998).

The biological effects of pore-forming toxins will be influenced by: the susceptibility of the targeted cell, the number of high affinity binding sites, non-specific cell surface

characteristics such as organisation of charged molecules, its accessibility and the cellular reactions occurring within this cell. Two mechanisms have been identified which confer resistance to cells; cells may be able to prevent insertion of the pore-forming toxin domain (Valeva *et al.* 1997) or lesions may be repaired (Walev *et al.* 1994).

1.7 Cell Binding, Oligomerisation and Pore Formation of Thiol Activated Toxins

It is thought that pneumolysin and the other TATs cause cell lysis via similar mechanisms. Work on streptolysin-O suggests that the mode of interaction with cell membranes involves two steps. Firstly, interaction with the receptor in the target-cell membrane results in insertion of the toxin into the lipid bilayer. Then lateral diffusion and oligomerisation of toxin monomers results in the formation of arc and ring structures (at sufficiently high toxin concentrations) visible with electron microscopy. These are thought to be the transmembrane pores (Palmer *et al.* 1993). Probably only a small number of pores, maybe even one, is sufficient to induce lysis of erythrocytes (Bhakdi *et al.* 1984).

In common with most pore-forming toxins, the four different domains of pneumolysin (Figure 1.3) have been linked to different activities. Domains 1, 3 and 4 have been implicated in oligomerisation (De Los Toyos *et al.* 1996). Removal of the N-terminus (in domain 1) of perfringolysin-O produces a molecule that can bind, but not permeabilise membranes (Iwamoto *et al.* 1990). The second domain seems to act as a linker molecule. Domain 3 has been observed at a range of angles in relation to the other three domains. The fourth domain is expected to contain the site involved in cell binding, as mutations affecting binding to membranes occur in this domain. Proteolytic fragments of perfringolysin-O and pneumolysin that consist mainly of the fourth domain can bind to cholesterol and cholesterol-containing membranes (Nakamura *et al.* 1994). Truncation of the C-terminal end (in domain 4) of pneumolysin by 21 residues abolished cell binding and therefore haemolytic activity. C-terminal deletions of 6 and 11 residues decreased the binding activity of pneumolysin by 98% (Owen *et al.* 1994). The precise orientation of the monomer during cell binding, with respect to the membrane is still unclear.

Baba *et al.* (2001) made truncated versions of pneumolysin. A molecule comprising of domain four retained the ability to bind to the cell membrane and to cholesterol, whereas domains one to three had no binding activity. At high concentrations, domain four could block the binding and subsequent haemolytic activity of the full-length toxin. A domain four

fragment of streptolysin-O was also found to bind membranes but did not form oligomers or cause cell lysis (Weis and Palmer 2001). Again, at high concentrations, this fragment was found to decrease haemolytic activity and interfere with oligomerisation of the wild type toxin. These results provide evidence that domain four is essential for binding to membrane cholesterol and that this interaction leads to subsequent membrane damage by thiol-activated toxins.

Cell binding and oligomerisation seem to be independent events. Binding of pneumolysin to cell membranes is not dependent on temperature, whereas oligomerisation is. Some monoclonal antibodies block oligomerisation but have no effect on cell binding (De Los Toyos *et al.* 1996). Analytical ultracentrifugation techniques have shown that pneumolysin dimers can form at low concentrations and oligomerisation is concentration dependent regardless of the presence of cell membranes (Gilbert *et al.* 1998).

De Los Toyos *et al.* (1996) raised monoclonal antibodies (mAbs) to pneumolysin to identify regions of the pneumolysin sequence involved in the lytic mechanism. On the basis of cross-inhibition ELISAs they divided the antibodies into five groups and used one member from each group for further study (Table 1.3). The toxin was proteolytically nicked to produce a 45-kDa N-terminal fragment of the first 142 amino acids and a C-terminal 37-kDa fragment. All the mAbs except PLY4 recognised the C-terminal fragment. mAb PLY4 recognised only the whole toxin. From this study, the authors proposed there were three epitopes that elicited antibodies capable of neutralising the lytic action of pneumolysin. Two of these epitopes stop cell binding by binding to the C-terminal end of the toxin. A third site not involved in cell binding at residues 142-143 was involved in oligomerisation.

Oligomers formed by TATs dissociate in SDS but are stable in non-denaturing detergents such as deoxycholate. They can be detected by gel-exclusion chromatography, sucrose density centrifugation, or by native gel electrophoresis in the presence of detergents. Appropriate detergents such as sodium deoxycholate can induce oligomerisation. This was first shown for *S. aureus* α-toxin in 1981 (Bhakdi *et al.* 1981). Pore formation can be detected by haemolysis assays or by measuring efflux and influx of markers such as K^+ and ATP.

Table 1.3 – Activities of Monoclonal Antibodies raised against Pneumolysin

ANTIBODY	REGION OF PLY RECOGNISED	NEUTRALISING ABILITY	ACTIVITY
PLY4	Amino acids 142 or 143	<0.5	Prevented pore formation in liposomes. Highly neutralising
PLY5	Extreme C-terminal end	1	Inhibits erythrocyte binding. Highly neutralising
PLY7	Around residue 419	31.2	Inhibits erythrocyte binding.
PLY8	Six C-terminal amino acids	-	Non-neutralising
PLY9	Epitope exposed during cell binding	-	Non-neutralising

Data taken from De Los Toyos *et al.* (1996). Neutralising ability = nanograms of antibody required to neutralise 1 haemolytic unit (HU) of pneumolysin activity.

Using the crystal structure of perfringolysin-O, a hypothesis for cholesterol interaction with the Trp-rich loop found in the conserved sequence of thiol-activated toxins was proposed (Rossjohn *et al.* 1997). The orientation and location of the tryptophan at residue 464 (Trp464) is suggestive of a potential binding site for cholesterol if the Trp-rich loop is displaced. The sterol rings would fit convincingly against the aliphatic portions of the long surface side chains. Binding here could trigger membrane insertion because the extended loop is predominantly hydrophobic and together with the protruding aliphatic side chain of the cholesterol molecule, could act as a dagger for membrane penetration. The same mechanism was proposed for membrane insertion by pneumolysin (Rossjohn *et al.* 1998). Experiments have demonstrated that cholesterol binding induces and triggers conformational changes in the environment of some tryptophan residues without a change in overall secondary structure (Nakamura *et al.* 1995). Therefore the Trp-rich motif is close to, but not directly involved in cholesterol binding, and maintenance of hydrophobicity of the loop is critical for lysis.

The majority of pore-forming toxins self-associate in membranes to form non-covalently bound oligomers. Membrane binding may serve to orientate molecules uniformly, so interacting surfaces contact during collision (Bhakdi *et al.* 1997). Pore formation leads to irreversible anchorage in the lipid bilayer (Bhakdi and Tranum-Jensen 1987). In order to insert into membranes the pores must be amphiphilic, possess a lipid-binding surface and have a hydrophilic face that lines the aqueous channel. The predicted primary amino acid

sequence shows no major regions of hydrophobicity so the observed hydrophobic nature of thiol-activated toxins (Johnson *et al.* 1982) probably reflects the generation of an amphiphilic secondary structure.

Kinetic studies on streptolysin-O (Palmer *et al.* 1995) led to a model in which the following events occur: 1) Spontaneous association of two membrane-bound monomers. This stage displays second order kinetics and is the rate-limiting step, 2) Addition of monomers to the currently growing oligomers which gives rise to the arc shape structures seen by electron microscopy and finally, 3) Completion of the oligomer. It takes less time to complete a just initiated oligomer than for a given monomer to find a partner for dimer formation. This reinforces the idea that the second order kinetics observed at low concentrations reflects the initial association of two monomers.

Oligomerisation leads to the formation of rings and arcs of about 30-40 nm when inserted into cell membranes. These structures have been reported for many of the thiol-activated toxins and were shown for pneumolysin by Morgan *et al.* (1994b). Electron microscopy techniques enabled detailed analysis of the oligomer structures (Morgan *et al.* 1994a). The oligomer is made up of approximately 40 subunits and is thought to be the pore-forming structure that leads to cell lysis.

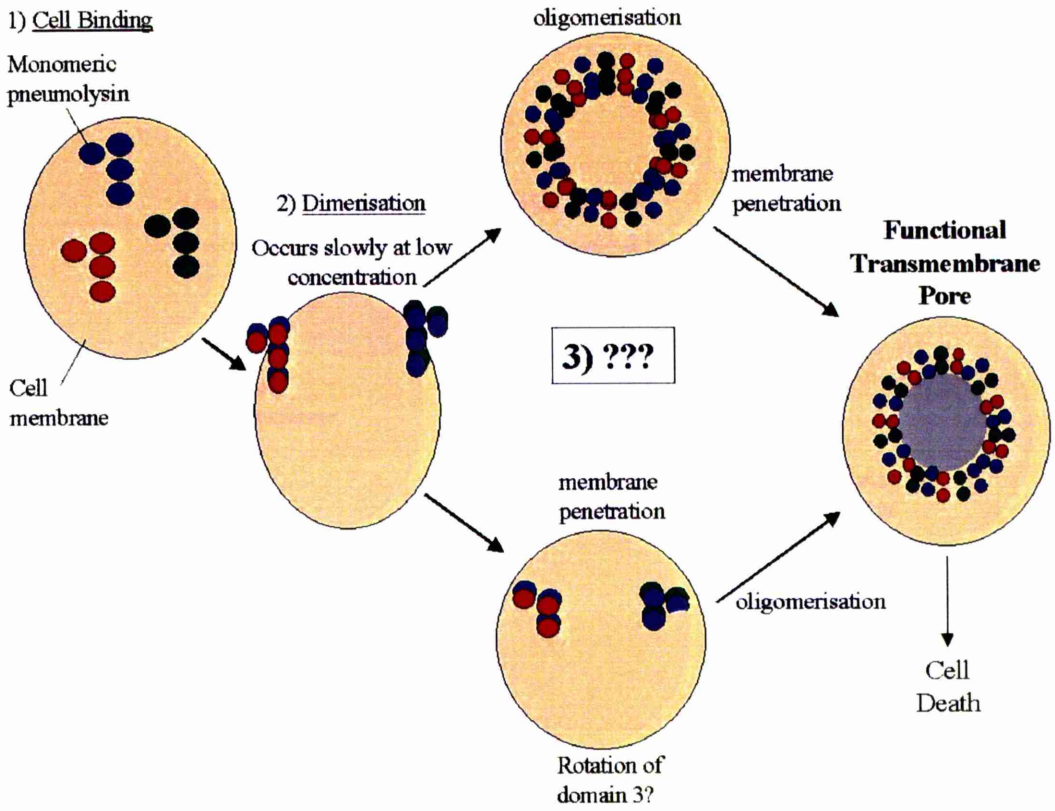
Examination of perfringolysin-O oligomers suggested that the ring structure consisted of two domains. A compact inner domain formed close contacts with the next monomer and an elongated outer domain extended outward from the inner ring with less contact between neighbouring monomers. This outer ring was proposed to be composed of the third domain of the monomer. Side-on, the oligomer appeared to be mushroom-shaped with the bottom half of the mushroom stalk spanning the membrane bilayer. In order for the width of the ring to match with electron microscopy data, a rotation of domain 3 needs to occur (Rossjohn *et al.* 1997). Scanning mutagenesis and fluorescence emission experiments suggest that part of domain 3 also enters the bilayer (Palmer *et al.* 1996). In the De Los Toyos study, monoclonal antibodies to an epitope in domain 3 inhibited pneumolysin oligomerisation. Their group hypothesises that the antibody recognised residues 142 and/or 143 in the protein and that these could be involved in oligomerisation.

Gilbert *et al.* (1999) determined a three-dimensional structure of membrane-bound pneumolysin pores by cryo-electron microscopy. Fitting the four domains from the crystal structure of perfringolysin-O revealed major domain rotations during pore assembly. This publication hypothesised that oligomerisation results in expulsion of domain three from its original position in the monomer. However during membrane penetration the third domain re-associates with the other domains. In contrast to previous studies, this model of pore formation places domain three in the internal channel of the pore.

The mechanism of how this molecule inserts into cell membranes is not understood (Figure 1.4). It is not known if oligomerisation occurs prior to membrane penetration or if membrane penetration is an early event. Membrane leakage can be induced by small oligomers (Korchev *et al.* 1992) suggesting membrane penetration is an early event although membrane penetration may not be necessary to cause membrane leakage or even cell lysis. An extracellular toxin secreted by *E. coli*, α-haemolysin, which binds and lyses eukaryotic cells, was thought to be a pore-forming toxin (Bhakdi and Tranum-Jensen 1986; Bhakdi *et al.* 1996). However, theoretical and experimental studies by Soloaga *et al.* (1999) presented α-haemolysin as an integral protein, interacting with only the outer phospholipid monolayer. The insertion of one or more molecules into the outer layer could induce transient breakdown of the monolayer and subsequent leakage of contents.

The fate of membrane constituents during pore-formation has not been clarified. Membrane damage occurs via physical perturbation of the bilayer integrity evoked by the formation of transmembrane pores (Bhakdi and Tranum-Jensen 1987, 1988). One model suggests that the hydrophilic surfaces of the inserted amino acids laterally repel membrane constituents (Valet and Opferkuch 1975). Cell death is the most obvious outcome of transmembrane pore formation if a lesion cannot be removed or repaired. Death could occur due to: rapid ATP loss caused by efflux of nucleotides through the pores (Bhakdi *et al.* 1989), an influx of water through the pores, the cell being unable to cope with the ionic disequilibrium or through apoptosis. Pneumolysin-induced cytotoxicity appeared to be closely associated with accelerated phospholipid hydrolysis and release of arachadonic acid (Rubins *et al.* 1994).

Figure 1.4 – A Model of Pore Formation by Pneumolysin



This figure shows the possible steps leading to pore-formation in eukaryotic cells. There are many mechanisms which are not understood such as those indicated at step 3. Oligomerisation may be a prerequisite to membrane penetration or this could occur the other way round. Although this figure shows cell binding as the first step, there is evidence that dimer and oligomer formation can occur in solution (Gilbert *et al.* 1998). Also one model of pore formation places the third domain of pneumolysin at the internal surface of the pore (Gilbert *et al.* 1999).

1.8 Studies on Pneumolysin Mutants

Much work has been carried out studying the effects of mutations within the pneumolysin protein. The main reason for many of these studies has been to try and find a form of pneumolysin that could potentially be used in a vaccine without having toxic side effects. The current polysaccharide vaccine is not effective in children or the elderly and the new conjugate vaccines still have limited serotype coverage. One way of improving the polysaccharide vaccine would be to include appropriate protein antigens from the pneumococcus. Ideally these would be stable proteins present in all serotypes. However, it is also important to consider other effects that pneumolysin has on the immune system as mentioned in sections 1.9 and 1.10.

The only cysteine in the pneumolysin sequence is situated near the C-terminus at residue 428. This residue was thought to be important, as it is part of the conserved region, found in thiol-activated toxins. This cysteine is important functionally in several of the other TATs. Substitution of the cysteine residue in pneumolysin with alanine had no effect but substitution for serine or glycine reduced cytolytic activity (Saunders *et al.* 1989). Derivatisation of the cysteine residue with a benzyl group inhibited cell binding (Iwamoto *et al.* 1987) and self-association of the toxin (Gilbert *et al.* 1998). A proline at position 463 in pneumolysin is also conserved in many thiol-activated toxins and changing this to a serine reduced cell binding by 90% and haemolytic activity by 27% (Owen *et al.* 1994).

All three of the tryptophan residues in the conserved region seem to contribute to haemolytic activity. Mutation of the tryptophan at position 433 has the greatest effect. Changing this residue to a phenylalanine (W433F) affects channel formation at a point subsequent to membrane binding and oligomer formation (Korchev *et al.* 1998) causing more than a 99% loss in cytotoxicity. This mutant shows a difference in cation sensitivity compared to the wild type toxin. It is thought that W433F forms larger pores that are less sensitive to bivalent cations (Korchev *et al.* 1992). Although differences in pore size could not be seen by electron microscopy, there may be differences in the internal structure of the channel (Korchev *et al.* 1998). The lack of cytotoxicity of this W433F mutant has been exploited as a possible vaccine candidate. When given to mice this pneumolysin toxoid conferred non-serotype-specific protection against *S. pneumoniae* (Mitchell *et al.* 1992).

Tryptophan residues are rigid, hydrophobic and polarisable and could potentially form a stable complex with the rigid ring system of cholesterol. This idea has been proposed for a unique cluster of four tryptophans in the pore-forming antibiotic gramicidin A (De Kruiff 1990). Modification of the cysteine residue would disturb the tight packing of cysteine leading to conformational changes of the Trp-rich loop; these changes could inhibit the triggering of the loop required for cholesterol binding and membrane insertion (Rossjohn *et al.* 1997). This would explain why chemical modification of cysteine causes toxin inactivation (Alouf and Freer 1999) but why this residue is not essential for toxin function (Saunders *et al.* 1989).

Pneumolysin contains eight histidine residues. These seem to be important, as treatment of pneumolysin with the histidine-modifying reagent diethyl pyrocarbonate resulted in loss of

activity (Mitchell *et al.* 1994). The histidine at residue 367 (within the Trp-rich motif) in pneumolysin appears to be essential for oligomer formation (Mitchell *et al.* 1992). The histidine residue at position 156 in domain 3 is thought to interact with the cell membrane during pore formation. Mutation of this residue results in severely reduced toxin activity (Hill *et al.* 1994). The region surrounding this histidine has a helical structure in the monomeric toxin but undergoes a transition to an amphipathic β-sheet on membrane insertion that may form the basis of a pore (Shepard *et al.* 1998).

Alexander *et al.* (1998) looked at the contribution of both cytotoxic and complement activating properties in the pathogenesis of pneumonia by inoculating mice with *S. pneumoniae* containing amino acid changes in the pneumolysin protein. After intranasal inoculation, mutations affecting haemolytic activity led to an increased survival time but did not improve the percentage of animals that survived. When complement activity was abolished, survival time was increased and 30% of the mice survived. When both activities were abolished the survival time was further increased and 30% of the mice survived. After intra-peritoneal injection, only changes affecting haemolytic activity altered virulence. It was concluded that during bronchopneumonia, complement activity is important during the first 24 hours. During lobar pneumonia, haemolytic activity is important in the first six hours after infection and the complement-activating activity becomes important after this time.

Different pneumococcal mutants containing various alterations to the pneumolysin gene to abolish cytotoxic and/or complement activating ability were still more virulent than a D39 derivative in which the whole pneumolysin gene was disrupted. This suggests that there is another property of pneumolysin, which acts as a virulence factor but which is distinct from the cytotoxic and complement activating activity. This property contributes significantly to the pathogenesis of disease (Berry *et al.* 1999). A mutant in which both haemolytic and complement activating properties were abolished, can still bind to the Fc fragment of IgG, although to a lesser extent than wild type pneumolysin. This binding may help to block establishment of a non-specific protective inflammatory response independent of classical complement pathway activation.

1.9 Effect of Pneumolysin on Host Cells and Tissues

During pneumococcal infection, antibiotic treatment may cause a dramatic, temporary increase in pneumolysin levels due to bacterial lysis. Pneumolysin is able to injure a range of

cells (Table 1.4) including bronchial epithelial cells (Steinfort *et al.* 1989), alveolar epithelial cells (Rubins *et al.* 1993), and pulmonary arterial endothelial cells (Rubins *et al.* 1992). Ring and arc structures have been seen by electron microscopy in these cells suggesting a similar mechanism to haemolysis is involved. These cytotoxic interactions may account for the histopathology seen during early pneumococcal pneumonia such as alveolar flooding and haemorrhage (Wood 1941).

At sublytic concentrations, pneumolysin has been shown to cause inhibition of neutrophil respiratory burst (Paton and Ferrante 1983), inhibition of antibody synthesis by B-cells (Ferrante *et al.* 1984) and release of pro-inflammatory mediators such as nitric oxide (Braun *et al.* 1999a), TNF-α and IL-1 (Houldsworth *et al.* 1994). Regions in the toxin that are important for cell lysis are also involved in these sublytic activities (Saunders *et al.* 1989). However a recent study has presented data to suggest that IFNγ production is induced by a separate mechanism to pore formation (Baba *et al.* 2002).

Pneumolysin is a potent activator of phospholipase A in pulmonary artery endothelial cells (Rubins *et al.* 1994). This activation requires the toxin to form functional trans-membrane pores. Activation of phospholipase A during an infection could contribute to tissue injury and promote the inflammatory response. Direct toxicity could result from the release of free fatty acids and lysophosphatides. Arachidonic acid released by phospholipase A can cause chemotaxis and respiratory burst in neutrophils (Badwey *et al.* 1984; Curnutte *et al.* 1984). This activation could underlie a connection between the lytic activity of the pneumolysin and its ability to cause inflammation in models of pneumonitis (Feldman *et al.* 1991).

Calcium-dependent cytoskeletal dysfunction has been observed in endothelial cells under attack by pore-forming toxins. Cellular contraction leads to the formation of intercellular gaps that permit leakage of macromolecules across confluent monolayers. This could be a significant event in the development of pulmonary oedema during severe pneumonia (Suttorp *et al.* 1988; Suttorp *et al.* 1990). Separation of tight junctions also promotes adhesion and bacterial invasion of mucosa (Dowling and Wilson 1998).

Cilia protect the brain and respiratory tract by forming a physical barrier and by mucociliary clearance. These cilia may protect the neuronal tissue from damage during infection by allowing continual movement of CSF, and by preventing margination of bacteria during

meningitis. This requires the cilium to beat in a co-ordinated fashion. Pneumolysin is entirely responsible for the ability of pneumococcal culture filtrates to slow ciliary beating and disrupt the surface integrity of human respiratory epithelium in organ culture (Steinfort *et al.* 1989; Feldman *et al.* 1990). This occurs due to the direct effect of the toxin and also indirectly via inflammation. Delayed clearance from the respiratory tract would be advantageous to bacteria by giving them time to multiply, produce virulence factors and establish infection (Mitchell and Andrew 1997). Brain cilia are more sensitive to pneumolysin than respiratory cilia (Mohammed *et al.* 1997).

A common complication of pneumococcal meningitis is sensorineural hearing loss. When pneumolysin was perfused through the scala tympani of the guinea pig, widespread electrophysical and histological damage resulted (Comis *et al.* 1993). Use of D39 and PLN-A *S. pneumoniae* showed pneumolysin to be the principal cause of cochlear damage in an experimental pneumococcal meningitis model of meningogenic deafness (Winter *et al.* 1997). When the toxin was given by intracorneal injection (Johnson and Allen 1975) it induced pathology similar to that observed in natural infections with the organism. This pathology was reduced if the rabbits were made leukopenic before injection suggesting leukocytes could be a source of cornea damaging enzymes such as collagenase (Harrison *et al.* 1993).

Pneumolysin has effects on host tissue that could compromise non-specific host defence mechanisms. Tissue necrosis generates niches for bacterial survival and multiplication

1.10 Effect of Pneumolysin on Immune Cells

A direct effect of pneumolysin on immune cells is to kill phagocytes and lymphocytes by pore-formation. This aids microbial persistence and invasion. Pneumolysin also has many other effects on cells as are mentioned in Table 1.4 and will be described below. The exact mechanisms involved in these events are not known. The toxin probably has to bind to the cell but whether oligomerisation, membrane penetration and what kind of cell signalling is occurring are unknown.

Other ways in which pneumolysin can abrogate the immune response have been described. Very low doses (approximately 1ng/ml) of highly purified pneumolysin significantly inhibited the respiratory burst of human neutrophils (Paton and Ferrante 1983). This was

associated with a reduced capacity to engulf and kill opsonised pneumococci. In addition, both chemotaxis and random migration of neutrophils was inhibited (Paton and Ferrante 1983). Similar inhibitory effects of pneumolysin on the respiratory burst, degranulation, bactericidal activity, and phospholipid methylation of human monocytes have also been reported (Nandoskar *et al.* 1986). Mice infected with sublethal doses of *Listeria monocytogenes* showed listeriolysin-O-dependent inhibition of both T-cell-dependent and T-cell-independent immune responses. (Hage-Chahine *et al.* 1992). Pre-treatment of human lymphocytes with low (1ng/ml) doses of pneumolysin abrogated lymphoproliferative responses to various mitogens, as well as the capacity to produce lymphokines and antibodies (Ferrante *et al.* 1984). This activity of pneumolysin could interfere with events in the development of resistance to *S. pneumoniae,* which is dependent on the acquisition of type-specific antibodies to capsular polysaccharide.

Several studies have also reported activities of TATs that increase the inflammatory response. This would enhance the host's ability to fight infection but may also damage host tissues and provide a favourable environment for bacterial multiplication. Increased cytokine and eicosanoid levels can lead to septic shock, so a balance needs to be reached.

Pore-forming toxins have been shown to induce rapid and massive shedding of CD14 and IL-6 receptor. The cleaved soluble IL-6 receptor was biologically active and was able to signal to bystander cells (Mackiewicz *et al.* 1992; Bazil 1995; Walev *et al.* 1996). This could contribute to a systemic inflammatory response during infection, which is important in host resistance and survival (Benton *et al.* 1998). In the presence of calcium, staphylococcal alpha-toxin leads to factor V(a) secretion by forming pores in platelets (Bhakdi *et al.* 1988). This mediates assembly of the prothrombinase complex on the platelet surface, which converts prothrombin to thrombin (Arvand *et al.* 1990) promoting coagulation and the acute phase response.

Low concentrations of pneumolysin caused an influx of Ca^{2+}, increased phospholipase A2 activity and complement receptor 3 expression in neutrophils. Pneumolysin sensitised these cells to cause enhanced superoxide production and release of elastase after activation. Pneumolysin also caused an efflux of K^+ and membrane depolarisation (Cockeran *et al.* 2001). The authors proposed that contrasting results found in earlier studies described above,

may be due to minor variations in the experiments such as the Ca^{2+} concentration of the media and the stimulus used to activate the cells.

Table 1.4 – Effects of Pneumolysin on different Cell Types

CELL TYPE	EFFECT OF PNEUMOLYSIN	AMOUNT USED	REFERENCE
Lymphocytes (human)	Decrease in mitogen-induced response	1-5HU/10^6 cells	Ferrante *et al.* 1984
Macrophages (mouse)	Increased nitric oxide production	2.6-20μg/5 x 10^5 cells	Braun *et al.* 1999a
Monocytes (human)	Decrease in oxygen-dependent respiratory burst	0.5-2.5HU/10^6 cells	Nandoskar *et al.* 1986
Monocytes (human)	Production of TNF and IL-1β	3pg-1ng/5 x 10^5 cells	Houldsworth *et al.* 1994
Neutrophils (human)	Decreased respiratory burst	<1HU/10^6 cells	Paton and Ferrante 1983
Neutrophils (human)	Ca^{2+} influx, K^+ efflux, increased phospholipase A2 activity, increased CR3 expression, increased superoxide production	0.02-42ng/10^7 cells	Cockeran *et al.* 2001
Pulmonary Alveolar Epithelial Cells (rat)	Increased alveolar permeability	10-1000HU/5 x 10^5 cells	Rubins *et al.* 1993
Pulmonary artery Endothelial Cells (bovine)	Ca^{2+}-dependent activation of phospholipase A	1μg/10^6 cells	Rubins *et al.* 1994
Pulmonary artery Endothelial Cells (bovine)	Disruption of cell integrity	Unknown*	Rubins *et al.* 1992
Respiratory Epithelium (human)	Epithelial disruption, slowing of ciliary beat	5ng-7.5μg/epithelial layer	Steinfort *et al.* 1989; Feldman *et al.* 1990
Spleen Cells (mouse)	Increased IFNγ and NO production	25 nM-800 nM (1.3-42μg/ml)	Baba *et al.* 2002

CR3 = Complement receptor 3. *The actual concentration of pneumolysin or numbers of cells used is not stated in this study - bacterial autolysate supernatants were collected from cultures of 10^8 *S. pneumoniae* and cells were used at confluence.

Pneumolysin is also capable of stimulating cells of the immune system *in vitro* to produce inflammatory mediators such as TNF-α and IL-1β (Houldsworth *et al.* 1994) and nitric oxide (Braun *et al.* 1999a). Less than 1ng of pneumolysin caused the same increase in these cytokines as 50ng of LPS. Intranasal inoculation of pneumolysin in mice induced a dose-dependent increase in neutrophils, macrophages, and lymphocytes in the BALF after six hours. Doses of 600 or 1000ng of pneumolysin caused an increase in concentrations of IL-6, and α-chemokines. The use of pneumolysin mutants in this study indicated that the cytolytic activity of the toxin was responsible for these effects (Rijneveld *et al.* 2002b).

The findings of these studies support the involvement of pneumolysin in the pathogenesis of inflammation-mediated tissue damage during pneumococcal infection.

1.10.1 Apoptosis

Apoptosis plays a critical role in tissue homeostasis (Allen *et al.* 1993; Abbas 1996). It may be mediated by death receptors, including Fas (Itoh *et al.* 1991), TNF receptor I and TNF-related apoptosis-inducing receptors (Ashkenazi and Dixit 1998); by caspase activation that is independent of death receptors (Thornberry and Lazebnik 1998), or by mitochondrial factors, such as apoptosis-inducing factor, that are independent of caspase activation (Susin *et al.* 2000). Monocytes and macrophages play an integral role in innate immunity, including the phagocytosis of micro-organisms (Aderem and Underhill 1999). They express pro-apoptotic ligands (Kiener *et al.* 1997) and undergo apoptosis when survival factors are withdrawn (Scheuerer *et al.* 2000).

Modulation of apoptotic cell death plays a role in the pathogenesis of a diverse group of infectious diseases. The mechanisms involved in apoptosis during bacterial infections remain incompletely characterised and vary between pathogens. Apoptosis may be a pathogen-directed mechanism of immune escape or may represent an appropriate host response to infection. Induction of apoptosis in macrophages by *Shigella flexneri* (Zychlinsky *et al.* 1992; Hilbi *et al.* 1998) and *Salmonella typhimurium* (Hersh *et al.* 1999) is associated with increased survival of intracellular bacteria. However, macrophage apoptosis could be an appropriate host immune response to infection with *Mycobacterium tuberculosis* (Rojas *et al.* 1997; Keane *et al.* 2000).

Bacteria use a variety of strategies to induce apoptosis in directly infected cells, and the same bacteria can use different mechanisms in different cell types (Gao and Kwaik 2000). Multiple host cell targets have been implicated in bacteria-associated macrophage apoptosis: activation of caspase 1 (Hilbi *et al.* 1998; Hersh *et al.* 1999) or caspase 3 (Gao and Kwaik 1999), inhibition of NFκB and TNF (Ruckdeschel *et al.* 1998), signalling via toll-like receptors (TLRs) (Aliprantis *et al.* 1999) or Fas (Baran *et al.* 2001) and caspase-independent activation via mitochondrial factors (Ojcius *et al.* 1998).

Apoptosis of a variety of cell types has been associated with streptococcal infections. Group B streptococci induce monocyte apoptosis (Fettucciari *et al.* 2000), *S. pneumoniae* induces neutrophil apoptosis (Zysk *et al.* 2000) and both have been shown to induce neuronal apoptosis (Leib *et al.* 1996; Braun *et al.* 1999b). Group A streptococci can induce apoptosis of lymphocytes and epithelial cells (Watanabe-Ohnishi *et al.* 1995; Tsai *et al.* 1999). Infection with *S. pneumoniae* results in apoptosis of human monocyte-derived macrophages at 20 and 36 hours after inoculation and is associated with internalisation and killing of the bacteria. This apoptosis was mediated at least in part by pneumolysin as a pneumolysin-negative pneumococcal mutant induced less apoptosis in macrophages than the wild type bacteria (Dockrell *et al.* 2001).

The pore-forming activity of pneumolysin may play a direct role in causing apoptosis. Small pores that allow passage of K^+ and Na^+, but restrict passage of Ca^{2+} and larger molecules have been shown to trigger programmed cell death in T-lymphocytes (Jonas *et al.* 1994). Efflux of K^+ from monocytes leads to activation of interleukin converting enzyme (ICE). ICE cleaves IL-1β releasing the mature cytokine and is also involved in the caspase cascade leading to apoptosis (Walev *et al.* 1995).

Pneumolysin-induced apoptosis in macrophages was shown to be Fas independent and mediated by caspases. However Fas was involved in apoptosis induced in bystander (uninfected) macrophages by fixed macrophages previously cultured with opsonised *S. pneumoniae* (Dockrell *et al.* 2001). In brain cells, the pneumococcus induces an apoptosis-inducing factor (AIF)-dependent form of brain cell apoptosis (Braun *et al.* 2001). Loss of both pneumolysin and H_2O_2 were necessary to abolish apoptosis and mitochondrial damage by pneumococci. Furthermore, pneumolysin and/or H_2O_2 could induce apoptosis in these cells *in vitro* (Braun *et al.* 2002). In this study apoptosis was caused by an increase in

intracellular Ca^{2+} and the release of AIF from mitochondria. Chelating calcium blocked AIF release and cell death.

1.11 Immunity to *Streptococcus pneumoniae*

Early studies demonstrated that circulating antibodies to capsular polysaccharide are critical for protection against invasive pneumococcal infection (Macleod *et al.* 1945). Resistance of normal mice to pneumococcal infection actually involves various components of the immune system including the acute phase response, antigen-specific adaptive immune responses, normal phagocyte function and inflammation (Benton *et al.* 1995; Briles *et al.* 1999).

Leukocytes can move into the alveolar space by two mechanisms. The leukocyte adhesion molecules (CD18) of the integrin family are responsible for approximately half the leukocytes (Cabellos *et al.* 1992). Additional leukocytes are recruited through a less well-understood, CD18-independent pathway that is unique to pulmonary inflammation induced by pneumococci (Tuomanen *et al.* 1995).

1.11.1 Macrophages

In lung infections, alveolar macrophages are the first line in defence and are ideally situated between the lung tissue and the airways (Lohmann-Matthes *et al.* 1994). They originate from monocytes in the blood and differentiate within the lung. Interstitial macrophages are located in lung connective tissue and have antigen presenting capacity (Lohmann-Matthes *et al.* 1994). Dendritic cells are specialised antigen presentation cells and are present, in low numbers, in the lung interstitium. Intravascular macrophages are attached to the endothelium of pulmonary capillaries (Dehring and Wismar 1989), they are highly phagocytic and remove bacteria from the blood stream (Warner *et al.* 1987). Alveolar macrophages express a range of surface receptors such as those for Fc, complement and lectin, that enable them to engulf bacteria rapidly (Lohmann-Matthes *et al.* 1994).

Macrophages require two signals for activation. The first signal is IFNγ, which sensitises the macrophage to respond to the second signal. IFNγ can be produced by inflammatory CD4 T-cells and CD8 T-cells. The second signal can be delivered by LPS, TNF, or by surface molecules on the T-cell upon interaction with specific antigen on the macrophage surface. Macrophage activation generates a series of biochemical responses that convert the macrophage into a potent antibacterial effector cell. Their lysosomes fuse more efficiently to

phagosomes, and they make oxygen radicals and NO, which have potent antibacterial activity. Specific surface molecules increase in number, making the macrophage a more effective antigen presenting cell and more sensitive to TNF.

Macrophages are stimulated by microbial components to synthesise cytokines including IL-1, IL-6, IL-8, IL-12, and TNF. Other mediators released by macrophages include NO, leukotrienes and platelet-activating factor (PAF). Activation of the complement cascade leads to the release of the inflammatory mediators C5a, C4a and C3a. These effects combine to form the inflammatory response which is characterised by pain, redness, heat and swelling. The heat and redness are caused by vasodilation which results in slower blood flow through the infected area allowing leukocytes to interact with the endothelium and enter the infected tissues.

1.11.2 Neutrophils

When required, neutrophils react vigorously to chemotactic stimuli and are sequestered rapidly from the blood to the site of infection. These motile, short-lived cells engulf bacteria and utilise both oxygen-independent and dependent anti-microbial mechanisms (Burnett 1997). Reactive oxygen intermediates (ROI) are generated following phagocytosis via the NADPH/cytochrome b electron donor system. The major species produced are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH.), all of which are rapidly microbicidal.

1.11.3 Lymphocytes

Specific immunity to infection is conferred by lymphocytes which carry receptors for, and recognise antigens. B lymphocytes are the precursors to antibody-secreting cells which are important in protection from pneumococcal disease. Lymphocytes within the lungs are found as aggregates below the mucosal layer of the airways (known as bronchus associated lymphoid tissue - BALT), within the air spaces and interstitium and in the intravascular compartment (Pabst 1997). The number of lymphocytes within the lungs of infected animals increases during pneumococcal pneumonia (Bergeron *et al.* 1998; Kadioglu *et al.* 2000). However the roles of these recruited lymphocytes are not fully understood.

1.11.4 Airway Epithelium

The airway epithelium acts as a physical barrier by protecting sensory nerves and smooth muscle from stimulation by inhaled irritants. If damaged, sensory nerves are exposed and reflex bronchoconstriction occurs. The epithelial layer also acts as far more than just a barrier. It can secrete mucus, cytokines, chemokines and epithelium derived relaxing factors such as NO and prostaglandins (Goldie *et al.* 1990). It can transport antibodies, proliferate in response to injury, actively propel mucus via surface cilia and can inhibit selected activities of activated macrophages including expression and/or secretion of several cytokines, growth factors and adhesion molecules (Hunter *et al.* 1985; Castranova *et al.* 1988; Holtzman *et al.* 1988; Shoji *et al.* 1990; Cromwell *et al.* 1992; Devalia *et al.* 1993).

Airway epithelial cells could be important in regulating airway NO. In humans, it is likely that NO production in the lung during inflammation comes from the epithelial cells as well as macrophages. Nitric oxide synthase (NOS) is constitutively expressed in endothelial cells and neurons and inducible (i)NOS can be induced by cytokines. *In vitro,* airway epithelial cells have been shown to produce NO after stimulation with the cytokine mixture TNF, IL-1 and IFNγ in the presence or absence of LPS (Asano *et al.* 1994; Robbins *et al.* 1994) and by respiratory syncytial virus (Kao *et al.* 2001).

1.11.5 Cytokines and Inflammation

One important function of the innate immune response is to recruit more phagocytic cells and effector molecules to the site of infection by the release of cytokines and other inflammatory mediators. Cytokines are low molecular weight molecules involved in regulating the amplitude and duration of an inflammatory response. They are usually transiently and locally produced and are extremely potent, acting at picomolar concentrations. Specific cell surface receptors are normally expressed at a relatively low number and interact with their ligand with high affinity. Cytokines interact in a complex network by inducing each other and transmodulating cell surface receptors. They can have synergistic, additive or antagonistic effects on cell function. Most cytokines have multiple and diverse biological functions.

IFNγ exerts several regulatory activities on the immune system, including activation of phagocytes, stimulation of antigen presentation, orchestration of leukocyte-endothelium interactions and stimulation of the respiratory burst (Boehm *et al.* 1997). It can be cytostatic

for many cells. As mentioned above, macrophages are activated by IFNγ to express increased antimicrobial activity (Nathan *et al.* 1984; Murray *et al.* 1985; Black *et al.* 1987; Jensen *et al.* 1987).

Interleukins are a group of cytokines involved in signalling between cells of the immune system. IL-1 activates vascular endothelium and increases the access of effector cells. It also activates lymphocytes and can cause local tissue destruction. IL-1 acts on macrophages to produce more IL-1 as well as IL-6 and TNF. IL-6 activates lymphocytes and increases antibody production.

Tumour necrosis factor is a cytokine produced by macrophages and T-cells and has multiple functions in the immune response. It is likely to be a key mediator near the top of the cytokine cascade. Amongst other things, TNF activates vascular endothelium and increases its permeability leading to increased access of antibody, complement and cells from the blood, and increased fluid drainage to the lymph nodes. Typical endotoxin effects can be inhibited by the administration of anti-TNF antibodies or provoked by the addition of TNF. Most cells carry TNF receptors and activation by TNF binding can lead to a cascade of responses, such as apoptosis, increased TNF synthesis and production of other cytokines such as IL-1, IL-6 and IL-8. The local effects of TNF induce an inflammatory response that helps to contain infection. Systemic effects though, can be harmful and cause vasodilation and loss of plasma volume owing to increased vascular permeability. In septic shock, TNF triggers disseminated intravascular coagulation leading to the generation of microthrombi and the consumption of clotting proteins, so the ability to clot blood appropriately is lost. This can lead to failure of vital organs such as the kidneys, liver, heart, and lungs. However the ability of TNF to control infection outweighs the possible consequences of its systemic release. IL-1, IL-6 and TNF-α are produced in response to similar stimuli and help coordinate the body's response to infection. They raise body temperature causing fever, which is believed to help eliminate infections. They activate hepatocytes to synthesise acute phase proteins and enhance recruitment of neutrophils from the bone marrow. They also help to initiate the adaptive immune response.

Chemokines are small cytokines released at infectious sites by several cell types including macrophages and endothelial cells. They play a central part in the inflammatory response and act mainly as chemoattractants, recruiting phagocytic cells from the blood to sites of

infection. The α- or CXC chemokines generally activate neutrophil functions and do not activate phagocytes. The β- or CC chemokines tend to affect monocytes and macrophages but not neutrophils. The α-chemokine, IL-8 (similar to MIP-2 in mice) is a chemotactic factor for neutrophils and leukocytes; it activates binding of leukocytes to vascular endothelium by β_2 integrins and increases access of effector cells.

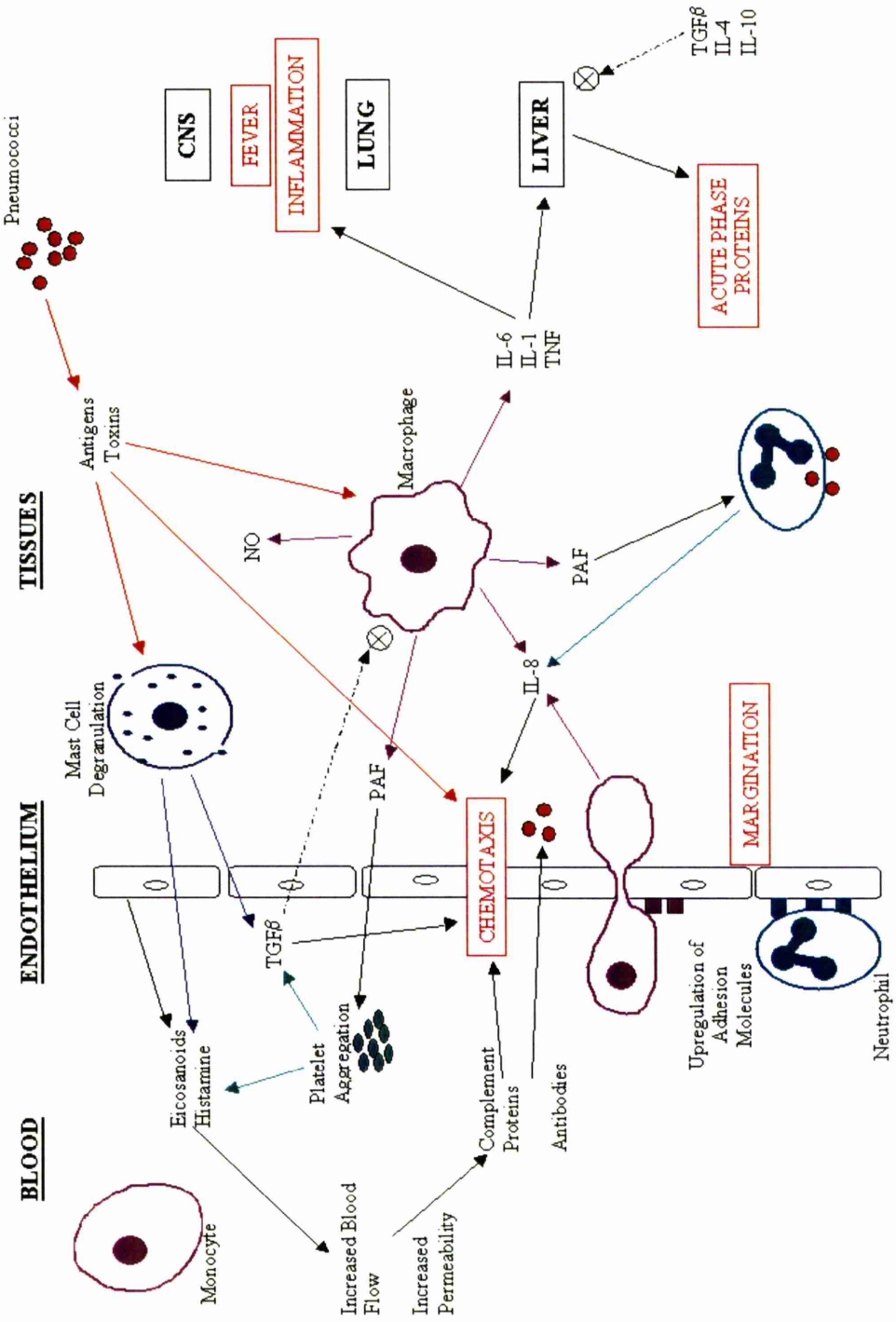
The acute phase response produces molecules that bind bacteria but not host cells. Acute phase proteins are produced by the liver in response to IL-6. Two of these, C-reactive protein (CRP) and mannose-binding protein, bind to the surface of bacteria (and other pathogens) where they act as opsonins and also activate the complement cascade. The complement proteins are also produced as acute phase proteins by the liver.

Eicosanoids are generated from arachidonic acid, which is released from membrane phospholipids by phospholipase A or C. Arachidonic acid may then be metabolised, either by the cyclooxygenase pathway to yield prostaglandins and thromboxanes or via the lipoxygenase pathway to yield the leukotrienes. Eicosanoids have numerous local effects and modulate inflammation by potentiating the effects of other mediators. An overview of some of the inflammatory responses that may be induced during pneumococcal infection is shown in Figure 1.5.

1.11.5.1 Nitric Oxide

Nitric Oxide is a small molecule that is synthesised by NOS (see Figure 1.6). It is an inter- and intra-cellular messenger molecule that plays a role in many physiological processes such as smooth muscle relaxation, vasodilation, platelet aggregation, immunoregulation, signal transduction and microbial stasis or killing. NO is a free radical and its effects are extremely rapid, local and potentially toxic. Since the half-life of NO is very short, of the order of seconds, direct measurement is difficult. NO production is usually assessed indirectly, by measuring the conversion of radiolabelled L-arginine to L-citrulline or by accumulation of nitrite (NO_2^-) or nitrate (NO_3^-) in biological fluids.

Figure 1.5 – The Inflammatory Response

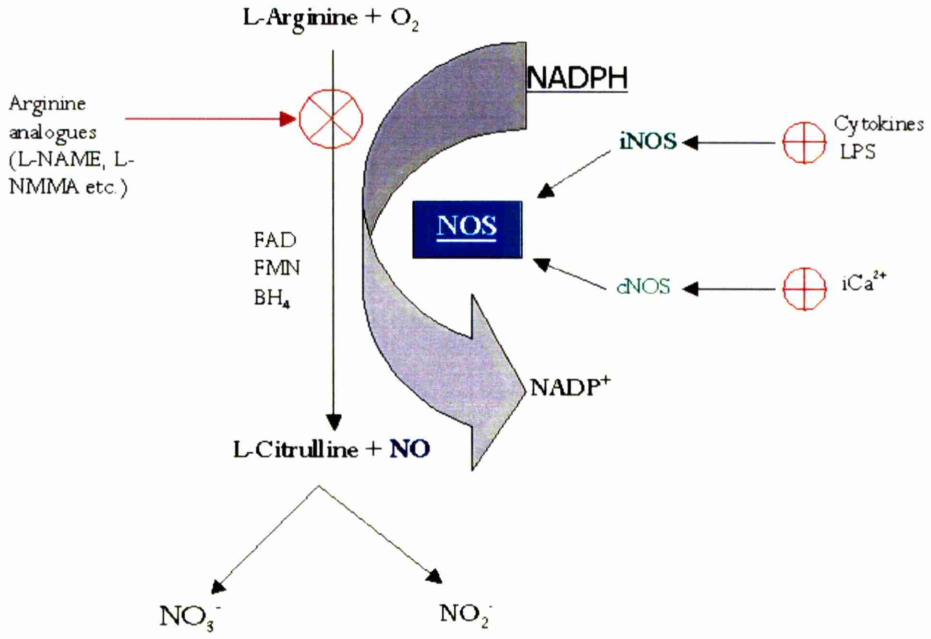


This figure shows some of the events leading to the acute phase response. The immune system can initiate inflammatory reactions either via the complement pathway activated by antibodies or antigens, or by degranulation of mast cells. The cytokines TNF, IL-6 and IL-1β act in synergy to produce a range of pro-inflammatory effects such as the up-regulation of adhesion molecules and macrophage activation. Regulation of these events by anti-inflammatory mediators such as IL-10 is important to reduce host damage. Abbreviations: CNS, central nervous system; TGF, transforming growth factor; PAF, platelet activating factor; IL, interleukin; TNF, tumour necrosis factor; NO, nitric oxide.

The three isoforms of NOS are: neuronal (nNOS), endothelial (ecNOS) and inducible (iNOS). Both ecNOS and nNOS are constitutively expressed in their tissues of origin. These are collectively known as constitutive NOS (cNOS) and produce lower NO levels (picomolar range) than iNOS (nanomolar range). NO has antimicrobial activity on its own but it can also form a number of oxidation products such as NO_2^-, NO_3^-, N_2O_3, and S-nitrosothiols. It can react with superoxide to produce the extremely reactive and microbicidal peroxynitrite anion, $ONOO^-$, which can also cause tissue damage.

Nitric Oxide is generated by many cell types in response to different inflammatory signals. NFκB plays an important role in the inflammatory process and may induce a number of biochemical mediator genes, including iNOS. In inflammatory conditions, lipopolysaccharide (LPS), staphylococcal enterotoxin B and cytokines such as IL-1β, TNF-α and IFNγ can activate iNOS production. Combinations of these are more potent stimulators of NO production than individual cytokines alone (Ralston *et al.* 1995).

Figure 1.6 – The L-Arginine Nitric Oxide Pathway



Abbreviations: BH_4 = tetrahydro-biopterin, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide, iCa^{2+} = intra-cellular calcium, NADP = nicotinamide adenine dinucleotide phosphate, NOS = nitric oxide synthase

NO and its derivatives are potent antimicrobial agents. These display microbiostatic or microbicidal activity against a wide array of pathogens including uni- and multi-cellular

parasites, fungi, bacteria and viruses. NO has a selective bacteriostatic effect on some bacteria, including *Staphylococcus epidermidis* and group B streptococcus e.g. *Streptococcus agalactiae* (Hoehn *et al.* 1998). In activated macrophages, a proportion of iNOS is associated with the cortical submembraneous actin cytoskeleton as well as in intracytoplasmic vesicles and in free cytoplasm. iNOS association with the actin cytoskeleton is ideally suited to deliver NO to micro-organisms in contact with the cell surface (Webb *et al.* 2001).

Different components of Gram-positive bacteria have been shown to induce NO production such as cell wall polyosides and rhamnose glucose polymers, from *Streptococcus mutans*, and LTA from *Staphylococcus aureus*, (Martin *et al.* 1997). Live pneumococci, oxacillin-killed pneumococci, and pneumococcal cell wall preparations stimulated the production of iNOS and NO from a murine macrophage cell line in the presence of IFNγ (Orman *et al.* 1998).

NO plays an important role in the induction of tissue injury and death during pneumococcal pneumonia. Overproduction of NO can be detrimental during infections and is associated with septic shock. Induction of NOS by LTA from *Streptococcus faecalis* can abrogate host cell differentiation (Elgavish *et al.* 1996). Treatments to reduce the amount of NO may help to relieve the pathogenesis of pneumococcal disease. When mice infected with *S. pneumoniae* were treated with the arginine analogue L-NMMA, a partial blockade of NO production was achieved. Compared to untreated mice, the L-NMMA treated mice displayed higher cytokine levels, preservation of lung ultrastructure and delayed onset of death (Bergeron *et al.* 1999). Antibiotics could be a useful tool in reducing NO levels, as was demonstrated in a rabbit model of pneumococcal meningitis. NO production in the CSF was significantly attenuated by the antibiotic ceftriaxone (Destache *et al.* 1998).

Sensorineural hearing loss caused by pneumolysin could be blocked by pre-treating the cochlea with a NOS inhibitor or by blocking receptors responsible for excess NO release. NO may therefore represent a significant link in the chain of events leading to the deafness resulting from bacterial meningitis. (Amee *et al.* 1995).

Not all NO production is harmful to the host; a deficiency in eNOS was found to be detrimental in a murine model of pneumococcal meningitis. The deficiency was related to increased expression of cytokines and adhesion molecules, leading to increased meningeal

inflammation, aggravated intra-cranial complications and an increased death rate (Koedel *et al.* 2001).

1.11.5.2 Role of Cytokines in Pneumococcal Disease

In mice, TNF plays an essential role in developing resistance to pneumococcal infection during the first two days. This early response seemed to be independent of IL-1β or IL-6 but IL-1β production was necessary for survival beyond five days (Benton *et al.* 1998). Immunisation of mice against TNF led to increased bacteraemia and earlier death compared to untreated mice after pneumococcal challenge (van der Poll *et al.* 1997). The reduced capacity of CBA/Ca mice to induce rapid TNF activity within airways following infection with *S. pneumoniae* may be a factor in their susceptibility to pneumococcal pneumonia (Kerr *et al.* 2002). However two studies in humans showed that TNF levels had no relationship with patient outcome (Marik *et al.* 1993; Moussa *et al.* 1994). In another study patients critically ill with pneumonia had higher TNF levels than non-critically ill pneumonia patients (Puren *et al.* 1995). However the compartment in which the cytokine response is mounted could be important. Although mice susceptible to pneumococcal pneumonia show decreased levels of TNF in the airways, they have increased TNF levels associated with the tissue compared with resistant mice (Kerr *et al.* 2002).

IL-6 is usually associated with its pro-inflammatory properties, especially its ability to induce the acute phase response. However it also appears to have regulatory properties as IL-6 knock out mice seem to mount an increased inflammatory response to *S. pneumoniae* compared to wild type mice (Feldman *et al.* 1991).

IFNγ plays a complex role in innate immunity during pulmonary infection. A recent study found that both IFNγ$^{-/-}$ and IFNγ receptor$^{-/-}$ mice had significantly fewer pneumococci in their lungs after intranasal inoculation compared to wild type mice (Rijneveld *et al.* 2002a) suggesting that endogenous IFNγ is not required for an effective pulmonary defence in pneumococcal pneumonia. However, in an earlier study IFNγ production in the blood of infected mice was increased during infection with a virulent strain compared to an avirulent strain. Furthermore, this group found a higher mortality rate in IFNγ$^{-/-}$ mice suggesting a protective role for IFNγ in disease (Rubins and Pomeroy 1997).

Caterall (1999) suggested that the compartmentalised cytokine response of pneumonia contributes to host defence and that interference with the cytokine network in such a way that pulmonary inflammation is reduced, is likely to be harmful to the host. Addition of IL-10 to an inoculum with *S. pneumoniae* results in decreased TNF and IFNγ production leading to increased levels of bacteria in the lungs and blood and shorter survival time (van der Poll *et al.* 1996)

The role of individual cytokines during pneumococcal disease is unclear and studies have produced conflicting and confusing results. It appears that the levels of cytokines may not be as important as the timing, regulation and compartment of cytokine production.

1.11.6 Signal Transduction by the Pneumococcus

In general, cytokines are not stored intracellularly and their secretion depends on new protein synthesis. The transcription factor NFκB is required for maximal transcription of many cytokines involved in inflammation including TNF, IL-1, IL-6 and IL-8 (May and Ghosh 1998).

CD14 is a surface glycoprotein abundant on the cell membranes of monocytes, macrophages and neutrophils. It binds the complex of LPS and LPS-binding protein allowing LPS to activate monocytes (Ulevitch and Tobias 1995). CD14 signalling is thought to occur through toll-like receptor (TLR) 2 and NFκB activation (van Furth *et al.* 1999; Yoshimura *et al.* 1999). It is now thought that Gram-positive bacteria and cell products may also be able to activate cells via CD14. Blocking of CD14 decreases TNF release from human monocytes stimulated with group B streptococcal cell walls (Cuzzola *et al.* 2000), and anti-CD14 antibodies block pneumococcal-induced TNF and IL-10 production. A different anti-CD14 antibody that blocked LPS-induced TNF production, did not block TNF induced by *S. pneumoniae* suggesting the bacteria binds to a different part of CD14 (van Furth *et al.* 1999).

The family of Toll proteins were originally identified in *Drosophila melanogaster* as proteins involved in specific immune recognition (Williams *et al.* 1997). A human homologue, TLR2, functions as an LPS transducer (Kirschning *et al.* 1998; Yang *et al.* 1998), although TLR4 is probably the major mammalian LPS signal transducer (Poltorak *et al.* 1998; Hoshino *et al.* 1999; Qureshi *et al.* 1999). TLR2 may be involved in the recognition of other bacterial ligands (Yoshimura *et al.* 1999). Heterologous expression of human TLR2, but not

TLR4, in fibroblasts conferred responsiveness to *S. pneumoniae* as evidenced by inducible translocation of NFκB. CD14 co-expression synergistically enhanced TLR2-mediated activation.

CD40 ligand (CD40L) influences epithelial cell function through up-regulation of adhesion molecule expression and cytokine production (Hwang *et al.* 2000). CD40L is also a key component of T-cell-dependent activation of macrophages and dendritic cells. CD40L is essential for antibody responses to non-encapsulated *S. pneumoniae* (Wu *et al.* 1999) and the pneumococcal protein PspA (Hwang *et al.* 2000).

It is only recently that research into pneumococcal signalling has begun to elucidate some of the mechanisms involved. Future work in this area will increase understanding of bacterial signal transduction in the host and perhaps reveal new ways of combating disease.

1.12 Conclusions

Pneumolysin is an important determinant of pneumococcal pathogenicity. It can influence homeostasis and trigger production and release of many potent mediators that may have roles in the pathogenesis of septic shock. Pneumolysin can cause host damage by direct killing of phagocytes and lymphocytes. It can promote spread of infection by killing and damaging tissue cells, and can liberate biologically active cellular mediators from intracellular compartments or from cell membranes.

As pore-forming toxins tend to be immunogenic, they could be used in the development of vaccines. Pneumolysin produces a protective antibody response and there is little antigenic variation between isolates from different serotypes (Paton *et al.* 1993). Immunisation of mice with 20μg of purified pneumolysin toxoid (W433F) plus adjuvant, conferred a significant degree of protection against all nine serotypes of *S. pneumoniae* used as a challenge (Alexander *et al.* 1994). Preliminary studies of pneumolysin derivatives conjugated to polysaccharides have produced a strong antibody response to both the protein and polysaccharide moieties in mice (Kuo *et al.* 1995).

1.13 Aims of this Project

Firstly a stock of pure pneumolysin protein will be created. This will ensure that the protein used in all experiments is of the same concentration and has the same specific activity. Previous studies have determined areas in the pneumolysin molecule likely to be involved in

oligomerisation. Data from the De Los Toyos *et al.* (1996) study suggests that the region containing residues 142 and 143 is important during oligomerisation. By mutating residues 142 and/or 143, construction of mutant pneumolysin proteins unable to oligomerise will be attempted.

Fluorescence techniques will be used to characterise oligomerisation in wild type and mutant proteins. By using a mutant pneumolysin protein unable to oligomerise, we aim to further define the mechanisms involved in oligomerisation and pore formation by pneumolysin.

The role of pneumolysin in the production of inflammatory mediators will also be investigated. The role of oligomerisation and pore formation in inducing inflammatory mediator production will also be investigated by using the mutant proteins. We chose to look at production of NO and TNF in particular as *in vivo* studies in our group have shown these mediators to have important roles in the outcome of disease. Two different cell types were used; macrophages and lung epithelial cells. These cells are important in the early immune response to pneumococcal infection in the lung and are likely to be involved in the production of mediators.

In order to characterise the role of pneumolysin in the context of the whole organism, the same assays using live *Streptococcus pneumoniae* and a mutant pneumococcus with the pneumolysin gene deleted will be used.

Finally DNA microarray techniques will be used to identify changes in macrophage gene regulation induced by pneumolysin. Analysis of this data will begin to elucidate the wider picture of gene regulation in mediator production macrophages induced by the toxin.

2. MATERIALS AND METHODS

2.1 Bacterial Cell Culture

For production of DNA or protein, *Escherichia coli* cultures were grown in 25 g/l Luria Broth (Miller's LB Broth Base) (Sigma) +/- 15 g/l agar (Agar Bacteriological No 1) (Oxoid) at 37°C. *Streptococcus pneumoniae* D39, serotype 2 was obtained from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London). *S. pneumoniae* cultures were grown on agar plates made from 40 g/l blood agar base (BAB) No2 (Oxoid) with 5% defribrinated horse red blood cells (E & O Laboratories) added when cooled to a hand-hot temperature. Liquid *S. pneumoniae* cultures were grown in a 37°C static incubator in 37 g/l brain heart infusion (BHI) broth (Oxoid). Cultures were grown aerobically unless otherwise stated. Bacterial strains were incubated with ampicillin, kanamycin or erythromycin (all Sigma) where appropriate (see appendix).

2.1.1 Preparing Glycerol Stocks

Bacterial strains and genetic constructs were stored at -80°C in glycerol. After streaking the culture onto an appropriate agar plate and growing overnight, a single colony was used to inoculate 20 ml broth. This culture was incubated at 37°C until it reached mid-log growth phase (OD_{600} = 0.7) and sterile glycerol was added to make up 20% of the stock. The culture was aliquoted into 1 ml cryotubes and stored at -80°C. Where necessary viable counts were made of the glycerol stocks after freezing.

2.1.2 Bacterial Viable Counts

If the sample to be counted was frozen it was first thawed for two minutes in a 37°C water bath and then centrifuged at 16000 x g to pellet the bacteria, which were resuspended in PBS (phosphate buffered saline).

As previously described (Kadioglu *et al.* 2000), 20 µl of the sample was added to 180 µl of sterile PBS in a 96-well plate and mixed well. Five more 1/10 dilutions were made, so in total, six dilutions were made, ranging from 10^{-1} to 10^{-6}. The base of an appropriate agar plate was divided into six sections and labelled. In each segment, 20 µl was spotted three times into the appropriate segment. Each sample was plated out in duplicate so altogether there were six spots for each sample. The plates were air dried and incubated overnight at 37°C.

The colonies were counted in the sector where there were between 40 and 250 colonies per spot. The average number of colonies for each sample was multiplied by 50 to give the number per ml and then by the dilution factor to give the number of colony forming units per ml (cfu/ml).

2.2 Production of Crude Pneumolysin

A wild type pneumolysin stock was produced from M15(pRep4) *E. coli* with a pKK233-2 vector containing the pneumolysin gene from the *Streptococcus pneumoniae* strain D39. The pKK233-2 vector contains an IPTG-regulated promoter and is suitable for high-level expression of foreign proteins (Amann and Brosius 1985; Straus and Gilbert 1985). The pREP4 repressor plasmid prevents "leaky" expression of the toxin before induction. All cultures were grown in the presence of 100 μg/ml ampicillin and 20 μg/ml kanamycin to ensure the presence of the vectors. Overnight cultures grown from a single colony were used to inoculate larger volumes of LB at a 1:100 ratio. These were grown at 37°C until an OD_{600} reading of 0.7 was reached. Gene expression was induced by adding IPTG (Isopropyl-β-D-Thiogalactopyranoside) (Melford Laboratories Ltd) to a concentration of 1 mM. Cultures were grown for a further 1.5 hours and cells were harvested by centrifugation for 15 minutes at 3000 x g. The pellet was resuspended in buffer A (appendix). Cells were disrupted using 0.25 mm cups on the One Shot 0.75 W 20 k PSI Max Benchtop Cell Disrupter (Constant Systems Ltd) at a pressure of 12 K. Proteins in the soluble fraction were recovered by centrifugation at 20000 x g for 30 minutes.

2.3 Methods used to Purify Pneumolysin

Pneumolysin was purified from the soluble fraction of crude cell extracts using the BioCAD® 700E Perfusion Chromatography Workstation (Applied Biosystems). See appendix for preparation of buffers.

2.3.1 Hydrophobic Interaction Chromatography

A protocol for hydrophobic interaction chromatography (HIC) was developed by Dr. N. Rai. This method of chromatography separates biomolecules based on the hydrophobic groups on their surfaces. The column used was 4.6 mm in diameter and 100 mm in length with a capacity of 1.662 ml. For the duration of the method, the flow rate was set to 4 ml/minute. The column was equilibrated with 4 column volumes (CV) of 1 M NaCl in buffer A. NaCl was added to the sample to 1 M and up to 5 ml was added to the column. The column was

washed with 1 M NaCl for 1 minute followed by six CV of water. This had the effect of washing the salt from the column thereby producing a reduction in salt concentration. As the salt concentration dropped, the pneumolysin was eluted from the column. The column was then washed with a further 5 CV of water.

2.3.2 Ion Exchange Chromatography

Ion exchange chromatography (IEC) separates molecules based on differences in their accessible surface charges. A protocol for this method was also developed by Dr. N. Rai. The column used was 4.6 mm in diameter and 100 mm in length with a capacity of 1.662 ml. For the duration of the method, the flow rate was set to 6.5 ml/minute. The column was equilibrated with 8 CV of buffer A and then up to 5 ml of sample was loaded onto the column. In order to wash off contaminants and elute the protein, a salt gradient was set up. This started with 100% of buffer A and over a period of 10 minutes the salt was increased to a final concentration of 1.2 M. The column was then washed with 10 CV water.

2.3.3 Metal Chelate Chromatography

Metal chelate (MC) chromatography was used to purify proteins containing a six-histidine tag. The column used was 4.6 mm diameter and 50 mm in length with a capacity of 0.831 ml. For the duration of the method, the flow rate was set to 4.15 ml/minute. First the column was equilibrated with 10 CV of 300 mM NaCl in 50 mM Hepes and 5 CV of 500 mM extra pure imidazole (BDH) in the equilibration buffer. Then for 10 CV the imidazole was lowered to 1 mM. This low imidazole concentration prevents non-specific binding of background proteins but still allow the 6xhistidine-tagged proteins to bind strongly. Up to 5 ml of the sample was then loaded onto the column.

Three washes were used to elute contaminants in the sample. The first wash was 10 mM imidazole with 0.5 M NaCl in 0.5 M Hepes for 5 CV. The next wash was 10 CV of 20 mM imidazole in 480 mM NaCl in Hepes plus 15% ethanol. In the final wash the imidazole was increased to 50 mM. Adding imidazole to the washes decreases the incidence of non-specific or weak histidine binding (i.e. proteins in the sample that may be rich in histidines). The salt helps to decrease ionic interactions with the column and also helps to remove nucleic acids from the sample. The ethanol decreases hydrophobic interactions of molecules with the column. The wash steps ensure that the only molecules that stay bound to the column are the six-histidine tagged proteins. To elute the protein, a step-wise increase of imidazole in H_2O

was used. In the first elution step 5 CV of 100 mM imidazole was used. The amount of imidazole was increased by 50 mM in each subsequent step. Pneumolysin eluted between 150 and 200 mM of imidazole. The protein was then dialysed against PBS to remove the imidazole from the solution.

To re-use the column, the imidazole was removed by washing with 20 CV of water. Then the metal ions were stripped from the column with 30 CV of 50 mM EDTA in 1 M NaCl. The column was then washed with water for 20 CV and stored in 20% ethanol or recharged. To recharge the column, 30 CV of 100 mM $NiSO_4$ was used followed by 10 CV of water to remove excess metal ions and 10 CV of 200 mM NaCl.

2.3.4 Dialysis

Dialysis was used to change the buffering solution of the protein, for example to remove the salt after HIC and the imidazole after MC chromatography. The dialysis tubing was boiled for thirty minutes in 10 mM sodium bicarbonate/1 mM EDTA to remove chemical contaminants from the manufacturing process. Boiled tubing was stored in 1 mM EDTA at 4°C until required.

One end of the tubing was sealed with a dialysis clip. The protein solution was pippetted into the tube and the other end clipped. The bag was placed in >10 volumes of buffer and stirred gently at 4°C overnight.

2.4 Analysing Pneumolysin Preparations

2.4.1 Haemolytic Assay

Pneumolysin activity was determined by a haemolytic assay. 50 µl of PBS was added to every well of two round-bottomed 96-well plates. 50 µl of samples and controls were added to the first column and double dilutions were made in the PBS across two plates. Each sample was plated in duplicate. 50 µl of 2% defribrinated sheep's blood (E+O Laboratories) was added to every well and the plates were incubated for thirty minutes at 37°C. In negative wells, the red blood cells roll down the sides of the wells and form a visible red pellet in the bottom of the well. In wells containing pneumolysin, the cells are lysed and cannot form a pellet. The haemolytic unit (HU) is calculated from the number of dilutions required to cause 50% cell lysis. The end-point is taken as the well in which the pellet is half the size of the control wells. In the first well, pneumolysin is in a 1 in 2 dilution in PBS. When 50 µl of

blood is added this is another 1 in 2 dilution, therefore in the first well, the pneumolysin has been diluted by a factor of 4. If the endpoint is in this well, there are 4 HU in that well in 100 µl, so in 1 ml there are 40 haemolytic units. The haemolytic titre for lysis in each well is given in Table 2.1. The activity of pneumolysin can also be expressed as HU/mg.

Table 2.1 – Haemolytic Assay Titres of Pneumolysin

WELL NO.	TITRE (HU/ML)	WELL NO.	TITRE (HU/ML)
1	40	**13**	1.6×10^5
2	80	**14**	3.2×10^5
3	160	**15**	6.4×10^5
4	320	**16**	1.3×10^6
5	640	**17**	2.6×10^7
6	1280	**18**	5.2×10^7
7	2560	**19**	1×10^8
8	5120	**20**	2×10^8
9	1×10^4	**21**	4×10^8
10	2×10^4	**22**	8×10^8
11	4×10^4	**23**	1.6×10^9
12	8×10^4	**24**	3.2×10^9

The well in which 50% haemolysis occurs is used as the endpoint and relates to the activity represented in the table as haemolytic units (HU) per ml.

2.4.2 SDS-PAGE

SDS-Polyacrylamide gel electrophoresis (PAGE) was used to determine the size and purity of the protein. Biorad minigels were assembled, a 10% Separating gel was poured (appendix), overlayed with dH_2O and allowed to set. A stacking gel (appendix) was then poured on top of the separating gel, a comb was inserted to make 10 or 15 wells and the gel allowed to set. Samples were boiled in sample buffer (appendix) for five minutes and loaded onto the gel. Gels were run using a Biorad power pack 300 at 200 V. Gels were stained in Coomassie stain for 3-5 hours then destained until bands were clearly seen (appendix). Gels were dried between two sheets of cellophane (Biorad) for one hour in a Biorad GelAir dryer and cooled for thirty minutes.

2.4.3 Absorbance Assay

The relationship between absorbance at 280 nm and protein concentration is linear. Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 220 nm, Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 220 nm. Nucleic acids absorb UV light with an absorbance peak at 260 nm. Absorbance assays were used to determine protein concentration.

The UV lamp on a Unicam UV/Vis Spectrometer UV2 was allowed to warm up. Scan parameters were entered to measure absorbance from 190 to 350 nm. Clean quartz cuvettes were filled with the buffer solution (usually PBS) and the spectrophotometer was calibrated to zero. 100 µl of protein solution was added to the sample cuvette and the scan was carried out. If the absorbance was greater than two, the solution was diluted in buffer.

Absorbance values were recorded at 260 nm, 280 nm and 320 nm. The concentration of pneumolysin was calculated by dividing the absorbance at 280 nm by the extinction coefficient. The extinction coefficient for pneumolysin was calculated from the amino acid sequence and is 0.75. A high 260 nm reading suggested possible nucleic acid contamination. Readings at 320 nm were taken as an indication of aggregate scatter, and were all close to zero.

2.4.4 Bradford Assay

The Bradford protein assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when protein binding occurs. Both the hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible colour change.

This assay was used to confirm the protein concentrations determined by A_{280} readings. Standards of BSA (Sigma) were made covering a range of concentrations from 18-1500 µg/ml. 10 µl of sample, blank (PBS) or standard were added in triplicate to a flat-bottomed 96-well plate (Iwaki) and 200 µl of Bradford reagent (Sigma) was added to every well. The absorbance was read on a plate reader (Dynatech) at 595 nm.

A standard curve was created by plotting the average absorbance reading of each standard against its concentration. The average reading of the triplicates for each sample was then read from the curve to give a concentration for each sample.

2.4.5 Western Transfer

Gels from SDS-PAGE were equilibrated in transfer buffer. Biorad blotting apparatus was assembled. The gel and a Hybond-C Super membrane optimised for protein transfer (Amersham Life Sciences) were sandwiched between filter paper (all cut to size) and fibre pads, all pre-soaked in transfer buffer (appendix). An ice pack and stir bar were placed in the tank and the gel was blotted at 100 V for one hour, or 30 V overnight.

2.4.5.1 Anti-Pneumolysin Western Blot

Western blots using a rabbit polyclonal antibody against pneumolysin were carried out to confirm that protein bands visible on SDS gels were pneumolysin. The membrane was blocked in 3% skimmed milk in Tris-NaCl (appendix) for one hour on a shaker at room temperature. The primary antibody, polyclonal anti-pneumolysin serum from rabbit, was diluted 1:1000 in 3% skimmed milk in Tris-NaCl and the membrane was incubated at 37°C with shaking for 2-3 hours, or overnight at room temperature. The membrane was then washed four times with Tris-NaCl for five minutes each. The secondary antibody (anti-rabbit Ig HRP-linked whole antibody from donkey (Amersham Life Sciences)) was diluted 1:1000 in 3% skimmed milk in Tris-NaCl and the membrane was incubated at 37°C with shaking for one hour. The membrane was then washed as before and developed (appendix).

2.4.5.2 Anti-PentaHis Western Blot

Western blots using a monoclonal antibody against five histidine residues were carried out, where appropriate, to check that the protein bands visible on SDS PAGE contained a histidine-tag. The membrane was washed twice in TBS buffer (appendix) and blocked with 3% BSA in TBS for one hour on a shaker at room temperature. The membrane was washed twice with TBS buffer and incubated with the primary antibody (anti-pentaHis (Qiagen)) diluted 1:200 (to give 1 μg/ml)) in 3% BSA in TBS for one hour at room temperature. The membrane was then washed twice with TBS-Tween/Triton buffer (appendix) and once with TBS. The secondary antibody (anti-mouse Ig HRP-linked whole antibody from sheep (Amersham Life Sciences) was diluted 1:1000 in 3% BSA in TBS and the membrane was

incubated at 37°C with shaking for one hour. The membrane was then washed four times with TBS-Tween/Triton buffer and developed (appendix).

2.4.6 Analytical Ultracentrifugation

Analytical ultracentrifugation (AUC) is a robust technique for characterising macro-molecular interactions. Sedimentation equilibrium runs using AUC were used as a quality control method to check the size and self-association of pneumolysin. Sedimentation equilibrium can determine the absolute molecular weight of a molecule and no instrument calibration or shape assumptions are required. By measuring the average molecular weight of pneumolysin at different concentrations, the presence of oligomers would manifest itself by raising the average molecular weight of the toxin.

Pneumolysin was dialysed against buffer to remove the glycerol from the frozen stock, and the concentration was measured using a spectrophotometer tuned to a wavelength of 280 nm. Nine concentrations of pneumolysin were made ranging from 0.49-2.3 μM. Centrifuge cells with six sectors were loaded with 80 μl of pneumolysin in one half (three sectors) and with buffer as a reference solvent in the other three channels.

Measurements were made using the Beckman Optima XL-A analytical ultracentrifuge at speeds of 3000 (3 k) rpm, 16 k rpm, 20 k rpm and 40 k rpm, in this particular order. The concentration of pneumolysin was checked at 3 k rpm from its absorbance readings and was monitored for a short period to ensure that there was no leakage. The centrifuge speed was increased to 16 k rpm and once equilibrium had been established was increased to 20 k rpm. Scans were taken periodically at both speeds for approximately 20 hours by which time the absorbance readings remained constant indicating that equilibrium had been reached. The speed was then increased further to 40 k rpm to pellet all large particles, with a scan taken after a period of four hours to measure the baseline. This was subtracted from the equilibrium measurements taken at 16 k rpm and 20 k rpm.

2.4.7 Endotoxin Testing

Endotoxin levels were measured in pneumolysin samples using the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit from BioWhittaker. Endotoxin (from Gram-negative bacteria) catalyses the activation of a proenzyme in the LAL assay and the rate of activation is dependent on the amount of endotoxin present. This enzyme then splits p-nitroaniline

(pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA release is measured photometrically at 405 nm throughout the incubation period. Samples are mixed with the LAL substrate and a colour change is measured over time. The reaction time is inversely proportional to the amount of endotoxin present. The concentration of endotoxin in samples is calculated from a standard curve.

E. coli 055:B5 Endotoxin was reconstituted to 50 EU/ml with endotoxin-free water and vortexed thoroughly for ten minutes. Four 1/10 dilutions were made, between 0.005 and 50 EU/ml, to use as the standard curve. The assay can only detect endotoxin levels within this range so endotoxin levels in the sample must fall within these concentrations. Samples were diluted as necessary with endotoxin-free water. Samples and standards were added to a flat-bottomed 96-well plate (Costar) at 100 µl per well in duplicate. To ensure the samples were not interfering with the assay, 5 EU of standard endotoxin was added to duplicate samples as a "spike". The plate was then warmed in the plate reader at 37°C for ten minutes. The LAL Kinetic-QCL Reagent was reconstituted in endotoxin-free water and then 100 µl was added to each well. Readings were taken every 2.5 minutes for 1.5 hours.

2.5 Endotoxin Removal

2.5.1 Ultracentrifugation

After the cells were disrupted, the lysate was put into ultracentrifuge tubes and centrifuged using the Sorvall OTD-Combi Ultracentrifuge (Dupont) at 38 k rpm (rotor T865) for two hours at 4°C. The supernatant was kept overnight at 4°C.

2.5.2 Ammonium Sulphate Precipitation of Pneumolysin

The supernatant from the ultracentrifugation was made up to 20 ml with PBS and incubated on ice and stirred. Ammonium sulphate was added to make a 60% solution (6.7 g) and the solution was stirred for a further thirty minutes on ice. The solution was then centrifuged at 10000 x g for ten minutes and the supernatant retained for purification.

2.5.3 Polymyxin B Agarose

Polymyxin B binds to endotoxin and therefore can be used to remove it from solutions. Detoxi-Gel™ Endotoxin Removing Gel Columns (Pierce) contain polymyxin B immobilised on agarose. The columns were regenerated before each use by washing with 5 CV of 1% sodium deoxycholate, followed by 3-5 CV of water to remove the detergent. The column

was equilibrated with PBS and then the sample was added. After incubation for one hour at 4°C, the sample was collected by gravity flow by adding aliquots of water. The column was regenerated by washing with sodium deoxycholate as described above.

All solutions were made using endotoxin free water and PBS was made using tablets (Oxoid) by addition of one tablet to 100 ml water. Sodium deoxycholate was used to strip any bound endotoxin from the agarose and was also made using endotoxin-free water. The water used for the washes to remove the detergent was also endotoxin-free.

2.5.4 Biobeads

This experiment was carried out by Mr G. Meiklejohn. In this protocol Triton-X-114 is used to remove LPS from the sample, then SM-2 Biobeads (Bio-Rad) are used to remove the Triton-X. Triton-X was added to 50 µl of stock pneumolysin to a final concentration of 1%. This was then vortexed and incubated on ice for five minutes or until a homogenous solution was obtained. The sample was mixed, and incubated at 37°C for five minutes. Centrifugation at 1200 x g (no brake) at 37°C for five minutes isolated a top phase, which was transferred to a new tube. The Triton-X extraction was repeated three more times. To remove the Triton-X, the sample was added to 5 g of biobeads and mixed gently for two hours at room temperature. The sample was centrifuged at 1200 x g (no brake) to separate the beads and incubation with a fresh aliquot of biobeads was repeated.

2.6 Fluorescence Analysis

Fluorescence occurs when the absorption of a photon by molecule is followed by the emission of light of a longer wavelength. A fluorimeter can be used to excite a sample and measure any fluorescent light emitted. Fluorescent residues on the outside of a protein have a lower fluorescence than those on the inside as water quenches the fluorescence. We used this property to look at the fluorescent changes during oligomerisation of pneumolysin. ANS was used to amplify the signal from pneumolysin, so changes in fluorescence were more obvious.

All experiments were carried out in the JASCO FP-750 Spectrofluorometer at a constant temperature of 20°C. An excitation bandwidth of 5 nm and an emission bandwidth of 10 nm were used with a fast response. A scanning speed of 125 nm/min was used when appropriate. 120 µl of sample was added to 1 cm path-length quartz cuvettes and placed in the fluorimeter. Pneumolysin was excited at 285 nm and ANS (8-Anilino-1-naphthalene-

sulphonic acid) (Kodak Ltd.) at 370 nm. These excitation wavelengths were chosen since they provide the maximal signal change. Sodium deoxycholate (0.5%) was added to the sample to induce pneumolysin oligomerisation (BDH Laboratory Supplies). Titrations were performed by making additions (1µl) of concentrated ANS. Fluorescence emission was recorded either as a spectrum measurement or as a time course at an appropriate emission wavelength.

2.7 Site Directed Mutagenesis

2.7.1 Primer Design

Primers were designed and synthesised (Life Technologies) in order to clone the pneumolysin gene between the restriction sites BamH1 and Sac1 in the pET-33b(+) (Novagen) vector (See Figure 2.1). Transcription in pET vectors is controlled by the bacteriophage T7 that enables high levels of target gene expression. This vector also adds a six-histidine affinity tag to the N-terminus of the protein and this allows convenient purification of the protein on metal chelate columns. The primers were reconstituted in TE Buffer (appendix) to 1 nM/ml.

Figure 2.1 – Primer Design to clone Pneumolysin into pET-33b(+) vector

Primer 1: Forward Primer

	pET Sequence	Pneumolysin Sequence	
5'	CGG GAT CCG	GCA AAT AAA GCA GTA AAT GAC TTT	3'
	BamH1		

Primer 2: Reverse Primer

	pET Sequence	Pneumolysin Sequence	
5'	GAC GGA GCT CGA	CTA GTC ATT TTC TAC CTT ATC	3'
	SacI	Stop	

These primers were used to extract the pneumolysin gene from genomic *S. pneumoniae* DNA. The introduction of BamH1 and Sac1 restriction enzyme sites allowed the gene to be cloned between these sites in the pET-33b(+) vector.

Primers were also designed by Dr N. Rai and Ms L. Cunliffe to introduce mutations within the pneumolysin gene (See Figure 2.2). These primers were designed to create one mutant pneumolysin protein with a substitution of the asparagine residue at position 143 to an aspartic acid and another mutant with a deletion of the asparagine residues at positions 142 and 143. These mutations were chosen as monoclonal antibodies thought to bind to this region prevented oligomerisation of the toxin. A summary of the different primers used and

their function can be seen in Table 2.2. The forward and reverse primers that introduce each mutation (e.g. 3 & 4) would anneal to each other so each primer had to be used in a separate PCR to produce two fragments containing the mutation. These could then be annealed together in a fusion PCR (See Figure 2.3). A wild type pneumolysin construct was also made using primers 1 and 2 alone.

Figure 2.2 – Primer Design to introduce mutations into the Pneumolysin Gene

Primer 3: Forward Primer
5' CAG GTC AAT **GAT** GTC CCA GCT AGA

Primer 4: Reverse Primer
5' TCT AGC TGG GAC **ATC** ATT GAC CTG

Primer 5: Forward Primer
5' AT TAT GGT CAG GTC _ _ _ _ _ _ GTC CCA GCT AGA AT

Primer 6: Reverse Primer
5' AT TCT AGC TGG GAC _ _ _ _ _ _ GAC CTG ACC ATA AT

GAT/ATC – Introduces N143D Mutation; ------ – Represents deletion of asparagine residues at positions 142 and 143.

Table 2.2 – Primers used to make Pneumolysin Mutants in pET-33b(+) vector

PRIMER	FUNCTION
1 (9Y)	Forward primer to clone pneumolysin into pET33b(+). Introduces BamH1 Site.
2 (9Z)	Reverse primer to clone pneumolysin into pET33b(+). Introduces Sac1 Site.
3 (6O)	Anneals at bases 418-441 of pneumolysin, primes in forward direction. Introduces N143D mutation
4 (6P)	Anneals at bases 418-441 of pneumolysin, primes in reverse direction. Introduces N143D mutation
5 (8N)	Anneals at bases 410-443 of pneumolysin, primes in forward direction. Introduces N142/143 deletion.
6 (8O)	Anneals at bases 410-443 of pneumolysin, primes in reverse direction. Introduces N142/143 deletion.

Note: Numbers in brackets indicate number in the oligonucleotide library of Prof. Mitchell's research group.

2.7.2 Amplifying the Pneumolysin gene and introducing mutations

PCR reactions were made up using 3 µl (~500 ng) of genomic DNA from D39 *Streptococcus pneumoniae* (isolated by Ms J. Irvine), 100 pM of appropriate primers, 1 µl 10 xThermopol

PCR buffer, 2 mM $MgSO_4$, and 2 U $Vent_R^{®}$ DNA Polymerase (all obtained from New England Biolabs) and 200 μM dNTP (Promega), with dH_2O used to make up the volume to 100 μl per reaction. The reactions were heated to 94°C for five minutes and then run through thirty cycles of (94°C for 1 minute, 62°C for 30 seconds, 72°C for 1 minute) and then heated at 72°C for five minutes on the Hybaid Omnigene machine.

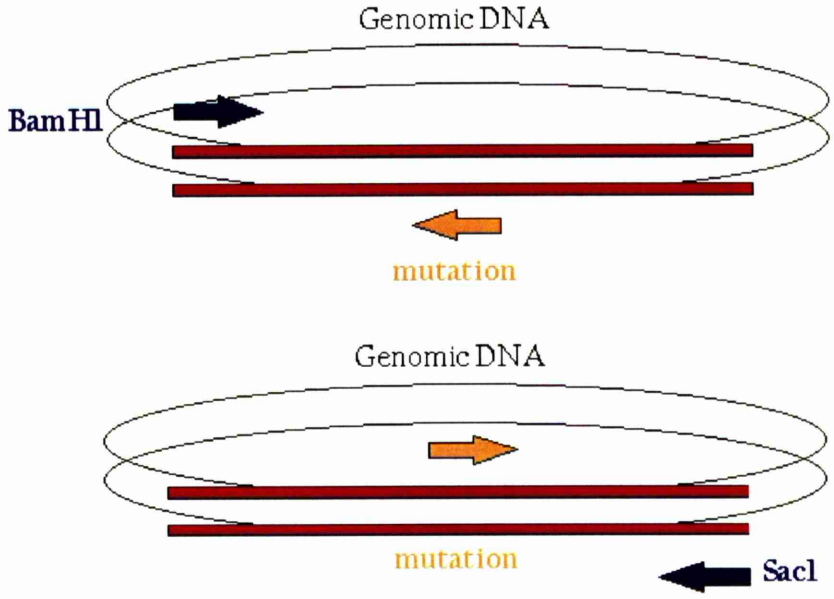
For the fusion PCR, 1 μl of each DNA fragment was set up as above with 0.5 μM of primers 1 and 2. The conditions for the PCR were; 94°C for five minutes followed by three cycles of (94°C for 1 minute, 50°C for 30 seconds, 72°C for 1.5 minutes) and 27 cycles of (94°C for 1 minute, 62°C for 30 seconds, 72 °C for 1.5 minutes) and 72°C for 1 minute. The first three cycles were carried out at a lower temperature to allow the 20 base pair overlaps between the fragments to anneal.

DNA from PCR were visualised by separating samples in DNA loading buffer (appendix) on a 1% agarose gel with 0.5 μg/ml Ethidium Bromide (Sigma). A 1 kb ladder (Promega) was run on all gels. DNA was extracted from gels using a Qiaquick Gel Extraction Kit (Qiagen) following the instructions provided.

2.7.3 Cloning the PCR products into suitable vectors

The DNA from the fusion PCR was ligated into the PCR-Script™ Amp SK(+) cloning vector (Stratagene) (appendix) and transformed into Novablue competent cells (Novagen) following instructions supplied (appendix). The cells were plated onto LB agar containing IPTG and X-gal to identify transformed cells containing inserts. Under these conditions, cells containing vector with no insert form blue colonies whereas cells with a vector containing insert produce white colonies. Cultures of white colonies were grown overnight and plasmid DNA was purified using the Qiaprep Spin Miniprep kit following the instructions provided.

Figure 2.3 – PCR Rationale



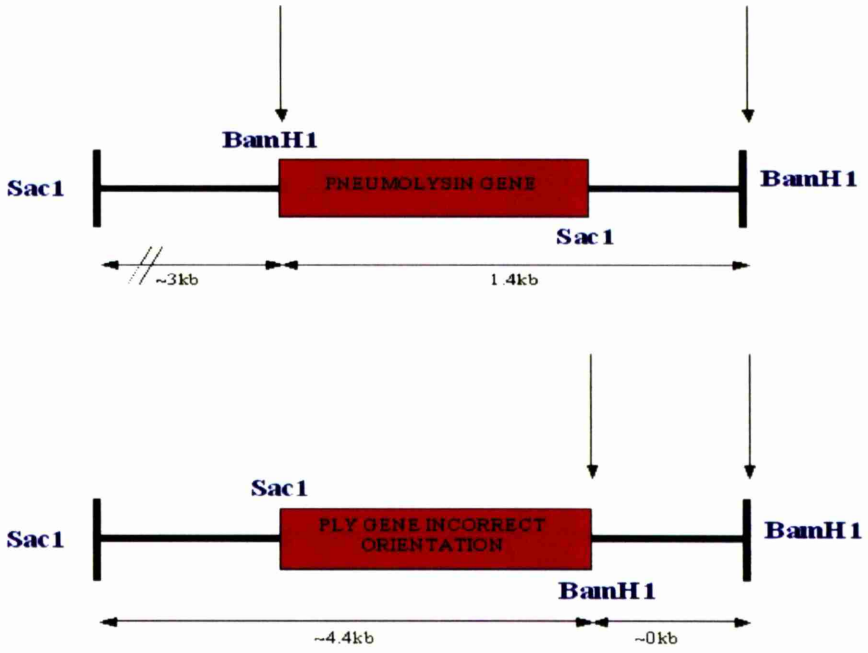
Two separate PCRs were carried to prevent the primers that introduce the mutations from annealing to one another. The resulting fragments were then joined using a fusion PCR. This produced pneumolysin gene containing a mutation and restriction sites to enable cloning into the pET-33b(+) vector.

The PCR products were cloned into PCR-Script™ as blunt-ended products using the restriction enzyme SrfI. Therefore, the pneumolysin gene could potentially insert into the plasmid in two different orientations. The plasmid DNA was cut using the restriction enzyme BamH1 to check which plasmids contained pneumolysin gene inserts in the correct orientation (see Figure 2.4). Arrows indicate where the restriction enzyme would cut the plasmid DNA. If the pneumolysin gene is inserted in the correct orientation two DNA

fragments would be seen on a gel. If the orientation was incorrect, only one larger fragment would be seen. In the incorrect orientation there is a risk of small fragments of DNA inserting between the two BamH1 sites during subcloning.

The pET33b(+) vector was digested with BamH1 and Sac1 to create "sticky ends" (appendix). Inserts cut from PCR-Script™ using the same enzymes were then ligated into the pET33b(+) vector and transformed into Novablue Singles (Novagen) (appendix). DNA was purified from overnight cultures using a Qiagen miniprep kit. A restriction digest with BamH1 and Sac1 (both Promega) was set up to check the inserts. Plasmids containing the correct insert were transformed into Epicurian coli BL21(DE3) cells (Stratagene) (appendix). These were used for protein expression.

Figure 2.4 – Rationale behind restriction digest of the PCR-Script™ plasmid



If the pneumolysin gene has inserted into the plasmid in the correct orientation then when it is "cut" with BamH1, two fragments of the sizes shown in the top picture will be obtained. In the incorrect orientation, only one large DNA fragment will be apparent when the restriction digest is separated on a gel.

2.7.4 DNA Sequencing

For DNA sequencing, plasmid minipreps of overnight cultures were linearised using the restriction enzyme BamHI for inserts in pET33b(+) or XhoI for inserts in PCRScript. The linearised DNA was separated on an agarose gel with a marker of known concentration so the amount of DNA could be estimated (appendix) and sent with the appropriate primers to

MBSU (Molecular Biology Support Unit) for sequencing. At MBSU, 500-600 ng DNA was mixed with 1 μl of the appropriate primer and 4 μl of Big Dye Terminators v.2 (Applied Biosystems) and made up to 20 μl with dH_2O. The primers used to sequence the pneumolysin constructs are shown in Table 2.3. The reaction was heated to 95°C for five minutes followed by thirty cycles of 50°C for five seconds, 60°C for four minutes and 95°C for ten seconds. DNA was precipitated by adding 2 μl of 1.5 M sodium acetate/0.25 M EDTA and 80 μl of 95% ethanol. After centrifugation at 16000 x g for one hour, the pellet was washed with 70% ethanol and centrifuged for a further twenty minutes. The ethanol was removed and the pellet left to air dry, resuspended in loading dye and separated on a 4.75% gel (Long Ranger) for 12 hours. The gel was loaded in a 377 DNA Sequencer (Applied Biosystems) and data collection and sequence analysis was carried out by computer software from Applied Biosytems.

Table 2.3 – Primers used to sequence the Pneumolysin Gene

IN PCR-SCRIPT	
T3	5' AAT TAA CCC TCA CTA AAG GG 3' Binds to T3 promoter of PCR-Script upstream of pneumolysin gene
T7	5' GTA ATA CGA CTC ACT ATA GGG C 3' Binds to T7 promoter of PCR-Script, downstream of pneumolysin gene
IN PET-33B(+)	
T7	5' TAA TAC GAC TCA CTA TAG GG 3' Binds to T7 promoter of pET-33b(+), upstream of pneumolysin gene
PlypET Term	5' GAC GGA GCT CGA CTA GTC ATT TTC TAC CTT ATC 3' Binds to the end of the pneumolysin gene and the portion of the pET33b vector immediately following.
WITHIN PNEUMOLYSIN GENE	
4T	5' GTT GAT CGT GCT CCG ATG AC 3' Binds between base pairs 274 and 293 of pneumolysin sequence

2.7.5 Gene Expression from pET-33b(+) vector in BL21(DE3) Epicurian coli

An appropriate glycerol stock was streaked onto an LB agar plate (+30 μg/ml kanamycin) to obtain single colonies and incubated overnight at 37°C. Late the next day, 20 ml LB broth (+30 μg/ml kanamycin) was inoculated with a single colony from the plate and incubated

overnight at 37°C with shaking. 1 litre of LB broth (+30 μg/ml kanamycin) in a baffled flask was inoculated with 10 ml of overnight culture and incubated at 37°C for two to three hours, with shaking, until the OD_{600} reached 0.6. IPTG was added to a final concentration of 1 mM and the flasks were incubated for a further two to three hours at 37°C. The flasks were placed on ice to prevent further bacterial growth and the cells were harvested by centrifugation for 15 minutes at 5000 x g. Cells were disrupted either by using the cell disrupter as described in section 2.2 or by using proprietary detergent formulations (Novagen or Pierce).

2.8 Tissue Cell Culture

RAW 264.7 murine monocyte macrophages were obtained from ECACC (European Collection of Animal Cell Cultures - number 91062702). They were cultured in Dulbeccos modified Eagle medium, supplemented with 10% Certified Foetal Bovine Serum (FBS), and 2 mM L-Glutamine (All from Life Technologies). Cells were incubated with 5% CO_2 at 37°C. To subculture, the cells were removed from flasks mechanically using a cell scraper (Griener). Cells were diluted one in five with media and reseeded into flasks (Iwaki or Griener).

The L929 murine connective tissue cell line was obtained from ECACC (number 85011425). These cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% of 5 U/ml Penicillin G Sodium/5 μg/ml Streptomycin sulfate in 0.85% saline, and 2 mM L-Glutamine (All from Life Technologies). To subculture cells, the media was removed and the monolayer washed with PBS. 1 ml of 1 x Trypsin in Hank's buffered saline solution (HBSS - without calcium or magnesium) with EDTA.4Na (Life Technologies) was added and the flasks placed at 37°C for up to five minutes until the cells became detached from the flask. Detached cells were diluted one in ten with fresh media and seeded into new flasks.

A549 cells, a human type II alveolar epithelial carcinoma cell line, were obtained from ECACC (number 86012804). They were maintained and passaged in F12k Kaign's modification nutrient mixture supplemented with 10% FBS and 2 mM L-Glutamine. For some experiments the cells were cultured in serum-free CHO-S-SFM II media. The cells were passaged using the same method as described above for the L929 connective tissue cells, except 0.25% Trypsin, 1 mM EDTA.4Na was used (All from Life Technologies).

Small airway epithelial cells and reagents were obtained from BioWhittaker. They are established from normal human tissue and have a finite lifespan *in vitro* of 15 population doublings. To make up culture media for small airway epithelial cells, singlequots® (appendix) were added to 500 ml of small airway basal media. The growth medium was changed the day after seeding and then every other day whilst examining the cells daily. The cells were fed with an increasing volume of medium as they became more confluent. If the cells were less than 25% confluent, they were fed 1 ml of pre-warmed fresh media per 5 cm^2. For cells 25-45% confluent, 1.5 ml of media per 5 cm^2 was used and 2 ml per 5 cm^2 when cells were greater than 45% confluent. At 60-80% confluence, the cells were subcultured. The medium was aspirated from the culture vessel and the cells washed twice with 2-3 ml of room temperature Hepes buffered saline solution. The cells were covered with 2 ml of 0.025% trypsin/0.01% EDTA and observed under a microscope until 90% of the cells had rounded up. The trypsin was neutralised with 4 ml of room temperature trypsin neutralising solution and the cells transferred to a sterile centrifuge tube. The cells were pelleted by centrifugation at 150 x g for five minutes. The supernatant was removed and the pellet resuspended in 4-5 ml fresh growth medium. The cells were counted and seeded into fresh flasks at 2500 cells/cm^2 in 1 ml of growth medium for every 5 cm^2 surface area of the flask (e.g. 15 ml for a 75 cm^2 flask). After 24 hours, the cells were examined and the culture medium changed.

Frozen stocks of cell lines were made by harvesting cells from a confluent 75 cm^2 flask and centrifuging for five minutes at 150 x g. The pellet was resuspended in 1 ml media supplemented with 20% FBS and 10% DMSO (Sigma) to act as a cryprotectant. Cells were placed at -80°C in an alcohol bath overnight, to reduce the temperature slowly, before being transferred to liquid nitrogen for long-term storage.

To recover cells from liquid nitrogen storage, a vial of cells was removed and defrosted quickly by immersion in a 37°C waterbath. The tube was swabbed with 80% industrial methylated spirits to ensure sterility. The cells were resuspended into 5 ml of pre-warmed growth medium in a 25 cm^2 flask and incubated at 37°C with 5% CO_2. The next day the media was changed to remove any DMSO and dead cell debris.

2.8.1 Cell Viability Assays

2.8.1.1 Trypan Blue Exclusion

200 µl of cells in suspension were added to 300 µl of media and 500 µl of trypan blue solution (0.4%, Sigma). The solution was mixed and left for up to five minutes. A coverslip was mounted onto the haemocytometer and approximately 12 µl of the cell suspension was pipetted into the space between the haemocytometer and the coverslip on each half. Live and dead cells in each of the four corner squares of each half were counted separately and an average number of cells per square was calculated. To work out the number of cells per ml, this value was multiplied by five (Cells are in a 1/5 dilution in trypan blue and media) and by 10^4.

2.8.1.2 MTT Assay (12-well tissue culture plates)

Cell viability was assessed by the ability of the cell mitochondria to breakdown MTT into a coloured formazan product (Levesque *et al.* 1995; Coote and Arain 1996). 1 mg of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) (Sigma) was added to tissue culture wells and incubated at 37°C for four hours. The supernatant was removed and 1 ml of lysing buffer (DMSO (Sigma)/ 0.04 N HCl) was added. 100 µl from each well was added in triplicate to a 96-well plate and the optical density was measured using an MRX Plate Reader (Dynatech Laboratories) at 540 nm with correction set at 630 nm. The amount of MTT and lysing buffer used was scaled as appropriate for different sizes of wells.

2.8.2 Experiments with RAW Macrophages

RAW cells were collected from flasks as described above and counted using the trypan blue exclusion method in a haemocytometer. Cells were seeded into 12- or 24- well tissue culture plates at 5 x 10^5 cells ml^{-1} and left to adhere to the plastic for two hours at 37°C. In some wells, macrophages were primed with 0.5 ng/ml IFNγ for four hours. After this, pneumolysin or other experimental substances (such as LPS) were added and the experiment started.

2.8.3 Experiments with Lung Epithelial Cells

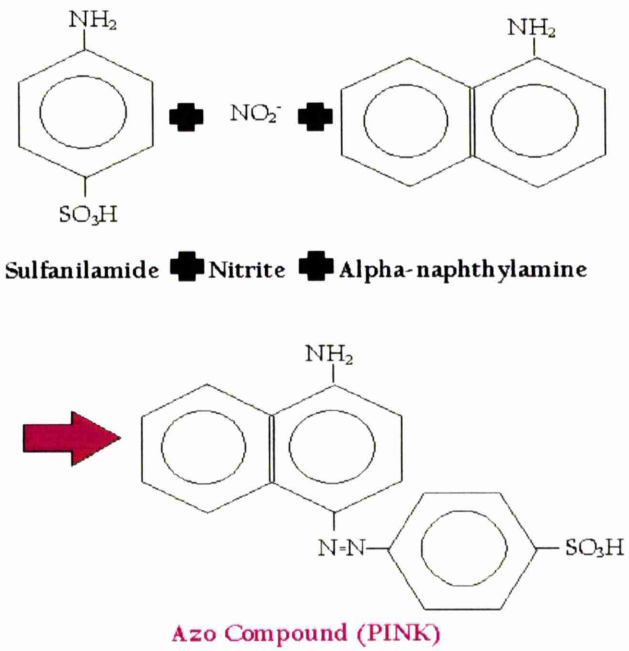
Lung epithelial cells were seeded the night before the experiment and allowed to adhere overnight at 37°C. A549 lung epithelial cells were seeded at 3 x 10^5 cells ml^{-1}, which had grown to 5 x 10^5 cells ml^{-1} by the start of the experiment. Small airway epithelial cells were seeded at densities recommended by Biowhittaker. These cells were then treated in the same way as the macrophages.

2.9 Nitric Oxide Measurement

Nitric Oxide is very difficult to measure directly as it has a half-life in the order of seconds. Nitrite (NO_2^-) and nitrate (NO_3^-) are stable end products and can be measured as an indication of NO production. Nitrite production from cell cultures was measured using the Griess reaction. 50 µl of supernatants from cell cultures and standards (sodium nitrite from 0.2 mM to 0.003 mM) were added in triplicate to a flat-bottomed 96-well plate. An equal amount of 0.1% alpha-naphtyl-amine in dH_2O and 1% sulphanilamide in 5% phosphoric acid (Griess reagent) were mixed and 50 µl added to every well of the plate. If nitrite is present a colour change occurs (see Figure 2.5) which can be read using a plate reader (Dynatech Laboratories) to measure absorbance at 540 nm.

In order to determine whether the tissue culture media contained a source of nitrate, a conversion assay was carried out by Dr A. Kerr. In this experiment, 50 µl of conversion buffer (appendix) was added to the media to convert any nitrate present to nitrite, which can be detected as described above, using the Griess reagent.

Figure 2.5 – Griess Reaction



When nitrite is present, a reaction occurs between the sulfanilamide and alpha-naphthylamine which results in the formation of a coloured azo compound. The colour can be measured in a spectrophotometer.

2.10 TNF Bioassay

A TNF bioassay was developed and optimised by Dr. A. Kerr from a method used at AstraZeneca Pharmaceuticals and published cytotoxity assays (Coote and Arain 1996; Levesque *et al.* 1995). The assay is based on the ability of TNF to kill cells from the TNF-sensitive L929 connective tissue cell line. Cell death was assessed by the MTT assay.

Late in the afternoon (to prevent cells proliferating to an unusable level before addition of samples), media was removed from confluent flasks of connective tissue cells and the monolayer washed with PBS. The cells were detached from the flask using trypsin and 10 ml of media was added. The cells were counted using a haemocytometer and adjusted to 3×10^5 cells per ml with medium. 200 µl of this cell suspension was added to each well of a flat-bottomed 96-well plate. The plates were then incubated overnight at 37°C. Early the next morning, these were checked to ensure the cells were not confluent or overcrowded. The medium was removed from all the plates and they were blotted dry on paper towels. 50 µl of 20 µg/ml Actinomycin D (Sigma) was added to each well to prevent connective tissue cell multiplication in wells without TNF activity. TNF standard (Sigma) was prepared to make a top standard of 2500 Uml^{-1} depending on the actual EC_{50} (1 U) information supplied with the standard. From this, eleven dilutions of one volume in three were made plus a zero standard to form a standard curve. Samples were prepared by making three 1 in 3 dilutions. 25 µl of the standards and samples – neat and dilutions - were added to the 96 well plates in triplicate. The plates were incubated overnight at 37°C. The next day, 100 µl of 1 mg/ml MTT was added to each well. Following four hours incubation with MTT, the supernatant was removed from all the plates and 100 µl of lysing buffer (DMSO/0.04 N HCl) was added to each well. The absorbance was measured using the Dynatech MRX Plate Reader (Dynatech Laboratories) at 540 nm with a correction filter set at 630 nm.

To interpret the results, a graph was plotted of A_{540} against $\log_{10}$TNF concentration. The absorbance when 50% kill occurs can be determined by subtracting the highest standard (lowest OD) from the zero standard (highest OD) and dividing by 2. The level of TNF at this OD can be read from the standard curve and should be close to 1 U. Graphs of positive samples can be plotted using the undiluted, 1/3, 1/9, 1/27 and the zero standard A_{540} values to give a sigmoid curve. The 50% kill OD can be extrapolated on the graph of the sample to

obtain the dilution of the samples where 50% kill occurs. This value is then multiplied by the level of TNF that gave 50% kill on the standard curve to give the level of TNF in the sample.

2.11 Propidium Iodide Staining of DNA

75 µl of cells in suspension were attached to Superfrost Plus slides (Menzel-Glaser) using a Cytospin-3 centrifuge (Shandon) at 600 rpm for four minutes. The slides were fixed in 50 µl of 4% formaldehyde in PBS at room temperature for ten minutes. The cells were washed in PBS and permeabilsed in 50 µl permeabilisation buffer for five minutes. After three more PBS washes, the cells were incubated in 50 µl of blocking buffer for ten minutes. 50 µl of 5 µg/ml propidium iodide containing RNaseA (200 µg/ml) (Calbiochem) was added to the cells, which were then incubated in the dark for thirty minutes. The cells were washed with 1% BSA in PBS three times for three minutes each. The slides were then allowed to dry and mounted in Vectashield (Vector Laboratories Inc.). The next day the slides were sealed with clear nail varnish.

2.12 Preparation of Samples for Microarray Analysis

2.12.1 Cell Culture and Stimulation

RAW 264.7 macrophages were seeded at 5 x 10^5 cells ml^{-1} in 24 well plates and allowed to adhere to the plastic for two hours. Thirty wells were seeded per experimental condition, which were as follows:

1) Media Alone
2) 100 ng Pneumolysin
3) 100 ng Pneumolysin plus 0.5 ng/ml IFNγ
4) 100 ng Pneumolysin – heat inactivated
5) 100 ng Pneumolysin – heat inactivated plus 0.5 ng/ml IFNγ
6) 0.5 ng/ml IFNγ

In keeping with other experiments, the macrophages in appropriate wells (for samples 3, 5 and 6) were primed with 0.5 ng/ml IFNγ for four hours. Pneumolysin was inactivated by heating to 56°C for ten minutes. A haemolytic assay confirmed that the protein was no longer active. 100 ng of wild type or heat-inactivated pneumolysin was added to appropriate wells, and PBS was added to control wells. The plates were incubated at 37°C for 24 hours. The experiment was carried out twice on different days and the RNA from each experiment

hybridised to different chips. Therefore for each experimental condition two datasets were obtained.

2.12.2 RNA Isolation

The supernatant was removed from the cells and 150 µl of lysis buffer from a total RNA miniprep kit (Stratagene) was added to each well. The lysis buffer for each of the thirty wells was then pooled into a 10 ml tube. Samples were stored at -80°C overnight at this stage if necessary. The RNA was purified according to the instructions supplied with the kit. The Stratagene miniprep kit was found to give better RNA yields than a Qiagen midiprep kit so six columns from the miniprep kit were used for each sample. Each RNA binding column was eluted with 2 x 30 µl of elution buffer into the same eppendorf to give a total of 360 µl of RNA for each sample.

2.12.3 Preparation of RNA for Affymetrix GeneChip® Protocol

The concentration of the RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. An absorbance of 1 at 260 nm corresponds to 40 µg of RNA per ml. The ratio of A_{260}/A_{280} was determined as an indication of the purity of the RNA sample. For each GeneChip® it is recommended to use between 5 µg and 40 µg. For these experiments 20 µg RNA was used at a concentration of 2.2 µg/µl.

Using the A_{260} readings, a volume of RNA estimated to contain ~30 µg was precipitated. 0.1 volumes of 3 M sodium acetate (pH4.8) and 0.8 volumes Propan-2-ol were added to the RNA sample and incubated on ice for thirty minutes. This was centrifuged at 16000 x g for thirty minutes at 4°C, the supernatant was removed and 100 µl of 70% ethanol was added to the pellet. The sample was centrifuged again for ten minutes. The pellet was air dried and resuspended in 12 µl of RNase free water.

Formaldehyde agarose (FA) gel electrophoresis (appendix) was used to check the integrity and size distribution of the RNA. 1 µl of concentrated RNA was added to 3 µl of depcH_2O and 1 µl of 5 x loading buffer. The sample was then incubated at 65°C for three to five minutes and chilled on ice before loading onto a 1.2% agarose gel. The samples were separated at 80 mV for approximately one hour.

The RNA samples were then sent to MBSU (Molecular Biology Support Unit) in the University of Glasgow for hybridisation to Genechip® arrays. Following protocols developed by Affymetrix, double stranded cDNA was synthesised from the RNA. A portion of the cDNA was then labelled and amplified using biotinylated nucleotides and *in vitro* transcription. Following the *in vitro* transcription reaction and quantification by standard methods, 20.5 μg of cRNA was fragmented and added to a hybridisation cocktail containing probe array control transcripts, BSA and herring sperm DNA. This mixture was hybridised with the probe array for 16 hours. The probe arrays were then washed and stained with Streptavidin-phycoerithrin conjugate using an automated protocol on the GeneChip® Fluidics Station 400 (Affymetrix). Each array was scanned twice using the GeneArray® scanner. Affymetrix GeneChip® software was used to calculate an average of the two images, define the probe cells and compute an intensity for each cell.

Analysis of data was carried out using Affymetrix® Microarray Suite 5.0 software and the Affymetrix website, www.netaffx.com.

2.13 Statistical Analysis

Results are expressed as median values with maximum and minimum value error bars unless otherwise stated. Levels of inflammatory mediators and cell viability were compared using non-parametric Mann Whitney U analysis with $p<0.05$ considered statistically significant for all analyses (Wardlaw 2000). A Bonferroni correction was used for multiple analyses.

All viability curves show mean values +/- the standard error of the mean. Curves were fitted in Microsoft Excel by χ^2 minimisation using a three-parameter model that matched the characteristic shape of the datasets. For one dataset, a four-parameter model was used to account for the fact that the data did not reach zero.

3. PRODUCTION AND PURIFICATION OF PNEUMOLYSIN

3.1 Purification of Pneumolysin Protein

A method to purify toxin from *E. coli* cell crude extracts containing pneumolysin was already available (Mitchell *et al.* 1989) and included the use of high performance liquid chromatography (HPLC). As HPLC was not convenient for producing large amounts of protein the method was adapted for use of perfusion chromatography technology using the BioCAD® 700E Perfusion Chromatography Workstation.

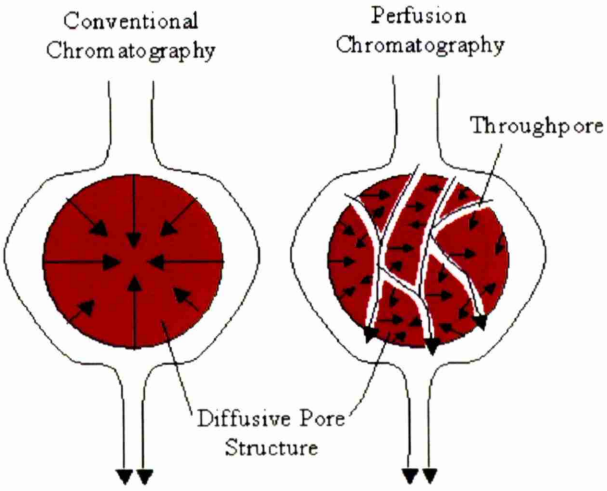
3.1.1 Perfusion Chromatography

Liquid Chromatography is used to purify proteins from potentially complex mixtures and allows recovery of biological activity of molecules. In order for chromatographic separation to occur, solute molecules must interact with the surface of the media particles. A chromatography particle is often highly porous to maximise the internal surface area for binding. Solute particles are carried to the perimeter of the particles by a liquid stream as it flows through the column bed. Transport of the solute molecules to the inside surfaces of the particles occurs by diffusion within the pores. Diffusion is a slow process and was the limiting factor in traditional chromatography separation. HPLC enables higher speed separations by a reduction in the particle diameter to the range 3-30μm. This reduces the distance for solute diffusion within particles and allows operation at higher flow rates. However it is not practical to reduce the size of particles below this range to further increase the speed.

Perfusion Chromatography technology was introduced by Perceptive Biosystems (Now called Applied Biosystems) in 1989. POROS™ Perfusion Chromatography media further increases the speed of intraparticle diffusion by incorporating two types of pores within each chromatography particle; large throughpores that transect the particle, and short diffusive pores that branch off from the throughpores (see Figure 3.1).

The length of the diffusive pores is small in comparison to the total particle diameter (typically less than 1μm), so the time required for sample molecules to diffuse to and from internal binding sites is very short. This increases the speed under which chromatography can be performed so large volumes can conveniently be produced (Regnier 1991).

Figure 3.1 – Perfusion Chromatography



Pore structure and mass transport in conventional and perfusive particles. Adapted from "The Busy Researcher's Guide To Biomolecule Chromatography" Applied Biosystems 1996.

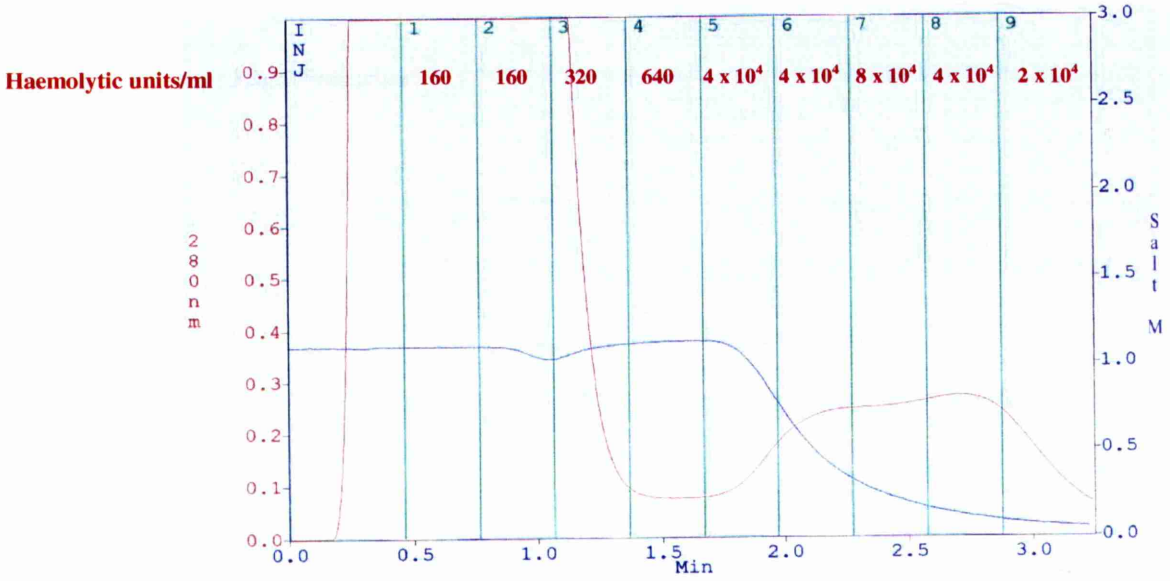
3.1.2 Purifying Pneumolysin using Hydrophobic Interaction Chromatography

Wild type pneumolysin was produced from M15(pRep4) *E. coli* with a pKK233-2 vector containing the pneumolysin gene from the *Streptococcus pneumoniae* strain D39. Figure 3.2 shows a typical example of protein recovery from a single purification using hydrophobic interaction chromatography (HIC) on the BioCAD. The chromatogram from the BioCAD in Figure 3.2(a) shows that most of the protein has passed through the column. As the salt concentration was decreased, the toxin was eluted. Fractions with haemolytic activity were analysed by SDS-PAGE (Figure 3.2(b)). The active fractions contained a band at approximately 53-kDa corresponding to the size of pneumolysin. This 53-kDa band was confirmed to be pneumolysin using a western blot (not shown).

3.1.2.1 Purifying Pneumolysin using Ion Exchange Chromatography

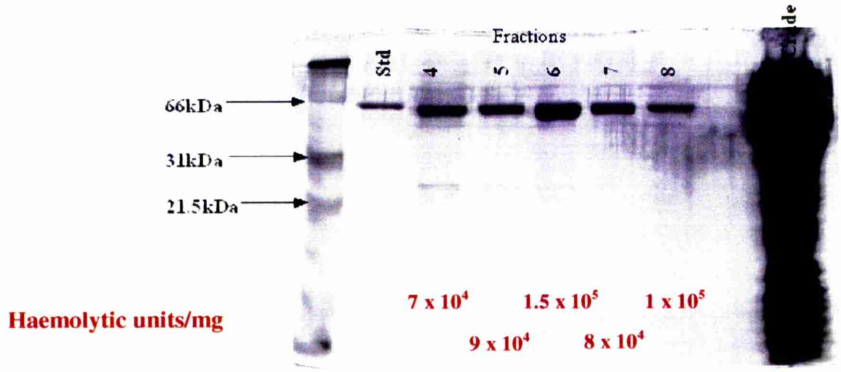
Other purification methods and combinations were used whilst optimising the pneumolysin purification. These included ion exchange chromatography (IEC) before and after the HIC column. By measuring the haemolytic activity at various stages during the purification process, it was apparent that these methods resulted in a substantial loss of protein. The HIC column provided a quick and efficient method of purifying large batches of pure protein.

Figure 3.2(a) – Purifying Pneumolysin using Hydrophobic Interaction Chromatography



A representative chromatogram is shown. This trace shows the 280 nm reading (red line), used to measure the protein passing through the detector. As the salt concentration is decreased (blue line), the protein is eluted from the column. The green lines and numbers represent the fractions collected from the column. The majority of the protein is eluted in fractions 5-9; this was confirmed by haemolytic assay. HU/ml are shown on the trace. Purity of these fractions was assessed by SDS-PAGE. INJ, sample injection onto column.

(b) – Purity of Fractions obtained using HIC



The Coomassie blue-stained gel shown is representative of pneumolysin recovered. Fractions from the HIC column do not contain the same extent of contaminating proteins seen in the crude extract shown in the far right lane. Fractions 4, 5 and 6 are contaminated with a protein of 23-kDa so fractions 7 and 8 were kept. The crude lysate had a haemolytic activity of 6.4 x 10^5 HU/ml, the fractions show HU/mg pneumolysin protein. Std, standard pneumolysin previously purified by Dr. N. Rai.

3.1.3 Production of Pneumolysin using Metal Chelate Chromatography

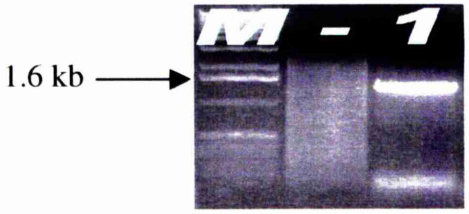
When the mutant pneumolysin protein with a modified cysteine residue at position 428 was purified it was noted that its retardation on the HIC column was not as strong as the wild type protein (Saunders *et al.* 1989). This indicated that the mutation might have altered the

hydrophobicity of the protein thereby making HIC a less effective method of purification. By using metal chelate chromatography, any mutations that may alter the behaviour of the protein, will not affect the binding of the protein to the chromatography column. As described in section 3.4, wild type and mutant pneumolysin affinity-tagged genes were cloned into the T7 expression system for high level production of protein in the pET-33b(+) vector. These proteins can be purified using the 6xhistidine affinity-tag (His-Tag) that this vector attaches to the N-terminus of the protein enabling the purification method to be standardised.

3.1.3.1 Isolating the pneumolysin gene from genomic DNA

The wild type pneumolysin gene was cloned into the pET-33b(+) vector and used to optimise metal chelate chromatography. Forward and reverse primers for the pneumolysin gene were used (Primers 1&2 in section 2.7) to amplify the pneumolysin gene from genomic DNA of D39 *S. pneumoniae*. Different concentrations of $MgSO_4$ were used to optimise the isolation of the wild type pneumolysin gene. Figure 3.3 shows the agarose gel of the PCR product using 2 mM $MgSO_4$. This concentration was used for all further reactions.

Figure 3.3 – Extraction of the Pneumolysin gene from *S. pneumoniae*

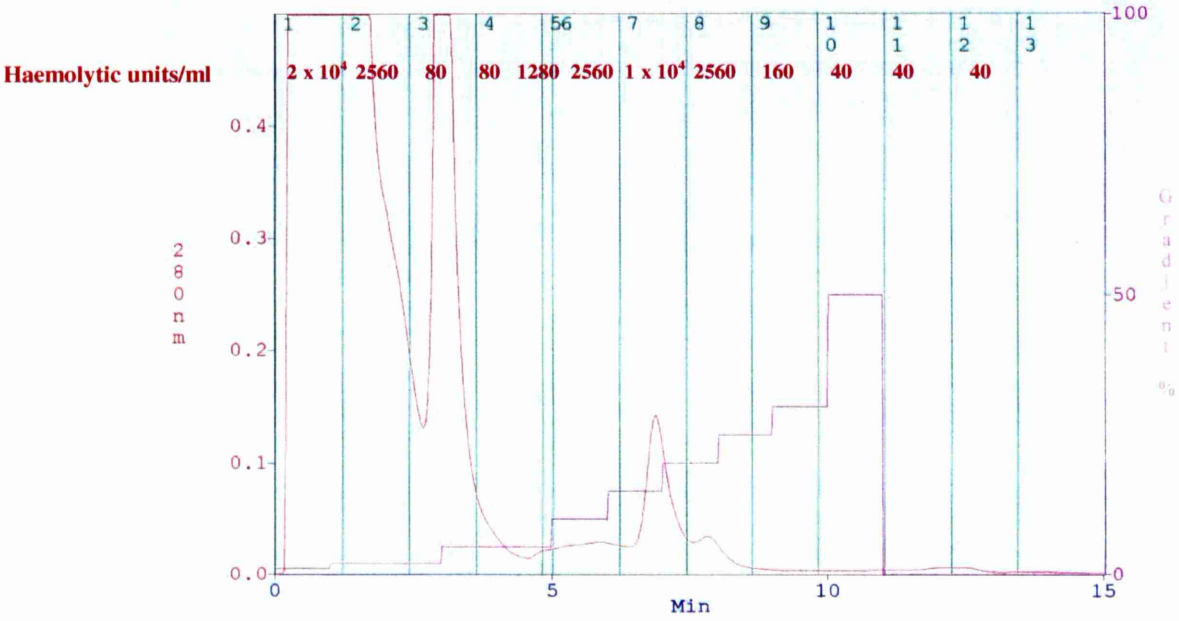


Primers were used to amplify the pneumolysin gene from *S. pneumoniae* genomic DNA. Lane 1 shows the PCR product of the correct size. M = 1 kb ladder, - = negative control

The size of the full pneumolysin gene is 1.4 kb so the DNA band on the gel in Figure 3.3 is the correct size. The DNA from lane 1 was extracted from the gel using a Qiagen gel extraction kit. The pneumolysin gene was cloned into the pET-33b(+) vector using the methods described in section 2.7. The results are shown in section 3.4 along with the mutant pneumolysin genes. Figure 3.4 shows a typical example of protein recovery of His-Tag pneumolysin using the metal chelate column on the BioCAD. A large peak in A_{280} can be seen in fraction 7 of Figure 3.4(a) when the pneumolysin protein is eluted from the column. Impurities from the crude lysate flowed straight through the column and can be seen as large peaks coming off the column in fractions 1-3. Figure 3.4(b) shows the single protein bands seen when fractions 5-8 were run on an SDS gel. The protein bands can be seen slightly

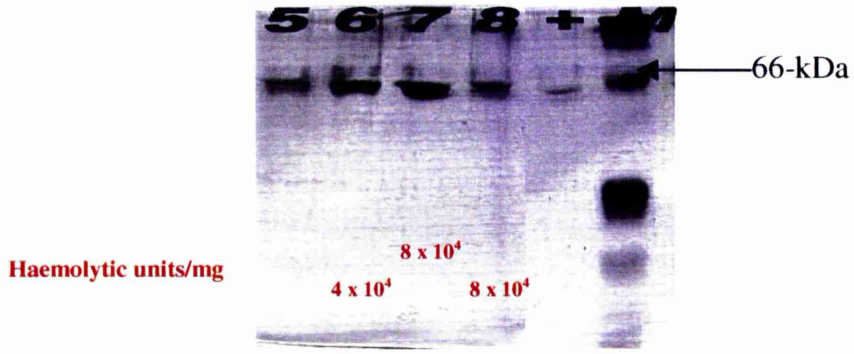
below the 66-kDa marker. The protein from the MC chromatography is slightly larger in size than that seen in Figure 3.2(b) as the His-Tag adds approximately 100 residues to the N-terminal end of the protein.

Figure 3.4 – (a) Purifying Pneumolysin using the Metal Chelate Chromatography



A representative chromatogram is shown. In this trace, the red line shows the protein absorbance at 280 nm. The magenta line shows the increase in the imidazole gradient used to elute the protein from the column. The protein is eluted when between 150 and 200 nM of imidazole is used in fractions 6-8; this was confirmed by haemolytic assay. HU/ml are shown on the trace. The green lines and numbers show the fractions collected.

(b) - Pneumolysin fractions from Metal Chelate Chromatography

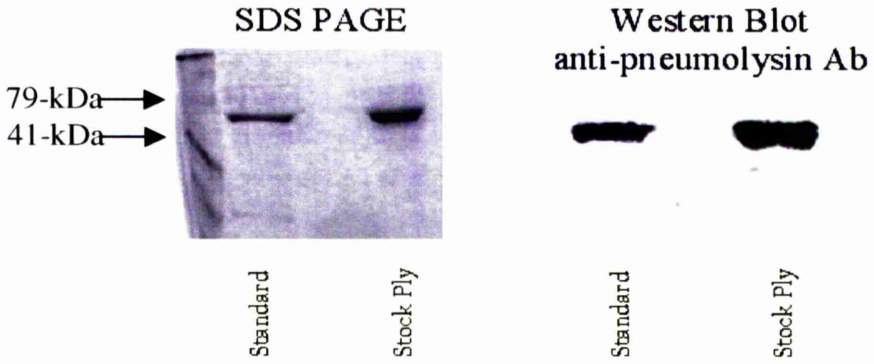


The Coomassie blue-stained gel shown here is representative of pneumolysin recovered. The purity of the fractions were analysed by SDS PAGE. The fractions show HU/mg pneumolysin protein. 5-8 = fractions from the metal chelate column. + = Pneumolysin previously purified using commercially bought, small scale MC column. M = Kaleidoscope Marker

3.2 Pneumolysin Stock

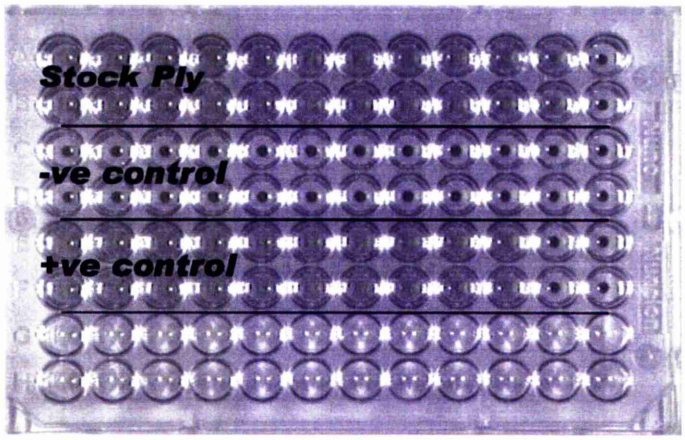
By creating a pneumolysin stock, we could ensure that for each experiment, the protein used was of the same concentration and specific activity. Stocks of pneumolysin and His-tagged pneumolysin were made by pooling fractions that had a haemolytic activity of more than 4 x 10^4 and showed no contaminating proteins on an SDS gel. The pooled pneumolysin fractions were run on an SDS gel and the bands at 53-kDa confirmed to be pneumolysin by western blot (see Figure 3.5).

Figure 3.5 – (a) Purity of Stock Pneumolysin



This figure shows the pooled pneumolysin fractions used to create a stock batch. The western blot confirmed the protein bands at 53-kDa to be pneumolysin. Standard pneumolysin was previously purified by Dr. N. Rai.

(b) - Haemolytic Assay



An example of a haemolytic assay is shown above. The stock pneumolysin is in duplicate (rows A and B) diluted 1/2 across the plate. The –ve control is PBS alone. The +ve control is previously purified pneumolysin obtained from Dr. N Rai. The end-point of the assay for the stock pneumolysin is in well 11.

The haemolytic activity of the stock pneumolysin is 4 x 10^4 HU/ml (Figure 3.5b). The concentration is 0.4 mg/ml so specific activity of the protein is 1 x 10^5 HU/mg. The stock of pneumolysin created using MC chromatography is 0.1 mg/ml and has a haemolytic activity of 1 x 10^5 HU/mg. The pneumolysin was aliquoted and stored with 20% glycerol at -80°C.

3.3 Quality Controls of Pneumolysin Protein

A number of experiments were carried out to assess the purity and state of the toxin stock. These are described below.

3.3.1 DNA Sequencing of pneumolysin clone

DNA sequencing of the His-Tag wild type clone at MBSU (Molecular Biology Support Unit – Glasgow University) confirmed the pneumolysin gene sequence and alignment was correct. The full sequence can be seen in section 9.1.

3.3.2 N-Terminal Sequencing of Pneumolysin Stock

Table 3.1 – N-Terminal Sequencing Results of Pneumolysin

NUMBER	STOCK PNEUMOLYSIN	KNOWN SEQUENCE	AMINO ACID
Start	(Met)	Met	Methionine
1	Ala	Ala	Alanine
2	Asn	Asn	Asparagine
3	Lys	Lys	Lysine
4	Ala	Ala	Alanine
5	Val	Val	Valine
6	Asn	Asn	Asparagine
7	Asp	Asp	Aspartic Acid
8	Phe	Phe	Phenylalanine
9	Ile	Ile	Isoleucine
10	Leu	Leu	Leucine

The stock pneumolysin was sent to PNACL (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester) and the N-terminal sequence of the protein was obtained using Edman degradation. This was performed by using an automated protein sequencer (Applied Biosystems 476). The first ten amino acid residues were sequenced, and matched the N-terminus of the known pneumolysin sequence. Two species were generated, one with

an N-terminal methionine and one with an N-terminal alanine. This is seen quite often in samples and probably occurs as a percentage of the N-terminal methionine is removed by aminopeptidase activity during purification (Pers Comm. Dr K. Lilley PNACL). The sequence generated can be seen in Table 3.1. As only one sequence was generated the stock is free from major contaminating proteins.

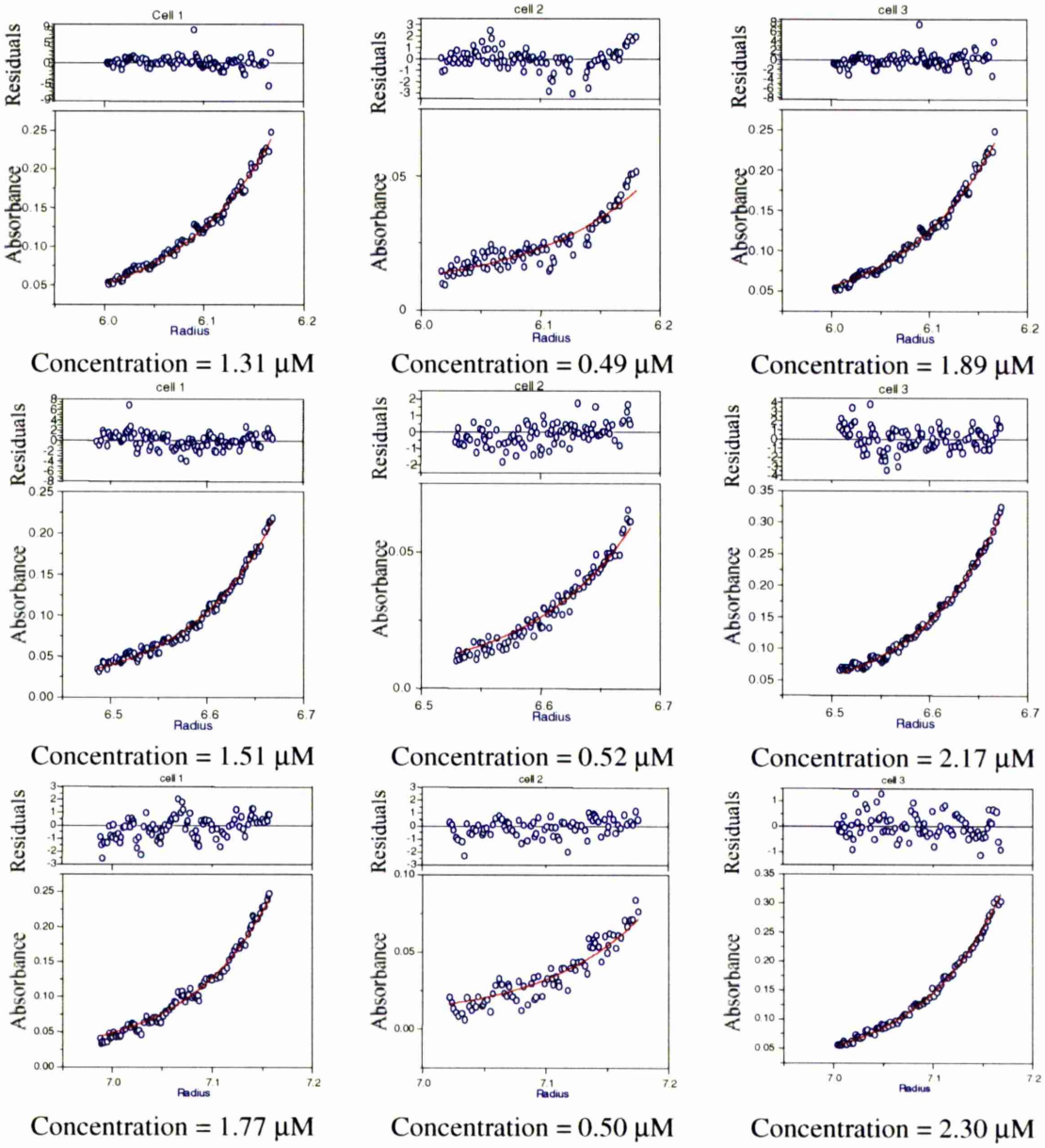
3.3.3 Analytical Ultra Centrifugation

By using appropriate modes of analytical ultracentrifugation (AUC), information about molecular weight, density and shape of a macromolecule can be obtained. In AUC, particles are made to move by centrifugal force. The distribution in concentration of the particles along the length of the centrifuge tube (or cell) is determined at one or more times as a function of time (in sedimentation velocity mode) or at thermodynamic equilibrium (in sedimentation equilibrium mode). The result of a sedimentation velocity run is a sedimentation coefficient, a number that gives information about the molecular weight and shape of the particle. At lower rotor speeds, when the concentration distribution no longer changes with time, the particles are said to have reached sedimentation equilibrium, which yields data about molecular weight, density and oligomer composition of the sample.

Pneumolysin has a tendency to aggregate in solution and form inactive species (Gilbert *et al.* 1998). We wanted to make sure that the pneumolysin stock was monomeric and stably stored at -80°C.

Sedimentation equilibrium data from nine concentrations of pneumolysin were obtained. This was done with the assistance of Dr O. Byron and Mr G. Campbell. Data was fitted to the ASSOC4 model from the Beckman Origin software according to the equation cited by Gilbert *et al.* (1998). A global fit of the apparent molecular weight of all nine concentrations was calculated using this software and calculated to be 54700-kDa (Figure 3.6). The actual molecular weight of pneumolysin is 52772-kDa. There is no increase in the apparent molecular weight indicative of dimer formation or toxin aggregation. This suggests that the stock pneumolysin is monomeric and storing at –80°C in glycerol does not induce oligomerisation. The highest concentration of pneumolysin used in this experiment is ~2.3 μM so we cannot be sure that at higher concentrations the pneumolysin remains monomeric. In experiments where <120 μg/ml of pneumolysin is used (2 μM is equivalent to ~100 μg/ml) we can, however, be confident that the protein is in a monomeric state.

Figure 3.6 – Sedimentation Equilibrium of Pneumolysin



These plots show the fitting of experimental data (at 16 k rpm) showing concentration radial distribution with minimal residual deviation. Using a global fit of all nine concentrations Mw, app was calculated to be 54700-kDa +/- 940.5. The actual molecular weight of pneumolysin is 52772-kDa. Variance = 1.256. Degrees of Freedom = 843

3.3.4 Endotoxin Removal from Pneumolysin

Endotoxins are part of the outer wall of Gram-negative bacteria and are shed from the membrane and released upon cell lysis. The biological activity is associated with the lipopolysaccharide (LPS). In aqueous solution, endotoxins exist in various states of aggregation, which are very stable. Individual molecules have to be explicitly created by

using detergents (Triton X-114), bile acids (deoxycholic acid) or chelators (EDTA) and have molecular weights between 10 and 20-kDa.

Due to the stability of endotoxin aggregates, they are difficult to remove from solutions. Boiling for thirty minutes will not destabilise the aggregates. Low levels of LPS can be found in commercially bought nutrient broth (0.035 ng/ml in C+Y broth used to grow *S. pneumoniae*), trypsin and RNase (Braun *et al.* 1999a). A study by Gao *et al.* (2001) found that commercial preparations of LTA contain endotoxin contamination that contributes to activation of NO from mouse macrophages *in vitro*. This activity could be inhibited by PMB, and they found that LTA alone could only induce NO from macrophages in the presence of IFNγ.

As pneumolysin is purified from a Gram-negative expression system i.e. *E. coli*, it is possible that endotoxin could contaminate the protein samples. LPS itself is not directly toxic but exerts its biological effects by stimulating host cells to produce inflammatory mediators which elicit various host responses. It can lead to the production of TNF, NO and lipid mediators such as prostaglandins and leukotrienes. In order to study pneumolysin-induced production of these mediators, it is important to be able to distinguish pneumolysin activity from LPS activity. The following methods were evaluated to reduce the levels of endotoxin.

3.3.4.1 Measuring Endotoxin Levels during Pneumolysin Purification

Endotoxin levels were measured at all stages of pneumolysin production using the HIC column (Table 3.2). Ultracentrifugation and ammonium sulphate precipitation steps were added to the usual purification process to see if these would have any effect on endotoxin levels.

These results show there are high levels of endotoxin present at all stages of purification. The Aldevron LAL assay has a limited detection range so samples with high levels of LPS must be diluted to fall within this range. The dilution factor of the samples is shown in the table and several samples were diluted further but still did not fall within the detection levels of the assay. This is why the EU levels are expressed as "greater than" a value. This makes it difficult to identify the stages of purification contributing to increased or decreased levels of LPS in the sample. As the levels were so high in these samples, further dilutions would not

help to identify which stages of purification could potentially reduce endotoxin contamination.

Table 3.2 – Results of Endotoxin Testing at Aldevron

NO.	STAGE OF PURIFICATION	MG/ML	ENDOTOXIN TESTING		
	Growth and induction of bacteria to produce pneumolysin.	Not tested.	Dilution Factor	EU/mg	EU/ml
	Harvest cell pellets by centrifugation	Not tested.			
	Disrupt Cells				
1	Soluble Fraction	5	500	>107	>535 †
2	Insoluble Fraction	11	500	>48	>528 †
	Ultracentrifugation				
3	supernatant	3	400	>143	>429
4	pellet	2	400	>215	>430 †
	Ammonium Sulphate precipitation				
5	supernatant	0.5	500	>1074	>537
6	precipitate	2.5	400	>172	>430
7	Filter Crude Protein	3.2	400	>134	>429
8	HIC	0.1	200	>1953	>215
9	Dialysis (to remove salt)	0.1	400	>3000	>430
10	Metal Chelate Chromatography*	0.4	20	<5	<5
11	PBS	0	20	-	<5

Total protein concentrations (mg/ml) were determined using the Bradford assay due to the turbidity of some samples. Samples were sent to Aldevron for endotoxin testing using the Limulus Amebocyte Lysate (LAL) Assay. EU = Endotoxin Units. Note: 5 EU is the lower limit of the assay. * This sample contains His-tagged pneumolysin that was purified using a B-Per 6xHis protein purification kit (Pierce). † The viscous nature of these samples may interfere with the assay.

A purified sample of His-tagged pneumolysin was also sent to measure the endotoxin levels (Sample 10 in Table 3.2). This sample had <5 EU/ml suggesting that metal chelate chromatography method may reduce endotoxin levels as well as providing a standardised method of purification. A more sensitive, in-house LAL assay (BioWhittaker) showed there

were still residual levels of LPS present in this sample. The following methods were evaluated to try to remove the remaining endotoxin.

3.3.4.2 Polymyxin B Agarose

Polymyxin B (PMB) is an antibiotic isolated from *Bacillus polymyxa*. It acts primarily by binding membrane phospholipids and disrupting the cytoplasmic membrane. Because of its affinity for bacterial cell membranes (lipopolysaccharides), PMB can be used to remove endotoxin from solutions (Mölvig and Baek 1987).

Detoxi-Gel endotoxin removing columns (Pierce) were used. These are ready-poured columns containing PMB agarose. Unfortunately these were not successful as the protein stuck to the PMB in the columns and was only released with the LPS, when the column was stripped with sodium deoxycholate.

3.3.4.3 Biobeads

This experiment was carried out by Mr G. Meiklejohn. Triton-X-114 is used to remove LPS from the sample, then SM-2 Biobeads (Bio-Rad) are used to remove the Triton-X. There was no pneumolysin detectable, by haemolytic assay, at the end of the protocol. The pneumolysin may have bound to the beads or inactivated in some way by the Triton-X. Triton-X lyses cells and therefore interferes with the haemolytic assay, making it difficult to see at what stage the protein loss occurred. Other methods of measuring the pneumolysin at different stages were not tried.

3.3.4.4 Different methods of lysing the cells

Trial runs of metal chelate chromatography using small pre-packed columns (Pierce) produced protein with low (<5 EU/ml) levels of endotoxin (Sample 10 in Table 3.2). When the purification was scaled up for use on the BioCAD, the endotoxin levels were higher than in the pneumolysin obtained using the Pierce columns. The major difference between the two protocols was the method of cell disruption. With the Pierce columns, the cells were disrupted using a lysis buffer supplied with the kit rather than the benchtop cell disrupter. For this reason, a protein preparation was made where half the batch was lysed using Bugbuster™ (Novagen), and half using the cell disrupter. After MC chromatography using the BioCAD, both methods produced similar amounts of pneumolysin with the same concentration (0.09 mg/ml) and haemolytic activity (1.1×10^5 HU/mg). The pneumolysin

produced using the cell disrupter had twice as much endotoxin (1.2 EU/100 ng pneumolysin) than the pneumolysin prepared using the Bugbuster™ (0.68 EU/100 ng pneumolysin).

The formulation of the Bugbuster™ lysis buffer is proprietary. It does contain non-ionic detergents that could help to remove the LPS by breaking bonds between endotoxin aggregates, which are very stable, and also between endotoxin and the pneumolysin molecules.

Conclusion: It was not possible to remove endotoxin further by use of polymyxin B or biobeads. By using Bugbuster™ to lyse the cells, there was a two-fold reduction in LPS levels.

3.3.5 Levels of endotoxin present in the pneumolysin samples

The levels of endotoxin in the stocks of pneumolysin were measured in a LAL assay (BioWhittaker) and are shown in Table 3.3.

Table 3.3 – Endotoxin Levels in stock Pneumolysin

CHROMATOGRAPHY METHOD	CONCENTRATION OF PLY	EU/MG	EU PER 100NG PLY	PG LPS PER 100NG PLY
HIC	0.4 mg/ml	20000	2	200
MC	0.1 mg/ml	6800	0.68	68

HIC, Hydrophobic Interaction Chromatography; MC, Metal Chelate Chromatography; EU, Endotoxin Units. 10 EU = 1 ng endotoxin (Information obtained from both BioWhittaker and NIBSC)

The amount of endotoxin present in 100 ng of pneumolysin is less than 5 EU. In vaccine development, the general rule is that there must be less than 5 EU per kilo, per hour in a dose of vaccine (Council of Europe 2002). However this varies between vaccines and can be between 10-100 EU per dose (Pers comm. Dr. S. Paul, NIBSC)

A study by Braun *et al.* (1999a) investigated pneumolysin-induced NO production. In their study they showed that incubation of RAW macrophages with up to 5 ng of LPS in the absence of IFNγ did not stimulate detectable nitrite production. They also showed that PMB, a strong inhibitor of endotoxin, significantly reduced LPS-induced NO production but did not significantly reduce pneumolysin-induced NO production. They also heat-inactivated the toxin and found the NO-inducing activity was reduced.

Table 3.4 – Examples of Endotoxin Levels in Reagents

SAMPLE	AMOUNT OF LPS	REF
C+Y media	0.35 EU/ml	Braun *et al.* 1999a
TNF standard	<1 EU/μg	Sigma Data sheet for product number T7539
Antibody preparation	<0.15 EU/ml	Rijneveld *et al.* 2002a
H. influenzae porin preparation	0.001% (0.5 EU per dose)	Galdiero *et al.* 2001
Recombinant pneumolysin purified from *E.coli*	4 EU/ml (0.2 pg/100 ng Pneumolysin)	Braun *et al.* 1999a
Recombinant pneumolysin purified from *E.coli*	<0.02 EU/ml	Cockeran *et al.* 2001
Recombinant pneumolysin purified from *E.coli*	0.1 EU/ml	Baba *et al.* 2002

This table gives examples of the level of endotoxin in protein preparations in published articles and in commercially available reagents.

Although the levels of endotoxin are not as low as those in previous publications (Table 3.4), they fall within the ranges acceptable as vaccine doses. Data will be presented later to show that these levels of LPS do not contribute to the activity of pneumolysin.

3.4 Production of Mutant Pneumolysin Protein

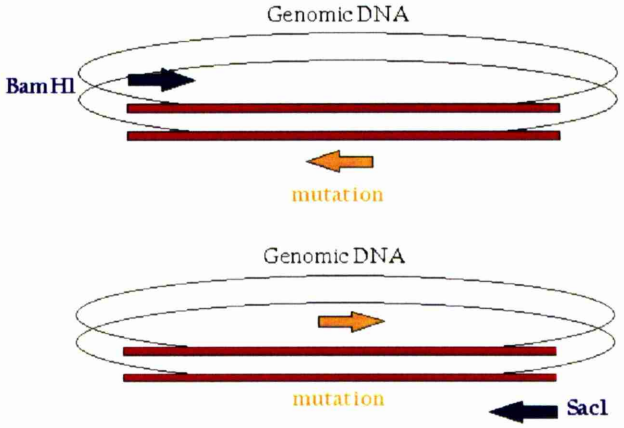
As described in section 1.4.4.3, De Los Toyos *et al.* (1996) raised monoclonal antibodies to various regions of pneumolysin. One of these antibodies was highly effective in its ability to neutralise haemolytic activity and prevented the assembly of pneumolysin pores in liposomes. This antibody recognised a region between the two asparagine residues at positions 142 and 143. One of the aims of this project is to evaluate the role of oligomerisation in pneumolysin-induced inflammatory mediator production. By mutating the region recognised by this neutralising antibody, we should produce a pneumolysin protein unable to oligomerise and form pores. Two mutations were designed:

1) A deletion of both asparagine residues 142 and 143
2) A mutation of the asparagine at position 143 to an aspartic acid.

Primers for each end of the pneumolysin gene (1 & 2 see section 2.7.1) were used to isolate the wild type pneumolysin gene from D39 *S. pneumoniae* genomic DNA. For each mutant, two fragments were isolated using the primers described in section 2.7.1. These fragments

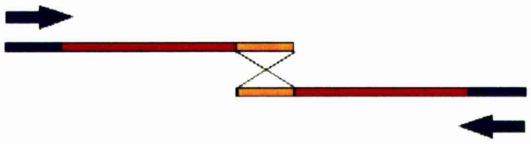
consisted of either the front or reverse end of pneumolysin plus the mutation. The different stages of introducing the mutations to the pneumolysin gene by PCR are shown in Figure 3.7. The conditions used for the PCR were the same as those used for making the full-length wild type gene and are described in the methods section 2.7.2.

Figure 3.7 – PCR Rationale



Design primers to introduce mutation and restriction sites. Produce two mutated fragments via PCR

These fragments are shown on the agarose gel in Figure 3.8

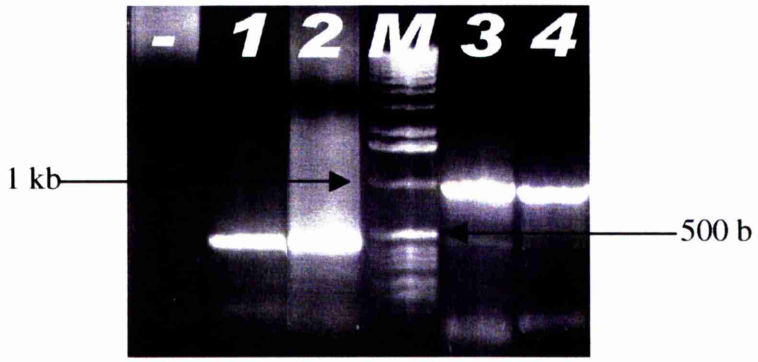


Join fragments in fusion PCR reaction

The products from the fusion PCR are shown in Figure 3.9

1.4 Kb PCR product containing pneumolysin gene plus restriction sites and mutation

Figure 3.8 – Pneumolysin gene fragments extracted from *S. pneumoniae*

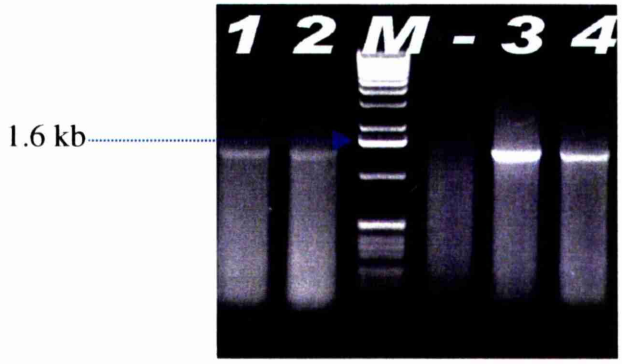


Pneumolysin gene fragments were extracted using primers to introduce mutations from D39 *S. pneumoniae* genomic DNA. M = 1 kb ladder, - = negative control, 1 = Front section of pneumolysin with substitution mutation, 2 = Front section of pneumolysin with deletion mutation, 3 = End section of pneumolysin with substitution mutation, 4 = End section of pneumolysin with deletion mutation.

The primers that introduce the mutations bind between bases 418-441 (substitution mutant) and between 410-443 for the deletion mutant. The pneumolysin gene is 1.4 kb long, so the PCR should yield front fragments of about 400 kb and end fragments of just under 1 kb. The fragments shown in Figure 3.8 are the correct size. The DNA from lanes 1 to 4 was extracted from the gel using a Qiagen gel extraction kit.

The next step was a fusion PCR to join the front and end sections of the pneumolysin containing each mutation. The amount of starting DNA, the annealing temperature, $MgSO_4$ concentration and the primer concentrations were all optimised before a successful PCR reaction was achieved; these conditions used are described in section 2.7.2. The PCR products obtained from this reaction are shown in Figure 3.9. These products contain the whole pneumolysin gene and are 1.4 kb.

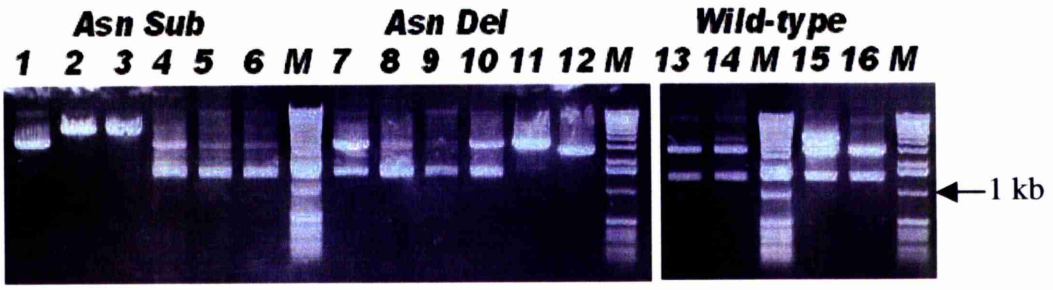
Figure 3.9 – Fusion PCR Products



M = 1 kb ladder, - = negative control, 1&2 = Fusion of the two fragments containing the substitution mutation, 3&4 = Fusion of the two fragments containing the deletion mutation

The DNA was extracted from the gel using a Qiagen gel extraction kit and ligated into the PCR-Script™ vector and transformed into competent cells. Blue/white screening was used to select transformants. Five suitable (white) colonies for each clone were picked from the plates and overnight cultures were grown by inoculating LB with a single colony. The plasmid DNA from each culture was isolated using a Qiagen DNA miniprep kit. The PCR products were cloned into PCR-Script™ as blunt-ended products using the restriction enzyme SrfI. This means that the pneumolysin gene could insert into the plasmid in two different orientations. The plasmid DNA was cut using the restriction enzyme BamH1 to check which plasmids contained pneumolysin gene inserts in the correct orientation (See section 2.7.3 and Figure 2.4).

Figure 3.10 – Restriction digests of transformed plasmids

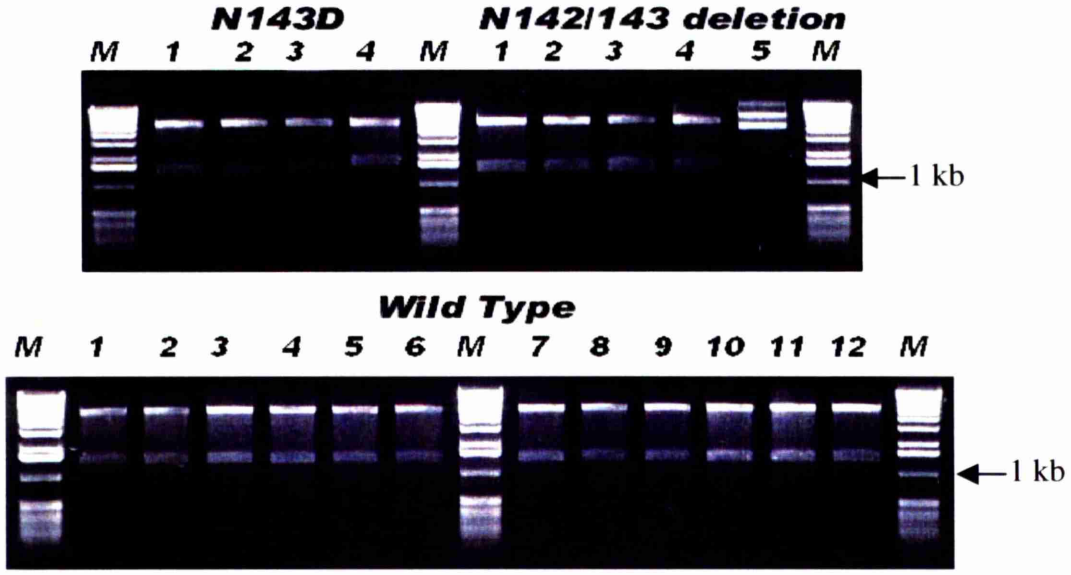


Restriction digests of the plasmid DNA was carried out to determine which plasmids contained the pneumolysin gene in the correct orientation. Asn Sub = N143D substitution mutant, Asn Del = 142/143 deletion mutant.

Figure 3.10 shows the results of the restriction digests. Some of the digests do not have inserts as there is only one band present e.g. lane 1, which is the size of the plasmid (~3 kb). Some may have the insert in the incorrect orientation e.g. 2 & 3, as there is only one band which is bigger than the plasmid alone. Digests with a band at ~1.4 kb and ~3 kb have an insert of the correct orientation. Two successful clones of each pneumolysin type were used to make glycerol stocks.

The forward and reverse primers used to extract the pneumolysin gene from the genomic DNA also introduce a BamHI site at the front of the gene and a SacI site at the end of the pneumolysin gene. This allowed the pneumolysin inserts to be "cut" from the PCR-Script™ plasmid using BamHI and SacI creating "sticky-ends". The pET-33b(+) vector was also treated with the same restriction enzymes. The inserts and vector were purified and ligation reactions were set up according to instructions in the pET-33b(+) manual. The ligated plasmids were transformed into Novablue competent cells (appendix). Transformants were selected using kanamycin resistance introduced by the presence of the pET-33b(+) vector. The plasmids were purified using a Qiagen miniprep kit and cut again with BamHI and SacI to check they had inserts of the correct size (Figure 3.11). One clone of each mutant was grown overnight at 37°C and transformed into BL21 cells ready for gene expression. Glycerol stocks were also made.

Figure 3.11 – Restriction Digest of Pneumolysin inserts in pET-33b(+)



The restriction enzymes BamHI and SacI were used to check the size of the pneumolysin inserts in the pET-33b(+) vector. Asn Sub = N143D mutant, Asn Del = N142/143 deletion mutant. All of the plasmids have inserts of the correct size except N143D 4 and N142/143 deletion 5.

3.5.1 Sequencing the Pneumolysin Clones

The clones were sequenced at MBSU (Molecular Biology Support Unit – Glasgow University) to check the pneumolysin gene and mutations were correct (Figure 3.12).

3.5.2 Gene Expression

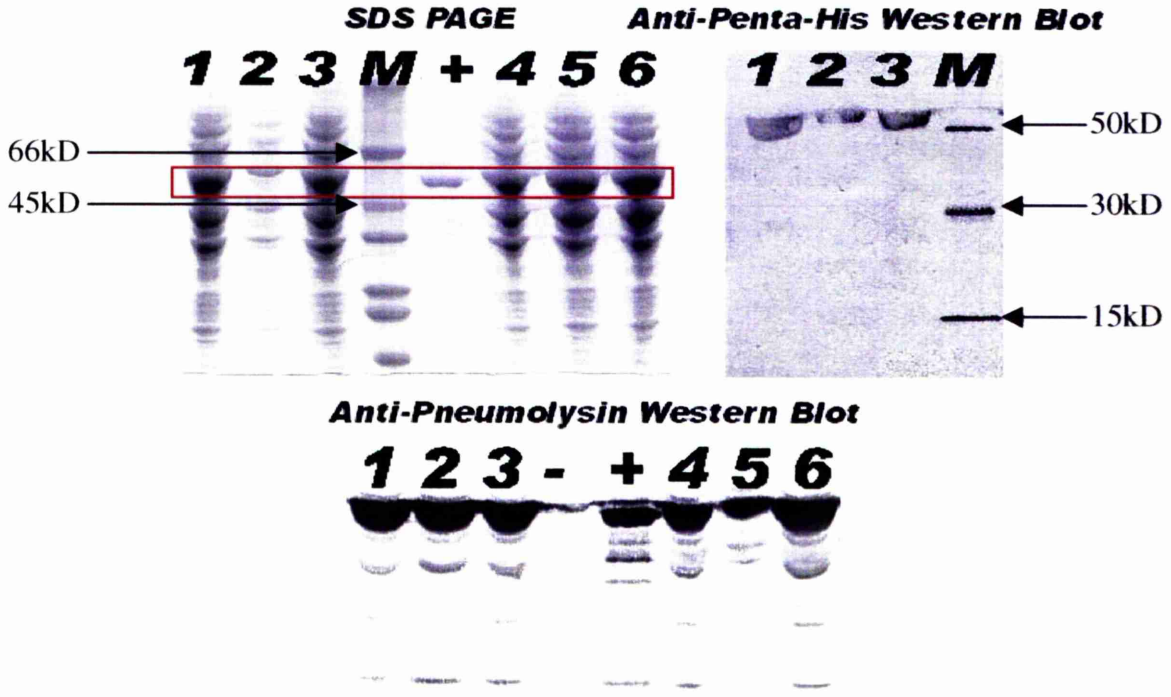
Trial expression of the toxin was carried out using several successful clones containing the wild type pneumolysin gene. 100 ml LB were inoculated with 1 ml of overnight culture and grown until the OD_{600} was 0.6. Gene expression from pET33b(+) had previously been optimised in our lab by Mr C. Bent. The cultures were induced by adding IPTG at 1 mM, for 2.5 hours and the cells were harvested by centrifugation. Pellets were lysed using B-Per™ reagent and run separated by SDS PAGE. Western blots were probed using a polyclonal antibody against pneumolysin and a monoclonal anti-penta-his antibody. The gels and blots can be seen in Figure 3.13.

Figure 3.12 – Sequence Alignment of Mutant Pneumolysin Clones

```
                 300        310        320        330        340        350
plyseq   attgatttgcctggtttggcaagtagcgatagctttctccaagtggaagacccca
AD7.4T    TTGATTTGCCTGgTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCA
AS.PPR                                          CCAAGtGGAAGACCCCA
AS.T7    ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCA
                 360        370        380        390        400
plyseq   gcaattcaagtgttcgcggagcggtaaacgatttgttggctaagtggcatcaaga
AD7.4T   GCAATTCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCAAAGTGGCATCAAGA
AS.PPR   gCAATTCAAGTgTTCgCGGAgCGGTAAACGATTTGTTGGCTAAGtGGCAtCAAGA
AS.T7    GCAATTCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGA
                 410        420        430        440        450        460
plyseq   ttatggtcaggtcaataatgtcccagctagaatgcagtatgaaaaaataacggct
AD7.4T   TTATGGTCAGGTC------GTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCT
AS.PPR   TTATGGTCAGGTCAATGATGTCCCAGcTAGAATGCAGTATgAAAAAATAACGGCT
AS.T7    TTATGGTCAGGTCAATGATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCT
                 470        480        490        500        510
plyseq   cacagcatggaacaactcaaggtcaagtttggttccgactttgaaaagacaggga
AD7.4T   CACAGCATGGAACAACTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGA
AS.PPR   CaCAGCATGGAACAACTCAAGGTCAAGTTTGGTTcTGACTTTGAAAAGACAGGGA
AS.T7    CACAGCATGGAACAACTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGA
```

Sequencing of the mutant pneumolysin clones was carried out to ensure the proteins had the mutation. plyseq = wild type pneumolysin sequence, AD7.4T = N142/143 deletion mutant sequence using forward primer (4T), AS.PPR = N143D mutant sequence using reverse primer (PlypET Term), AS.T7 = N143D mutant sequence using forward primer (T7).
Red box indicates region where mutations have successfully been introduced

Figure 3.13 – Gene Expression of His-Tagged Pneumolysin from pET-33b(+)



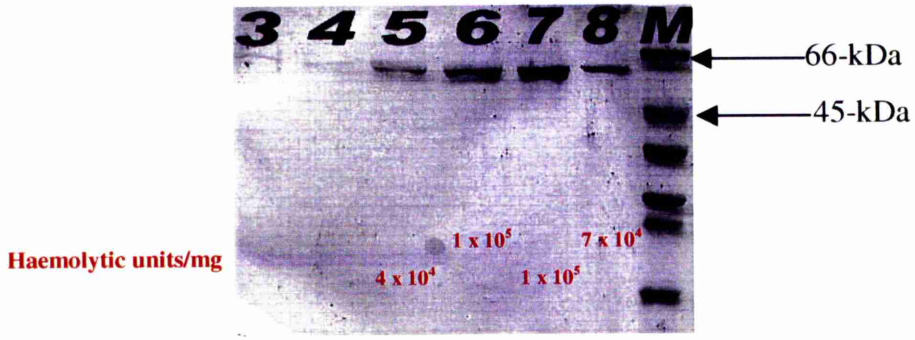
Crude lysates from 100 ml cultures of the pneumolysin clones were separated by SDS-PAGE to trial to check that over-expression of pneumolysin protein occurred after induction with IPTG. 1-6 = Different glycerol stocks of BL21 with pET-33b(+) vector containing the wild type pneumolysin gene. M = Dalton Mark VII-L (Sigma) on SDS PAGE, 6xHis Protein Ladder (Qiagen) on western blot. The red box on the SDS PAGE indicates the pneumolysin protein band.

The positive control is previously purified pneumolysin from the pKK233-2 vector so does not contain the His-Tag. The pneumolysin from the pET-33b(+) vector is a slightly larger size as it has about 100 extra amino acids due to the His-Tag. The anti-pneumolysin western blot confirms that the protein bands on the gel are pneumolysin. There are several smaller fragments recognised by the antibody that are likely to be breakdown products. Some of the samples analysed by western blotting were also probed with an anti-penta-His monoclonal antibody. This antibody detected the His-Tag on the protein band that is the correct size for pneumolysin (53-kDa). A haemolytic assay showed that the crude lysates also had haemolytic activity. Similar results were obtained with trial runs of the two mutant pneumolysin constructs.

The N142/143 deletion mutant should have a bigger effect on the pneumolysin molecule than the N143D substitution mutant. Haemolytic assays of the crude lysates showed that both the mutants had similar levels of haemolytic activity. To study the effects of changing this region of the protein, only the deletion mutant was expressed and purified further.

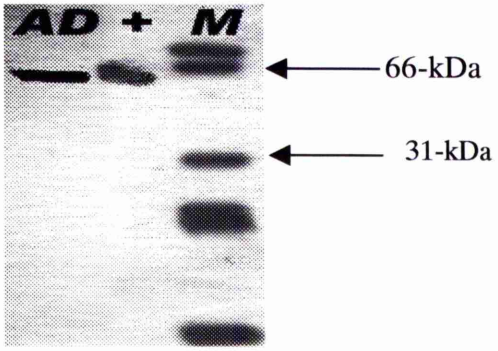
Purification using the metal chelate column was optimised with the wild type construct as described above. The BioCAD was then used to purify the N142/143 deletion mutant. SDS-PAGE was carried out to assess the purity of the fractions from the column and these are shown in Figure 3.14. Fractions showing a single band at 53-kDa on a gel and with a haemolytic activity greater then 4 x 10^4 HU/ml were pooled. The pooled fractions were then dialysed against PBS and separated on an SDS gel. Western blots using anti-pneumolysin antibodies confirmed the single protein band seen on the gels was pneumolysin.

Figure 3.14(a) – Purification of N142/143 deletion Pneumolysin Mutant



The N142/143 deletion pneumolysin mutant was successfully purified by MC chromatography. The purity of the fractions were analysed by SDS-PAGE. 3-8 = fractions from the metal chelate column. M = Dalton Mark VII-L Marker.

(b) - Anti-Pneumolysin Western Blot of pooled fractions



To confirm the protein band seen by SDS-PAGE was pneumolysin, a western blot was carried out using polyclonal anti-pneumolysin antibodies. AD = N142/143 deletion pneumolysin, + = wild type pneumolysin, M = Kaleidoscope Marker (brightness has been enhanced).

3.5 Quantification of N142/143 Deletion Pneumolysin

The concentration of the mutant pneumolysin protein is 130μg/ml and it contains 100pg of endotoxin per 100ng of pneumolysin.

The activity of the mutant pneumolysin is 8×10^4 HU/mg. The activity of the wild type pneumolysin is 1×10^5 HU/mg. If the mutant toxin did not oligomerise, we would expect to see no or very little haemolytic activity. This suggests that either this mutant does still form oligomers or that the monomeric pneumolysin can still disturb the cell membrane enough to cause lysis. The next chapter will describe the work carried out to assess whether or not this mutant protein is able to oligomerise.

3.6 Summary

In this chapter, the results of producing and purifying pneumolysin have been presented.

- A pneumolysin stock was produced using hydrophobic interaction chromatography with a specific activity of 1×10^5 haemolytic units/mg.
- In order to create a standardised method for purifying mutant proteins, the wild type gene was cloned into the pET-33b(+) expression vector which introduces a 6xhistidine affinity tag to the N-terminus of the protein. A stock of His-tagged pneumolysin was produced using metal chelate chromatography with a specific activity of 1×10^5 haemolytic units/mg.

- Two mutant pneumolysin proteins were cloned into the pET-33b(+) expression vector and transformed into competent cells for protein expression.
- The DNA and protein sequences and monomeric state of the protein have been confirmed.
- Endotoxin levels have been measured and are of an acceptable standard. All stocks contain <5EU/100ng pneumolysin.

The N142/143 deletion mutant has been purified and has the same haemolytic activity as the wild type toxin. The next chapter contains data describing the development of an oligomerisation assay using a spectrofluorimeter. This assay was then used to assess oligomerisation of the mutant protein.

4. FLUORESCENCE ANALYSIS OF PNEUMOLYSIN OLIGOMERISATION

4.1 Introduction to Fluorescence Studies

Fluorescence occurs when the absorption of a photon by a molecule (excitation) is followed by the emission of light of a longer wavelength (i.e. lower energy). Fluorescent molecules are rare and most are aromatic rings or ring systems. In biological systems, fluorescence quenching is usually the result of a collisional process or a long-range, radiative process called resonance energy transfer. Fluorescence measurements are sensitive to the environment of a molecule and can yield a lot of information about a molecule. For example, fluorescence measurements can tell us about the conformation, binding sites, solvent interactions, the degree of flexibility of a molecule and intermolecular distances.

Proteins contain three intrinsic fluors in the form of the amino acids tryptophan, tyrosine and phenylalanine. Tryptophan fluorescence is most commonly studied as phenylalanine has a low quantum yield (probability of fluorescence) and tyrosine fluorescence is frequently weak due to quenching by neighbouring amino acids.

Under certain circumstances, energy absorbed by one molecule (donor fluor) is transferred to another fluor (acceptor fluor) some distance away. This is known as resonance energy transfer and results in a decrease in the amount of fluorescence of the donor fluor when excitation is by a wavelength in the absorption spectrum of the donor fluor. The fluorescence spectrum then contains a new band at longer wavelengths.

1-anilino-8-naphthalene sulfonate (ANS) is a commonly used extrinsic fluor for proteins. In aqueous solutions ANS fluoresces very weakly, but in a non-polar environment the quantum yield increases markedly and the spectrum shifts towards shorter wavelengths. Both effects increase as polarity decreases.

These principles have been used to study oligomer formation and membrane penetration by other toxins, including the thiol activated toxin streptolysin-O (Palmer *et al.* 1996). In these experiments acrylodan was used as an extrinsic fluor.

Acrylodan is a sufhydryl-specific polarity-sensitive fluorescent dye. The emission spectrum of acrylodan is dependent on the polarity of its environment: the emission maximum is >500 nm in a hydrophilic environment and <470 nm in a hydrophobic environment (Prendergast *et al.* 1983). Staphylococcal alpha-toxin mutants containing single cysteines at positions 3-283 were derivatised with acrylodan whilst retaining functional activity. Acrylodan attached to

residues between 118-140 exhibited a marked blue shift in emission spectrum coinciding with oligomer formation in lipid bilayers (Valeva *et al.* 1996).

Palmer *et al.* (1996), used streptolysin-O to implicate residues in locally altered protein structure or membrane penetration. Like pneumolysin there is only one cysteine in the streptolysin-O molecule at residue 530 and replacement of this with alanine does not affect function (Pinkney *et al.* 1989). The mutant C530A was made and 19 further mutants were made which had an introduced cysteine at different positions. Acrylodan was attached to the engineered cysteine residues. One of the mutants (A213C) had maximal acrylodan emission at 516 nm when the toxin was in its monomeric form. When the molecule oligomerised, the acrylodan emission was blue-shifted and had a maximum emission at 489 nm. Indicating that the label had moved to a more apolar environment. Some of the other mutants also displayed a marked blue-shift upon oligomerisation. All of these mutations except one were between the amino acid residues 213-305. The group found that within the streptolysin-O oligomer, residues 274-305 form an amphiphilic helix that lines the aqueous lumen of the pore. This was sufficient to span the membrane twice. Residues 213-245 were found to be in a proteinaceous hydrophobic environment. This region may participate in pore assembly and become occluded between adjacent subunits of the oligomer or these effects could be related to an internal conformational change of the monomer accompanying oligomerisation.

In pneumolysin this is the region where monoclonal antibody binding prevented oligomerisation (De Los Toyos *et al.* 1996). A similar method could be used to assay oligomerisation in our mutant pneumolysin protein. First we needed to characterise oligomerisation of the wild type toxin. Pneumolysin has eight tryptophan residues and nineteen tyrosine residues that will contribute to the intrinsic fluorescence of the protein (Figure 4.1).

In an aqueous environment, fluorescent residues on the outside of a protein have a lower fluorescence than on the inside of a protein as water quenches the fluorescence. We can use this property to look at the movement of amino acids within a molecule, or during interactions with other molecules. For example; if there is a increase in fluorescence when pneumolysin oligomerises then we could hypothesise that some of the fluorescent residues either move to the inside of the molecule or are hidden by other pneumolysin molecules in the oligomer.

Figure 4.1 – Intrinsic Fluorescence of Pneumolysin

This homology model of pneumolysin shows amino acid residues that contribute to intrinsic fluorescence. The tryptophan residues are shown in red, tyrosines in yellow. The single cysteine residue near the trp-rich motif is shown in magenta. The figure was created using RasMol.

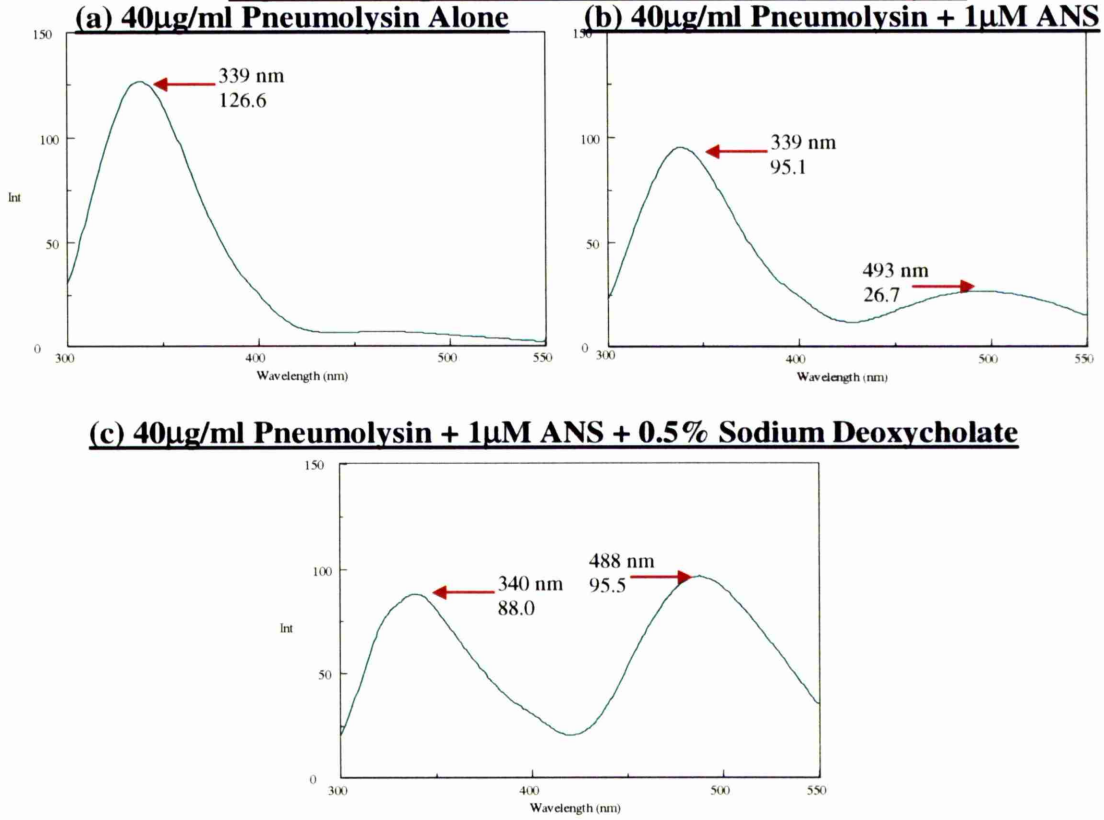
As mentioned above, fluorescence from tryptophans can be passed to ANS and the signal is amplified and transferred to a different emission wavelength. As the intrinsic fluorescence from pneumolysin is quite small it could be difficult to see any changes. We used ANS to amplify the signal from pneumolysin, so changes in fluorescence will be more obvious. When measuring fluorescence, the units used are arbitrary.

4.2 Oligomerisation of Wild Type Pneumolysin

Pneumolysin alone (40 μg/ml) has a peak emission around 340 nm. When 1μM ANS was added, the pneumolysin peak was quenched slightly and an ANS peak was observed at about 490 nm. Sodium deoxycholate is a detergent which has been shown to induce oligomerisation of another pore-forming toxin, staphylococcal alpha-toxin (Bhakdi *et al.* 1981). When 0.5% sodium deoxycholate was added to the cuvette, a slight increase was

observed in the pneumolysin peak and a large increase (close to 400%) was seen in the ANS peak. The results of representative experiments are shown in Figure 4.2.

Figure 4.2 – Spectrum Measurements of Pneumolysin



This figure shows changes in the spectrum measurements of pneumolysin after the addition of ANS and sodium deoxycholate. Peak emission and fluorescence intensity are marked on each graph by the red arrows. Int. = Relative fluorescence intensity. Data are representative of two independent experiments.

Figure 4.2(a) shows the emission spectrum of pneumolysin alone. The peak emission is at about 340 nm, (b) shows the emission spectrum of pneumolysin incubated with ANS. The pneumolysin emission at 340 nm has been quenched and the fluorescence emission is transferred to the ANS. The peak emission of ANS is about 490 nm. When 0.5% sodium deoxycholate is added to the pneumolysin and ANS (c), there is a large increase in the emission fluorescence of the ANS.

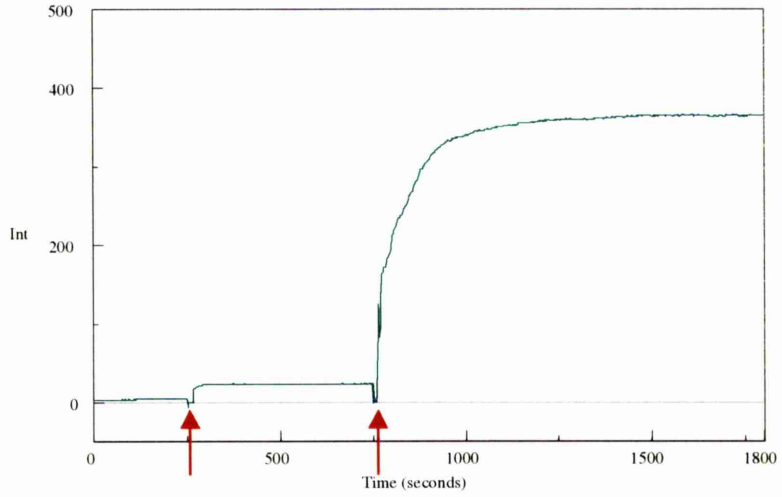
These results suggest that sodium deoxycholate has caused a change (presumably oligomerisation) in pneumolysin that has induced a large increase in ANS fluorescence. This could be caused by:

- ANS molecules becoming buried within the oligomer and therefore becoming more fluorescent.
- An increase in tryptophan fluorescence as the tryptophans move to a more hydrophobic environment. Fluorescence resonance energy transfer to the ANS results in increased ANS fluorescence.
- Or there could be an increased number of ANS molecules binding to the surface of the oligomer.

Movement of the ANS molecules or tryptophan residues to a more hydrophobic environment, would be likely to happen when oligomerisation occurs as surfaces of the pneumolysin molecule previously exposed to the fluorescence-quenching properties of water will now be occluded by other toxin molecules.

An experiment measuring fluorescence at 490 nm (the emission peak of ANS) over time was carried out to see how the ANS fluorescence increased with time after the addition of deoxycholate. Addition of 1 μM ANS to 40 μg/ml pneumolysin caused an immediate small increase in fluorescence that stayed constant. Further addition of 0.5% sodium deoxycholate resulted in a rapid increase to over 300 times the fluorescence of pneumolysin alone or pneumolysin with ANS (Figure 4.3).

Figure 4.3 – Fluorescence Emission of Pneumolysin over Time



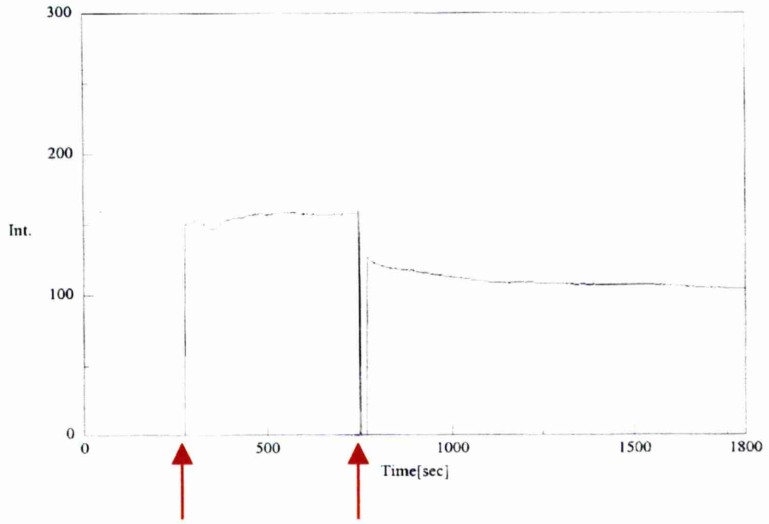
The fluorescence emission of 40 μg/ml pneumolysin was measured at 490 nm. The first red arrow shows the time when 1μM ANS was added. The second shows the time of the addition of 0.5% Sodium Deoxycholate. The plateau was measured for a further 1800 seconds and remained constant. Int. = Relative fluorescence intensity. Data are representative of two independent experiments.

4.2.1 Control Experiments

The experiments described above were repeated with BSA (bovine serum albumin) which does not oligomerise. When 1 μM ANS was added to 40 μg/ml BSA an increase in ANS fluorescence was seen indicating that ANS binds to BSA. When sodium deoxycholate was added there was no increase in fluorescence.

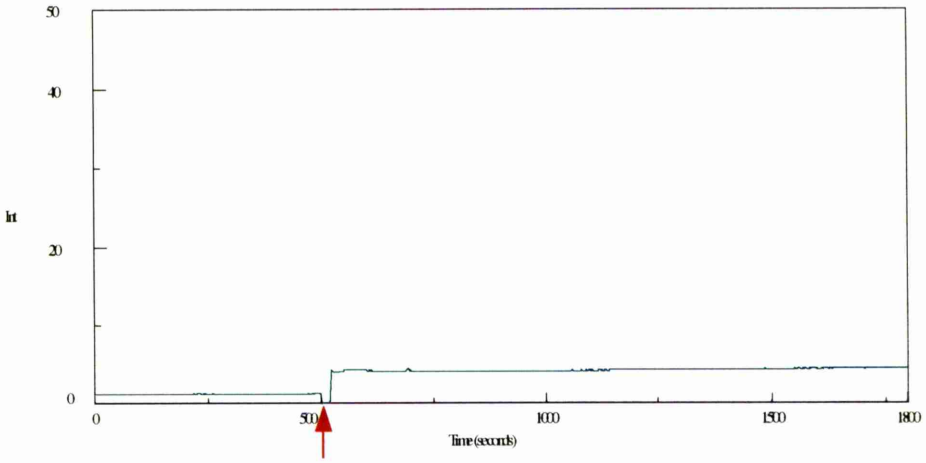
The effect of adding sodium deoxycholate to ANS is shown in Figure 4.5(a). When 0.5% sodium deoxycholate was added to 100 μM ANS, only a very small increase in fluorescence was seen. When sodium deoxycholate was added to pneumolysin, a small increase in fluorescence is observed (Figure 4.5(b)). This emphasises the benefit of using ANS as an acceptor probe to increase the magnitude of fluorescence changes.

Figure 4.4 – Fluorescence Emission of BSA



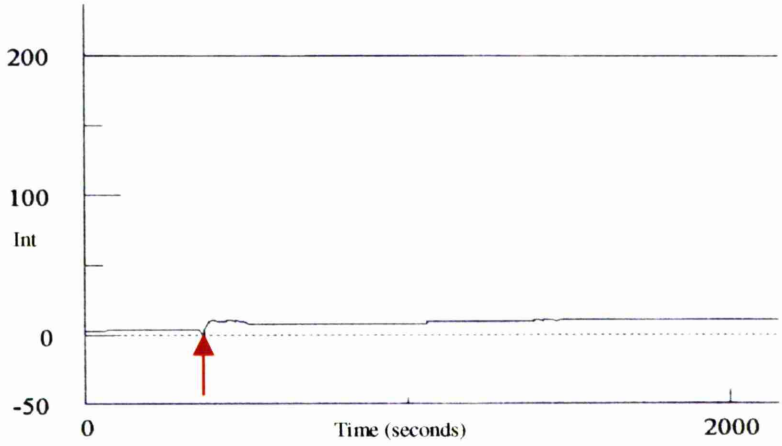
The fluorescence emission of 40 μg/ml BSA was measured at 490 nm. The first red arrow shows the time when 1μM ANS was added. The second shows the time of the addition of 0.5% Sodium Deoxycholate. Int. = Relative fluorescence intensity. Data are representative of two independent experiments.

Figure 4.5 (a) – The effect of adding Sodium Deoxycholate to ANS



Sodium deoxycholate was added to ANS to ensure the increase in fluorescence seen above (Figure 4.3) was not due to interactions between sodium deoxycholate and ANS. Red arrow indicates when 0.5% sodium deoxycholate was added. Int. = Relative fluorescence intensity. Note the scale on the y-axis is much lower than in the previous experiment (Figure 4.3). Data are representative of two independent experiments.

Figure 4.5(b) – The effect of adding Sodium Deoxycholate to Pneumolysin

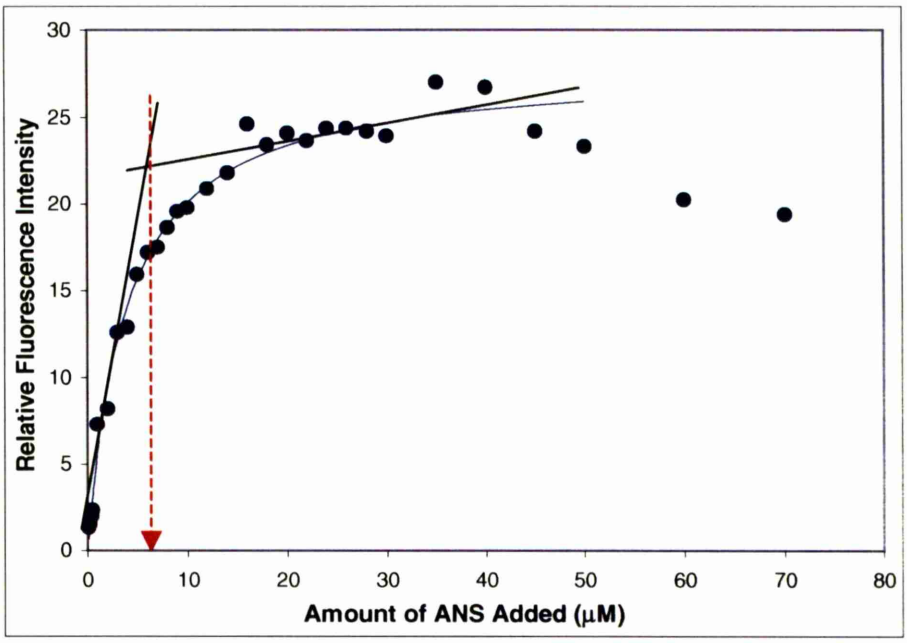


The addition of sodium deoxycholate to pneumolysin resulted in a small increase in fluorescence. This demonstrates the usefulness of an acceptor fluor to amplify the change in fluorescet signal. Red arrow indicates when 40 µg pneumolysin was added to 0.5% sodium deoxycholate. Int. = Relative fluorescence intensity.

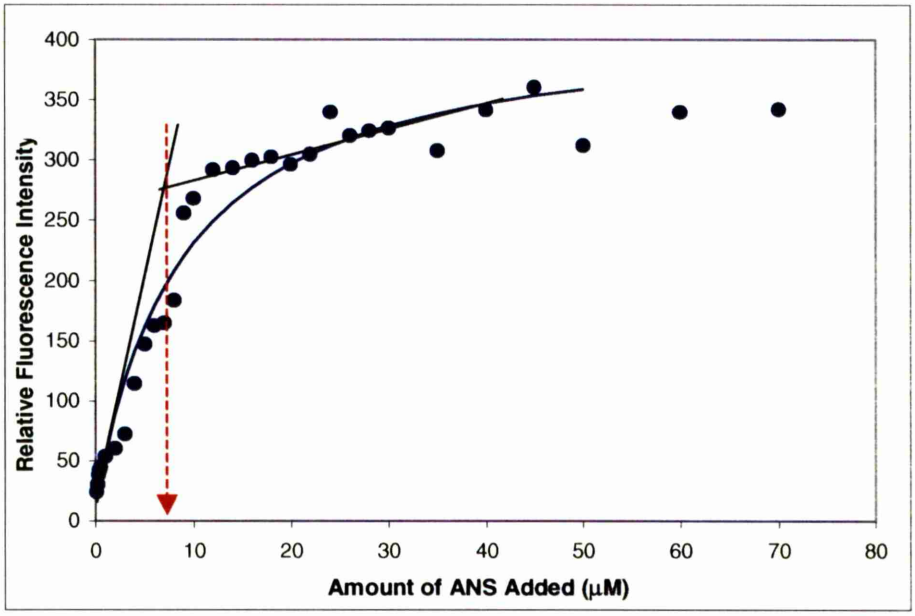
4.3 Ratio of bound ANS to Pneumolysin

As mentioned in section 4.2, the increase in ANS fluorescence may be due to an increase in the number of ANS molecules binding to the oligomer rather than the fluors moving to a more hydrophobic environment. In order to rule this possibility out two titrations of ANS and pneumolysin were carried out.

Figure 4.6(a) – Titration of ANS against 100 µg/ml Pneumolysin



(b) - Titration of ANS against 100 µg/ml Pneumolysin and Deoxycholate



0.5% sodium deoxycholate was added to 100 µg/ml pneumolysin and spectrum measurements were carried out at ten minute intervals for 60 minutes to ensure that the fluorescence intensity was stable. After this time, the ANS was added and spectrum measurements carried out as above. Adding ANS at different times after sodium deoxycholate had been added did not change the fluorescence readings.

A titration of pneumolysin against ANS can yield information about the ratio of ANS molecules bound to pneumolysin. A higher concentration (100 µg/ml) of pneumolysin was used to ensure there would be no ANS molecules free in the solution. Two curves are shown

in Figure 4.6, the first is a titration of ANS with pneumolysin; the second is a titration of ANS with pneumolysin and sodium deoxycholate.

A tangent was drawn at each end of the curve in Figure 4.6 to find out the concentration of bound ANS. In Figure 4.6(a) the concentration of bound ANS is approximately 8 μM. The molecular weight of pneumolysin is 52772 so 100 μg/ml = 1.89 μM. This works out as a ratio of 4 ANS molecules bound to every pneumolysin molecule. In Figure 4.6(b) the concentration of bound ANS is the same (8 μM). So, although the ANS fluorescence intensity is greatly increased when sodium deoxycholate is added, the amount of ANS molecules bound to each pneumolysin molecule remains the same. This suggests that the large increase in fluorescence is due to the movement of the fluors to more hydrophobic environments.

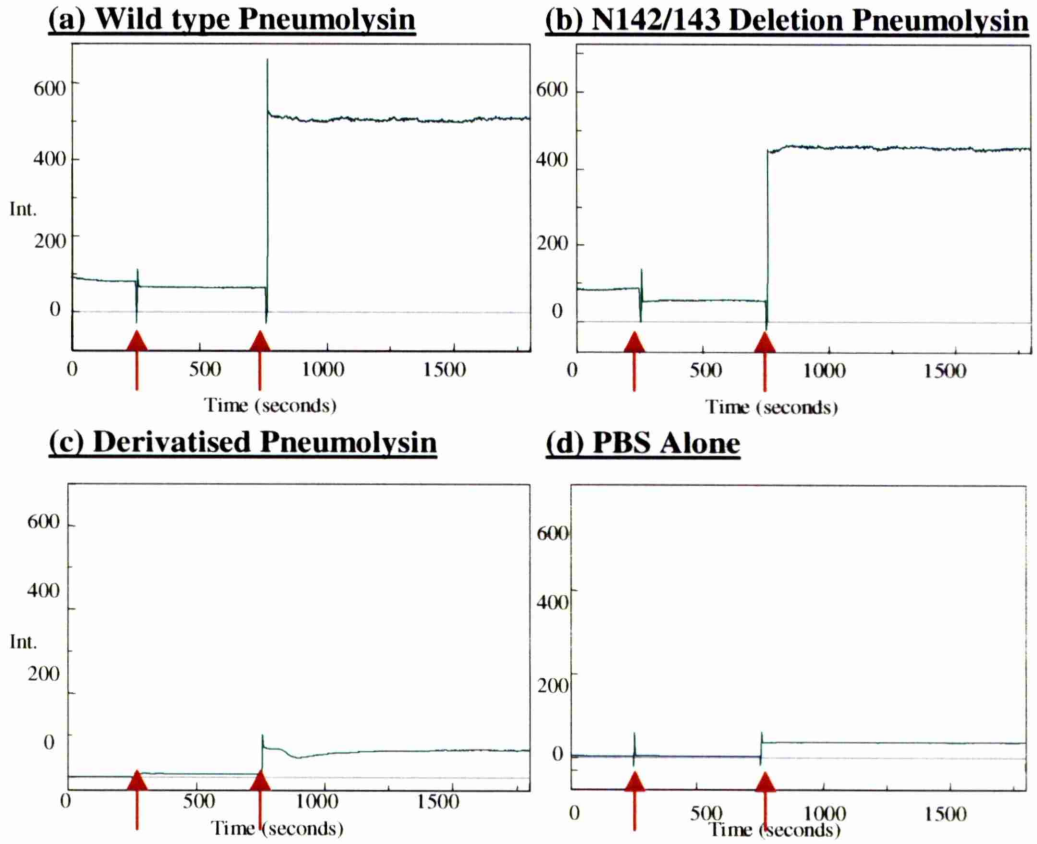
4.4 Oligomerisation of N142/143 Deletion Pneumolysin

As there was not much difference in the haemolytic activity between N142/143 deletion and wild type pneumolysin, the fluorescence activity of the two versions of pneumolysin was compared. A thionitrobenzoate derivate form of pneumolysin (Ply-TNB) was kindly donated by Dr. O. Byron. It is generated by reaction of dithio(bis)nitrobenzoate (DTNB) and pneumolysin in which the single cysteine residue becomes derivatised with a benzyl group via a disulphide bridge. Ply-TNB was used as a control as it has been shown not to self-associate (Gilbert *et al.* 1998).

When spectrum measurements were carried out with N142/143 deletion pneumolysin, very similar results to the wild type pneumolysin were obtained (data not shown) suggesting that N142/143 deletion pneumolysin was also forming oligomers. A time course experiment was also carried out, measuring ANS emission (490 nm). The results of these time courses are shown in Figure 4.7.

The time course experiments show that the N142/143 deletion mutant behaves in the same way as the wild type pneumolysin in the fluorimeter. The derivatised pneumolysin, which cannot self-interact does not show an increase in fluorescence when sodium deoxycholate is added. In fact, it behaves in a very similar way to the PBS alone. This data implies that the N142/143 deletion mutant does oligomerise.

Figure 4.7 – Time Course of Pneumolysin Fluorescence



The changes in fluorescence when ANS and sodium deoxycholate were added to pneumolysin were measured for the wild type pneumolysin, for a mutant pneumolysin protein (N142/143 deletion) designed not to oligomerise and a chemically modified pneumolysin protein (derivatised pneumolysin) which is unable to oligomerise. These data suggest that the N142/143 deletion mutant can in fact oligomerise as it behaves in the same way as the wild type toxin. Red arrows show where 1μM ANS (250 seconds) and 0.5% sodium deoxycholate was added (750 seconds). Int. = Relative fluorescence intensity.

This result is surprising as the monoclonal antibody data (De Los Toyos *et al.* 1996) and the streptolysin-O data (Palmer *et al.* 1996) suggested mutations in this region should abolish oligomerisation.

4.5 Summary

- An assay for detecting oligomerisation of pneumolysin was developed by measuring the increased fluorescence of tryptophan residues and/or ANS molecules as they move to a more hydrophobic environment.
- This fluorescence increase was shown not to be due to an increase in the number of ANS molecules binding to the oligomer or due to the interaction of ANS with sodium deoxycholate.

- Use of this assay with the N142/143 deletion mutant implied it was still able to oligomerise.

Although the mutant still oligomerises it is important to see if the mutation of these residues has other effects on behaviour, as sublytic effects may be different to lytic effects. Therefore we decided to still use the mutant pneumolysin in the viability and other assays to see if its activity has been changed in other ways.

5. EFFECTS OF PNEUMOLYSIN ON CELLS

5.1 Cell Viability in the presence of Pneumolysin

When this work was started, no information was available about the toxicity of pneumolysin on the cell types we planned to study. As cytokines are generally not preformed and require protein synthesis, cells need to be alive to produce them. In order to study the toxic effects of the protein, cell viability assays were carried out using a range of concentrations of pneumolysin.

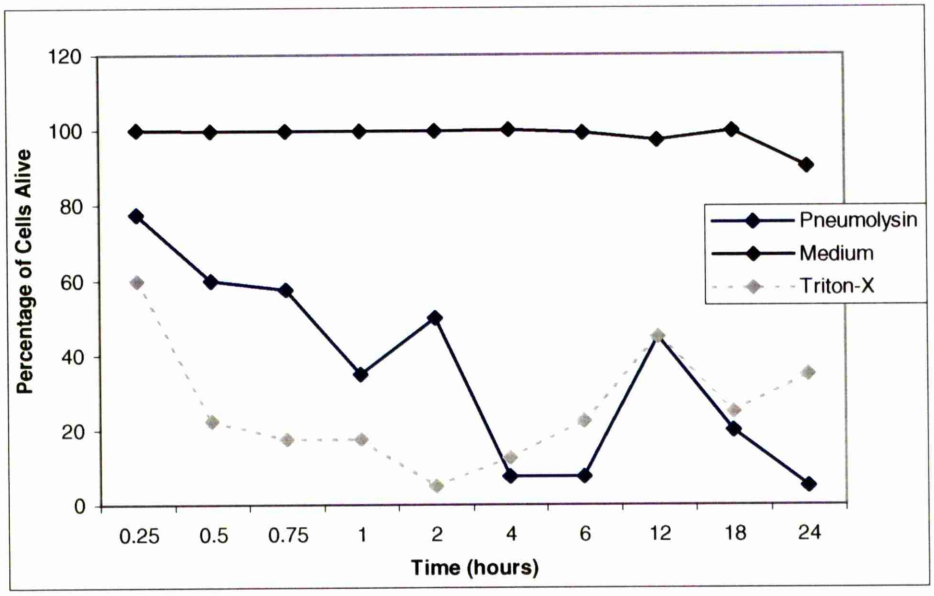
5.1.1 Viability of Macrophages incubated with Pneumolysin

When *S. pneumoniae* invades a host, one of the first cells it is likely to come into contact with is the macrophage. Macrophages are derived from blood monocytes that migrate into tissues and differentiate there. They are strongly phagocytic and possess microbicidal capacity and release an enormous number of biologically active substances including cytokines and microbicidal agents. To look at the effects that pneumolysin might have on these cells, a macrophage derived cell line (RAW 264.7) was used.

To find out how long it took for pneumolysin to kill the macrophages, a time trial was carried out. An arbitrary concentration of pneumolysin was used and the viability assessed at various times to obtain an indication of the time frame of toxin-induced cell lysis. Macrophages were seeded in a 12-well tissue culture plate and a high concentration (25 μg/ml) of pneumolysin was added. At each time point the viability of the cells was assessed by trypan blue exclusion (See Figure 5.1). Cells in medium alone were used as a negative controls and 1% Triton-X as a positive control.

The viability of the macrophages drops immediately (within 15 minutes) after addition of pneumolysin. After four hours the viability has decreased to the extent that greater than 90% of the cells are dead. The data at 12 hours are anomalous as there appears to be an increase in viability. This could be because the total number of cells incubated with pneumolysin or Triton-X has decreased compared to the cells incubated in medium alone so although the percentage of cells alive appears to increase, the total number of live cells is very much less than those incubated with medium alone. The percentage of viable cells in media over 24 hours did not drop below 90%. As the viability of cells incubated with pneumolysin dropped to <10% after four hours, this time was chosen to investigate how much pneumolysin is necessary to have a toxic effect.

Figure 5.1 – Viability of Macrophages incubated with Pneumolysin



Macrophages were incubated with 25 µg/ml pneumolysin. Viability was assessed by eye using trypan blue to stain dead cells.

A range of dilutions of pneumolysin was used to determine the concentration of pneumolysin required to kill 50% of the cells. In these experiments the MTT assay was used, as it is more convenient for large numbers of samples. This assay has been shown to be as sensitive as trypan blue exclusion (Coote and Arain 1996). It is also more quantitative as the coloured product produced by viable cells can be measured in a spectrophotometer.

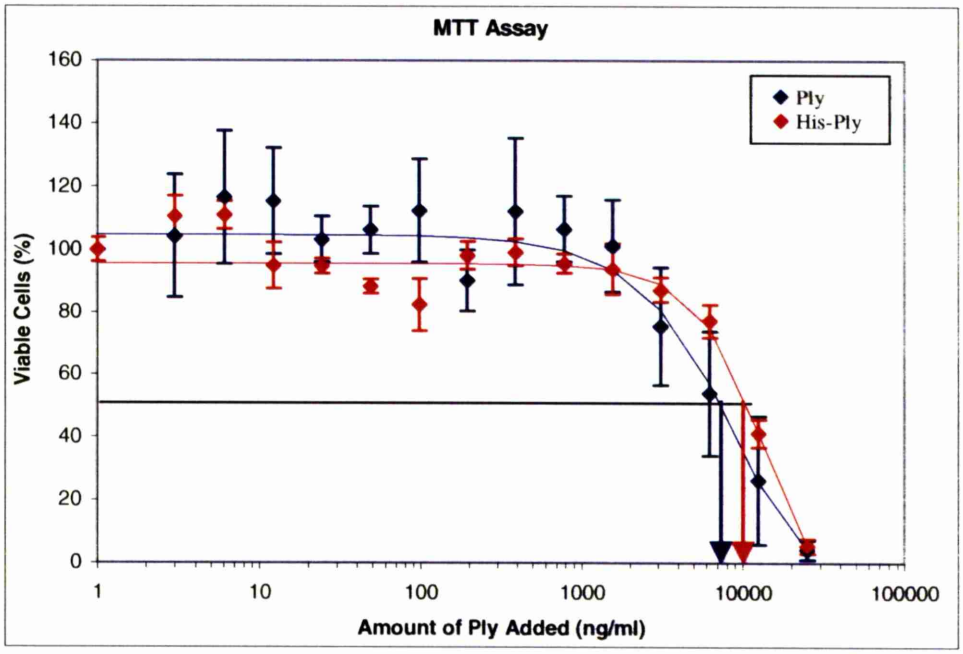
In order to compare MTT assays from different experiments, the absorbance of cells incubated in media alone was adjusted to 100% for each experiment. In some experiments the viability at certain points appears to be higher than 100%, this indicates there were more cells present under these conditions than cells incubated in the culture media alone.

As zero cannot be plotted on a logarithmic scale, the first point plotted on the x-axis at either 0.1 or 1 is the viability of cells in media alone (i.e. 0 ng/ml pneumolysin)

All viability curves show mean values +/- the standard error of the mean. Curves were fitted in Microsoft Excel by χ^2 minimisation using a three-parameter model that matched the characteristic shape of the datasets. In Figure 5.5, a four-parameter model was used to account for the fact that the data did not reach zero.

In Figure 5.2 the results of viability experiments using the wild type pneumolysin and the his-tagged pneumolysin stocks are compared. The graph shows that the concentration required to kill 50% of the macrophages is similar for both pneumolysin stocks. Between 7-11 µg/ml pneumolysin is required to kill 50% of the macrophages. For all future viability assays, the his-tagged pneumolysin was used.

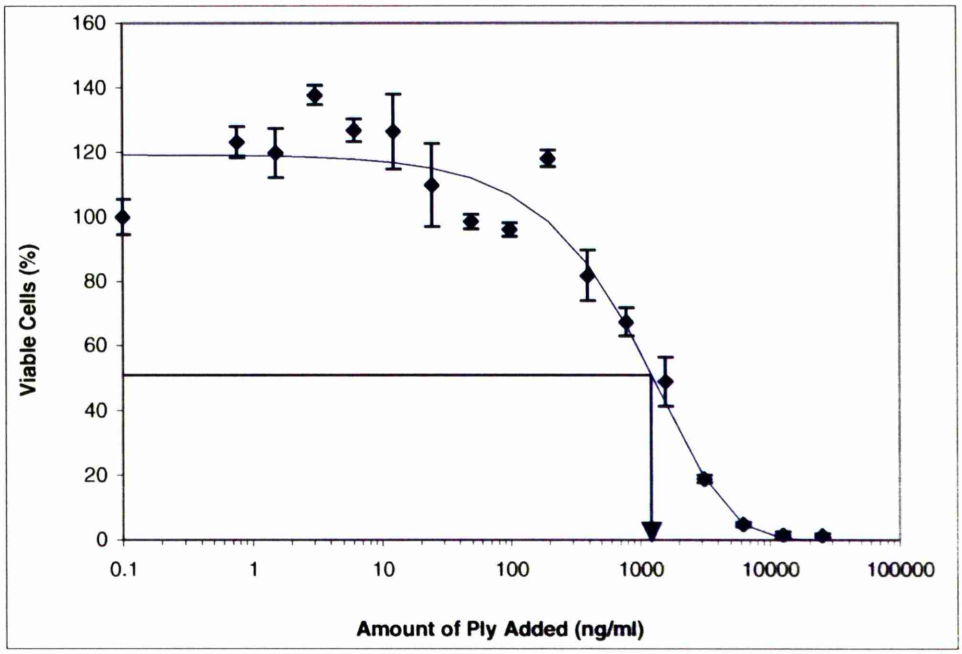
Figure 5.2 – Viability of Macrophages incubated with Pneumolysin for 4 hours



Macrophages (5 x 10^5 cells) were incubated with pneumolysin for four hours and viability was assessed by MTT assay. The activity of the pneumolysin stocks purified by different methods was compared. The graph shows mean values +/- 1 standard error of the mean. 50% kill = 7.3 µg/ml for pneumolysin purified by HIC (Ply); 10.8 µg/ml for the his-tagged pneumolysin purified by MC chromatography (His-Ply). Data from 3 separate experiments with duplicate samples are shown.

In later experiments when mediator production will be assessed, the cells were incubated with the toxin for 24 hours. Therefore the viability of the macrophages after 24 hours was also assessed. The results can be seen in Figure 5.3, only 1.3 µg/ml was required to reduce the viability by 50% after 24 hours. This five-fold difference could be because there is more time for pneumolysin molecules to attach to the receptors, maybe there is recycling of pneumolysin after cell lysis. Or perhaps the lower concentrations of toxin on individual cells could induce necrosis or apoptosis, which only becomes apparent at later time points.

Figure 5.3 – Viability of Macrophages incubated with Pneumolysin for 24 hours



Macrophages (5 x 10^5 cells) were incubated with his-tagged pneumolysin for 24 hours and viability was assessed by MTT assay. The graph shows mean values +/- 1 standard error of the mean. 50% kill = 1.3 µg/ml. Data from 2 separate experiments with duplicate samples are shown.

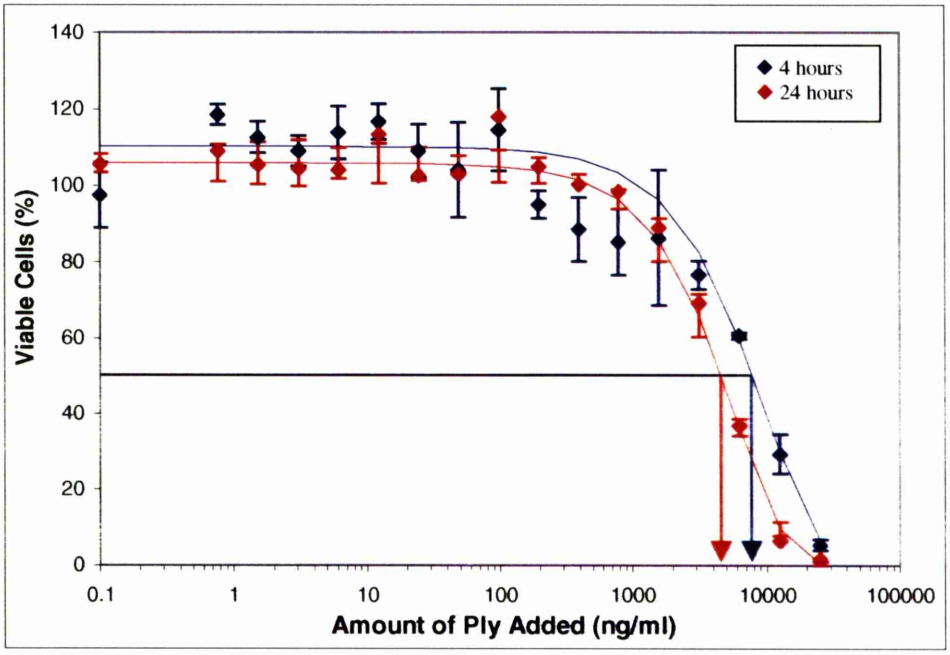
5.1.2 Viability of Airway Epithelial Cells incubated with Pneumolysin

During pneumococcal pneumonia when the bacteria invade the lungs, one of the cell types most likely to come into contact with the pneumococci is the airway epithelium. The airway epithelium is a physical barrier that protects sensory nerves and smooth muscle. In addition, these cells can also release mediators such as prostaglandins and nitric oxide (Folkerts and Nijkamp 1998). These cells may also act as a barrier during invasion of the bacteria into the blood stream. A primary isolate of human small airway epithelial cells (SAEC) were used for some experiments. These cells were difficult and expensive to handle, so most work was carried out using an epithelial-derived human lung carcinoma cell line; A549 cells.

After four hours, 8 µg/ml of his-tagged pneumolysin killed 50% of the lung epithelial cells (see Figure 5.4). Slightly less pneumolysin (5 µg/ml) was necessary to kill 50% of the cells after 24 hours. After four hours, there is not much difference between the susceptibility of the lung epithelial cells compared to the macrophages (50% kill required 7.5-11 µg/ml). When the macrophages were incubated with his-tagged pneumolysin for 24 hours, the viability dropped by 50% when five times less pneumolysin was added. The lung epithelial

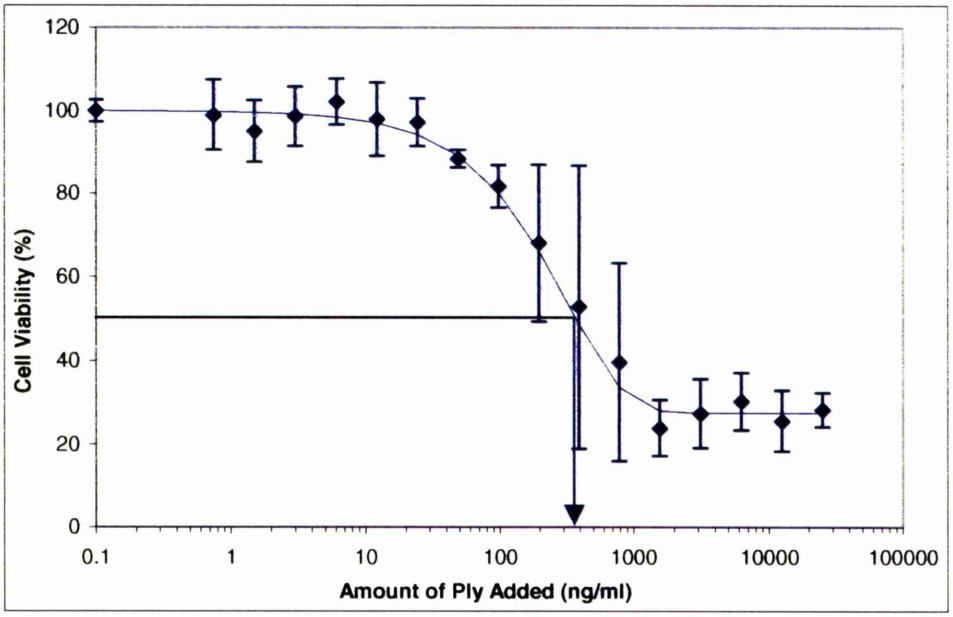
cells seem to be more resistant to the toxin than the macrophages and this difference is more marked after 24 hours.

Figure 5.4 – Viability of Lung Epithelial Cells incubated with Pneumolysin



Lung epithelial cells (5 x 10^5 cells) were incubated with his-tagged pneumolysin and viability was assessed by MTT assay after 4 and 24 hours. The graph shows mean +/- 1 standard error of the mean. 50% kill = 7.9 µg/ml after 4 hours. 4.6 µg/ml after 24 hours. Data from 2 separate experiments with duplicate samples are shown.

Figure 5.5 – Viability of Small Airway Epithelial Cells incubated with Pneumolysin



SAEC (2 x 10^4 cells) were incubated with pneumolysin for four hours. Graph shows mean +/- 1 standard error of the mean. 50% kill = 370 ng/ml. Data from 2 separate experiments with duplicate samples are shown.

Figure 5.5 shows that the SAEC are susceptible to lysis by pneumolysin. Only 370 ng is required to kill 50% of the cells after four hours. Interestingly, after four hours, the viability of the cells does not drop to less than 25%. When Triton-X was used to lyse the cells, the viability dropped to less than 2%. This suggests that about a quarter of the cells are able to survive concentrations up to 25 μg/ml pneumolysin.

The recommended seeding density for the small airway epithelial cells (BioWhittaker) is lower than that used for the A549 lung epithelial cells. If the amount of pneumolysin needed to kill 50% of 5 x 10^5 cells/ml is compared then the sensitivities of the two airway epithelial cell types are similar (7.9 μg to kill 50% of A549 lung epithelial cells compared to 6.2 μg/ml to kill 50% SAEC). As the sensitivities of the two cell lines are similar, the A549 lung epithelial cells were used in all further experiments, as they were much easier to use and maintain.

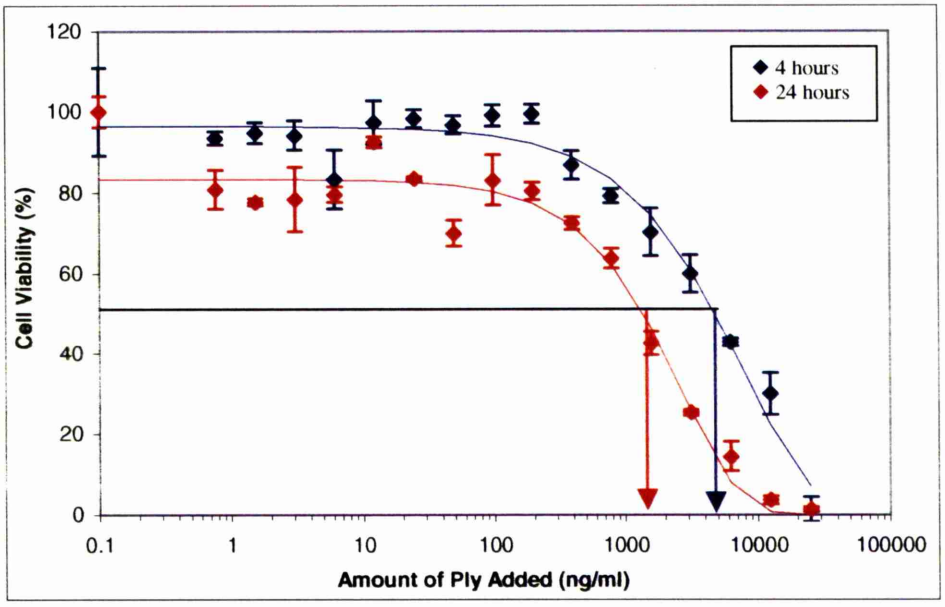
5.1.3 The effect of IFNγ on cell viability

As IFNγ was used to activate macrophages in later experiments it was important to check if addition of IFNγ has any effects on cell viability. Some studies (for example, Hirst *et al.* 2002) have noted that cells incubated with IFNγ are less susceptible to pneumolysin-induced lysis. If IFNγ does protect the macrophages from pneumolysin-induced lysis, any increase in mediator production may be due to an increase in the number of cells present rather than increased production from individual cells. The lung epithelial cells were activated with a mixture of the cytokines, TNF, IL-1β and IFNγ. The viability of these cells with IFNγ was also assessed. 0.5 ng/ml IFNγ was added to the cells for four hours, and was not removed before the addition of pneumolysin. The viability data for the cells primed with IFNγ for four hours and then incubated with pneumolysin are shown in Figure 5.6.

Priming the macrophages and the lung epithelial cells with IFNγ did not protect the cells from pneumolysin-induced death. In comparison to the viability of un-primed macrophages, IFNγ-priming led to lower amounts of toxin being required to lyse 50% of the cells. I.e. if anything, the cells have become more susceptible to the cytotoxic action of pneumolysin. This might partly be explained for the macrophages as a few of the IFNγ-activated cells had a tendency to lose adherence. When the MTT assay is carried out, the supernatant is removed so these cells would be lost and could lead to lower numbers of viable cells than

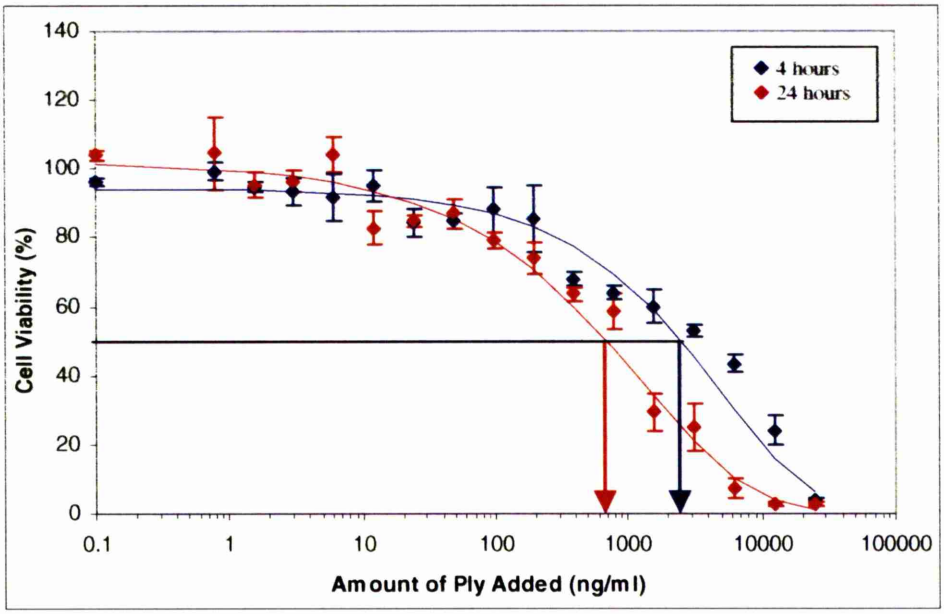
expected. Table 5.1 summarises the amounts of pneumolysin required to reduce cell viability by 50% under these different conditions. IFNγ had no effect on the viability of the SAEC (data not shown)

Figure 5.6(a) – Viability of Macrophages incubated with Pneumolysin and IFNγ



Macrophages (5 x 10^5 cells) were primed with 0.5 ng/ml IFNγ for four hours before the addition of his-tagged pneumolysin. Graph shows mean +/- 1 standard error of the mean. 50% kill = 4.8 µg/ml after 4 hours, 1.4 µg/ml after 24 hours. Data from 2 separate experiments with duplicate samples are shown.

(b) –Viability of Lung Epithelial Cells incubated with Pneumolysin and IFNγ

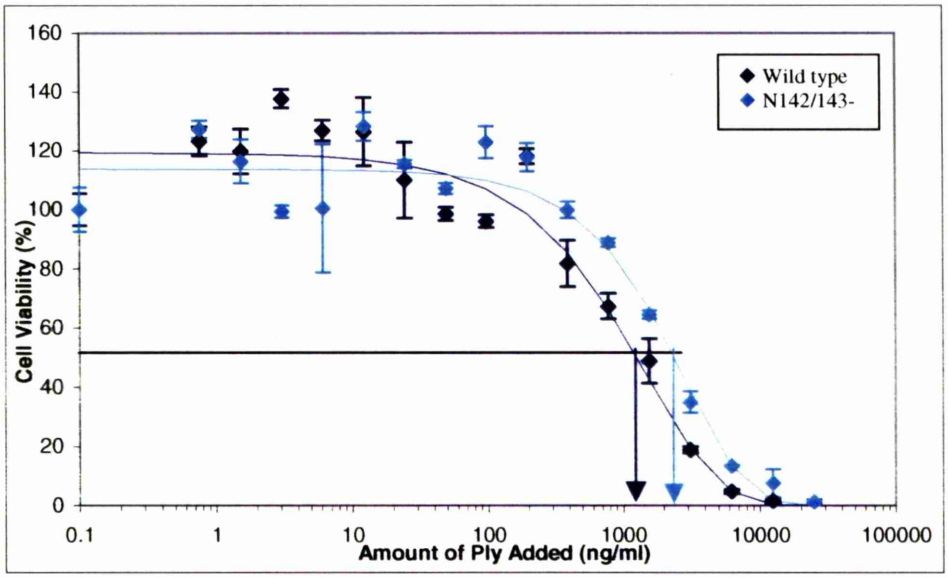


Lung epithelial cells (5 x 10^5 cells) were primed with 0.5 ng/ml IFNγ for four hours before the addition of his-tagged pneumolysin. Graph shows mean +/- 1 standard error of the mean. 50% kill at 4 hours = 2.5 µg/ml, 24 hours = 685 ng/ml pneumolysin. Data from 2 separate experiments with duplicate samples are shown.

5.1.4 Ability of Pneumolysin N142/143 deletion mutant to kill Cells

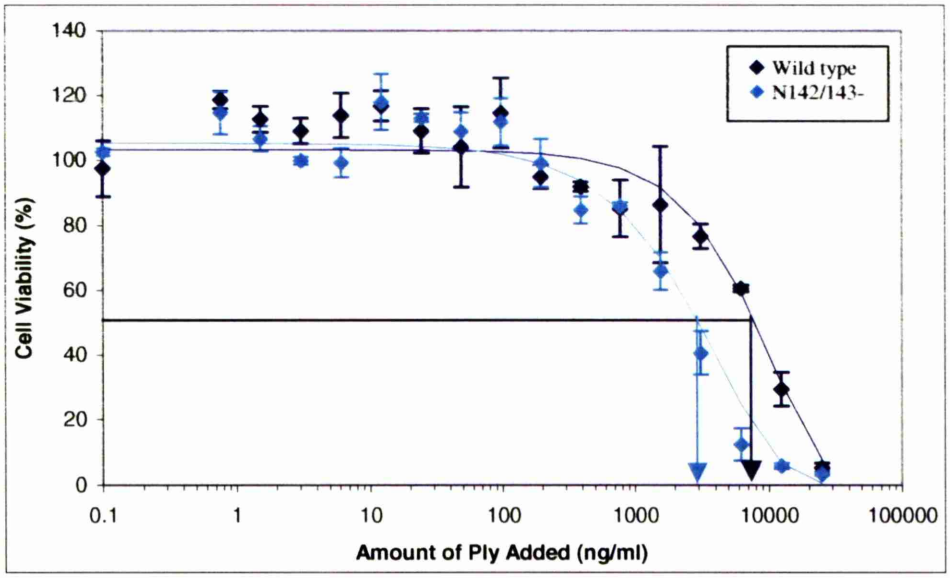
The same experiments were carried out using the N142/143 pneumolysin deletion mutant to see if altering this region had any effect on the lytic activity of the protein (Figure 5.7).

Figure 5.7(a) – Viability of Macrophages with N142/143 deletion Pneumolysin



Results shown here are from macrophages after 24 hours of incubation with his-tagged pneumolysin and IFNγ. The graph shows mean values +/- 1 standard error of the mean. Experiments at 4 hours and without IFNγ produced similar data. 50% kill with wild type pneumolysin = 1.3 µg/ml, N142/143 deletion = 2.4 µg/ml. Data from 2 separate experiments with duplicate samples are shown.

(b) – Viability of Lung Epithelial Cells after incubation with N142/143 deletion Pneumolysin



Results shown here are from lung epithelial cells after 4 hours incubation with his-tagged pneumolysin. The graph shows mean values +/- 1 standard error of the mean. 50% kill wild type = 8 µg, N142/143 deletion = 3 µg. Data from 3 separate experiments with duplicate samples are shown.

The mutation of the amino acids at positions 142 and 143 in pneumolysin did not abolish the ability of the protein to lyse either cell type. In fact half the amount of mutant protein was required to kill 50% of the lung epithelial cells (3 μg compared to 8 μg of wild type pneumolysin). A summary of the concentration of pneumolysin necessary to cause a 50% decrease in cell viability is shown in Table 5.1.

Table 5.1 – Amounts of Pneumolysin required to reduce Cell Viability by 50%

PNEUMOLYSIN	4H	24H	4H	24H
	No IFNγ		Plus 0.5 ng/ml IFNγ	
MACROPHAGES				
Wild Type	7.3	1.3	4.8	1.4
N142/143-	6.6	2.4	2.7	2.4
LUNG EPITHELIAL CELLS				
Wild Type	7.9	4.6	2.5	0.7
N142/143-	3	1.4	0.8	1.5
SMALL AIRWAY EPITHELIAL CELLS				
Wild Type	6.2	ND	7	ND

Pneumolysin concentration is given in μg/ml. All data given display the concentration of pneumolysin required to reduce viability of 5 x 10^5 cells by 50%. ND = Experiment not done.

The sublytic effects of pneumolysin are to be studied in future experiments. From these data, it was possible to choose concentrations of pneumolysin that did not affect the viability of the cells. Concentrations less than 100 ng/ml of pneumolysin should not reduce the viability of the cells. For some experiments higher concentrations of pneumolysin were used to see what effect cell lysis would have on mediator production.

5.2 Mediator Production in the Presence of Pneumolysin

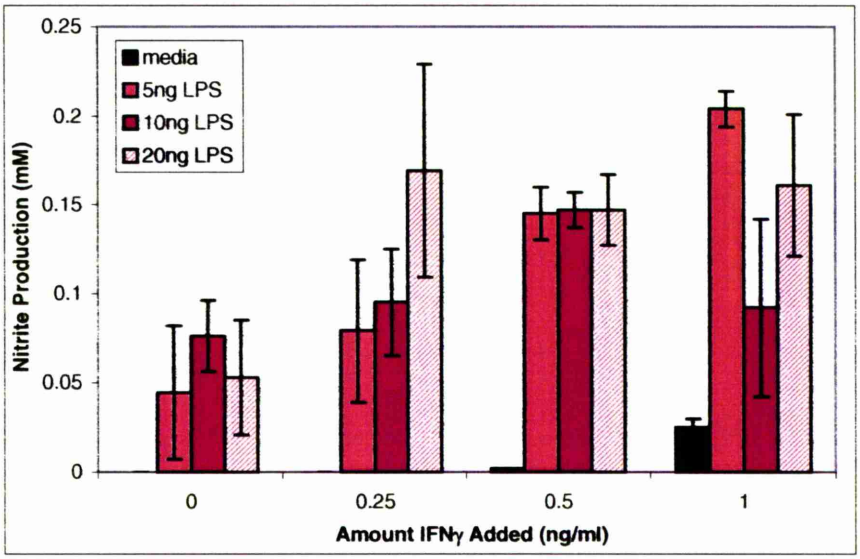
Many studies have been carried out into the production of various mediators by pneumolysin-stimulated cells. Pneumolysin has been shown to stimulate IL-1β and TNFα from monocytes (Houldsworth *et al.* 1994) and inhibit the respiratory burst in monocytes and neutrophils (Paton and Ferrante 1983; Nandoskar *et al.* 1986) amongst other activities (see sections 1.9 and 1.10 for more information). We decided to measure NO and TNF production. These mediators have many roles in the inflammatory response that can be both beneficial and harmful to the host. The correct regulation of these mediators is probably

crucial to the development of an effective immune response. During the course of this research, an article was published by Braun *et al.* (1999a) investigating nitric oxide production from RAW 264.7 macrophages. It will be interesting to compare the results from this study with the results presented below. This will be discussed in more detail in section 8.2.

5.2.1 Stimulating NO from Macrophages

A time course where macrophages were incubated for different lengths of time with different concentrations of LPS and IFNγ was carried out to find the optimum concentrations to use as a positive control. Most NO production was seen after 18 hours of incubation with LPS and IFNγ. There were no differences in NO production stimulated by different amounts of LPS and IFNγ (See Figure 5.8). 10 ng/ml LPS and 0.5 ng/ml IFNγ were used in subsequent experiments to act as a positive control.

Figure 5.8 – LPS and IFNγ induced Nitric Oxide Production from Macrophages



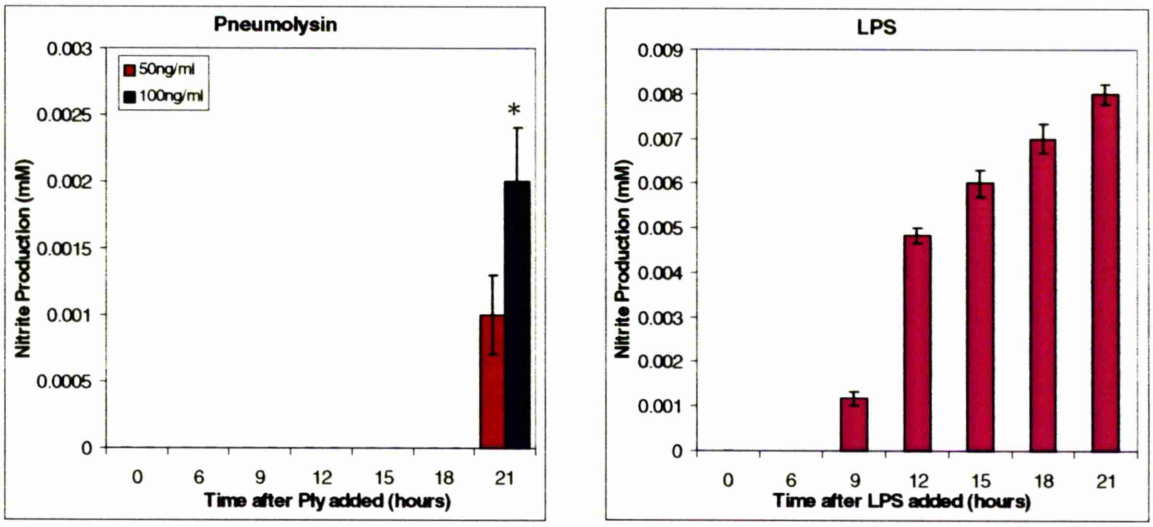
Macrophages were primed with IFNγ for four hours before addition of LPS. NO production is shown after 18 hours incubation with LPS. Mean values from two independent experiments are shown +/- standard error of the mean.

5.2.2 NO Production from Macrophages with sub-lytic concentrations of Pneumolysin

Time courses using four different concentrations of pneumolysin (100 ng/ml (10 HU), 50 ng/ml, 25 ng/ml and 12.5 ng/ml) were performed. These concentrations were chosen because they are sublytic and they have similar specific activities to preparations used in previous studies. For example 5 HU of pneumolysin was found to inhibit lymphocyte responses

(Ferrante *et al.* 1984). 10 ng/ml LPS was used as a positive control and pneumolysin buffer was added to negative control wells. All experiments were carried out using resting macrophages and with macrophages that had been primed with 0.5 ng/ml IFNγ for four hours. The results are shown in Figure 5.9.

Figure 5.9 – Nitric Oxide Production from Macrophages incubated with Pneumolysin and IFNγ



Macrophages were primed for four hours with 0.5 ng/ml IFNγ and incubated with different concentrations of pneumolysin. At various time points samples were taken and measured for NO production. *100 ng/ml pneumolysin stimulated a significant increase ($p<0.01$) in nitrite production after 21 hours compared to unstimulated cells. Data from 2 separate experiments with duplicate samples are shown.

The cells incubated with LPS and IFNγ showed a significant increase ($p=0.0025$) in NO production after nine hours compared to unstimulated cells. The nitrite production continued to increase throughout the experiment to a level of almost 0.01 mM. LPS alone did not stimulate as much NO production as LPS and IFNγ together. No nitrite production was detected when cells were incubated with pneumolysin alone (i.e. without IFNγ priming), or with the two lower concentrations of pneumolysin. 50 ng/ml of pneumolysin and IFNγ stimulated a small amount of nitrite after 21 hours. This was detectable in the Griess reaction but was not significantly different to cells incubated in media alone ($p=0.0578$). 100 ng/ml pneumolysin stimulated a higher level of NO production after 21 hours, which was significantly different to cells incubated in media alone ($p=0.0019$).

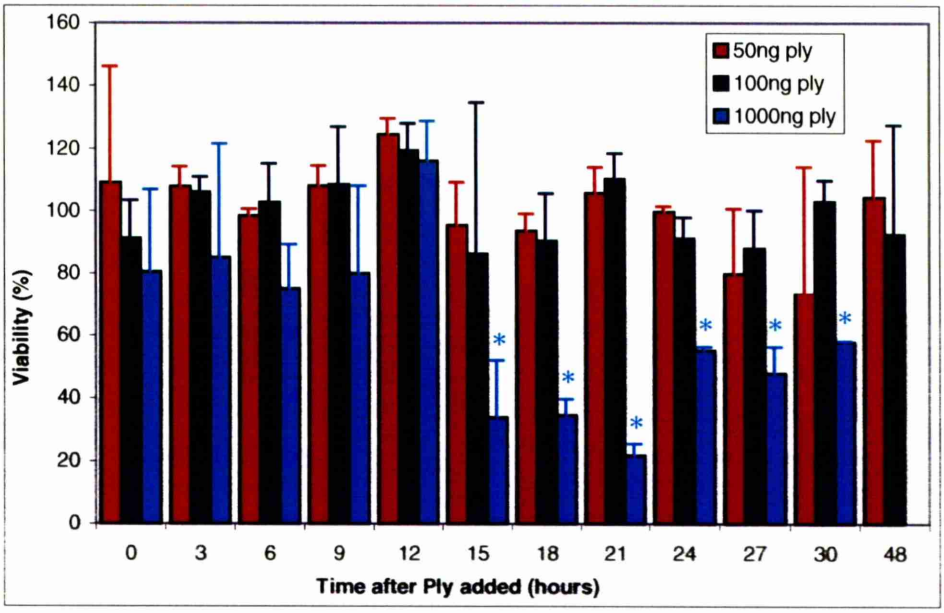
Next, three separate time course experiments were carried out, to see if the results in Figure 5.9 were reproducible. In the study by Braun *et al.* (1999a) concentrations of up to 30 μg/ml

were shown to induce high levels of NO production. These concentrations are very likely to be toxic, however the cell viability was not measured in their study. There is a small possibility that the toxin used in the Braun study has a different activity and did not have toxic effects at these concentrations. In order to compare their results with this study a higher concentration (1000 ng/ml) of pneumolysin was also used and its effects on viability measured. Figure 5.10(a) shows the viability of the macrophages after incubation with pneumolysin. The cell viability was measured using the MTT assay. Similar data was obtained when the cells were also incubated with 0.5 ng/ml IFNγ.

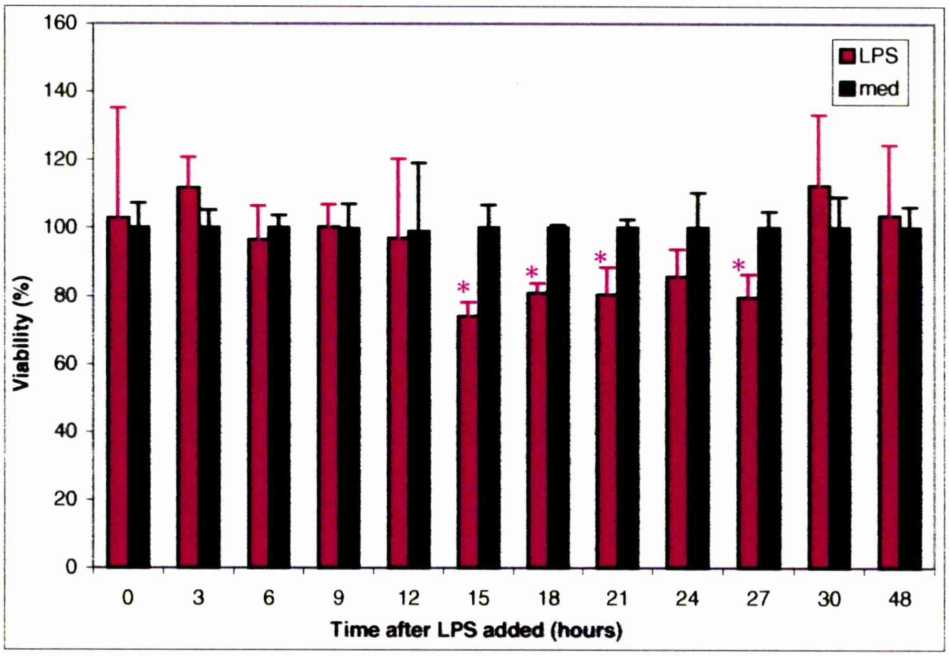
The viability of the macrophages does not change significantly when incubated with 50 ng or 100 ng/ml pneumolysin. When 1000 ng pneumolysin was added, the viability of the macrophages dropped after 12 hours of incubation and is significantly lower ($p<0.05$) at 15 hours and later time points compared to cells in media alone. The viability of cells incubated with LPS between 15 and 27 hours is significantly lower than cells in media alone. This might be because the cells stimulated with LPS have been activated and therefore may not be dividing as much as the cells in media alone. Growth inhibition of RAW 264 cells by LPS (0.5-10 ng/ml) has been reported previously (Ralph and Nakoinz 1977; Raschke *et al.* 1978).

The supernatants from these experiments were assayed using the Griess reaction to look for nitrite – a stable end product of NO production (Figure 5.11). Macrophages that had not been primed with IFNγ produced negligible amounts of NO regardless of the stimulus (data not shown). A slight increase over time was seen from cells incubated with LPS (but not IFNγ) but these differences were not statistically significant.

Figure 5.10(a) – Viability of Macrophages incubated with Pneumolysin

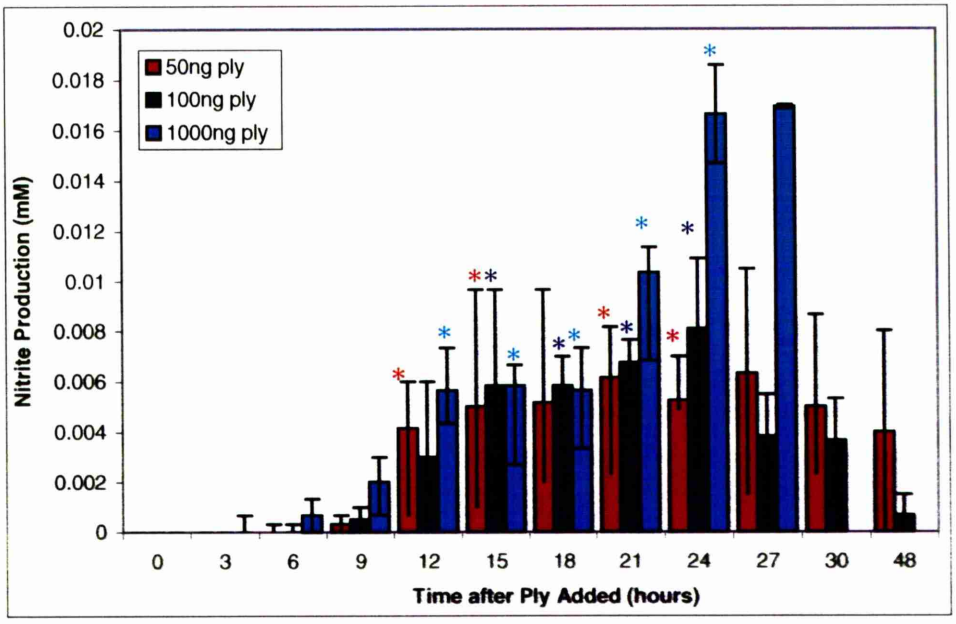


(b) – Viability of Macrophages incubated with LPS

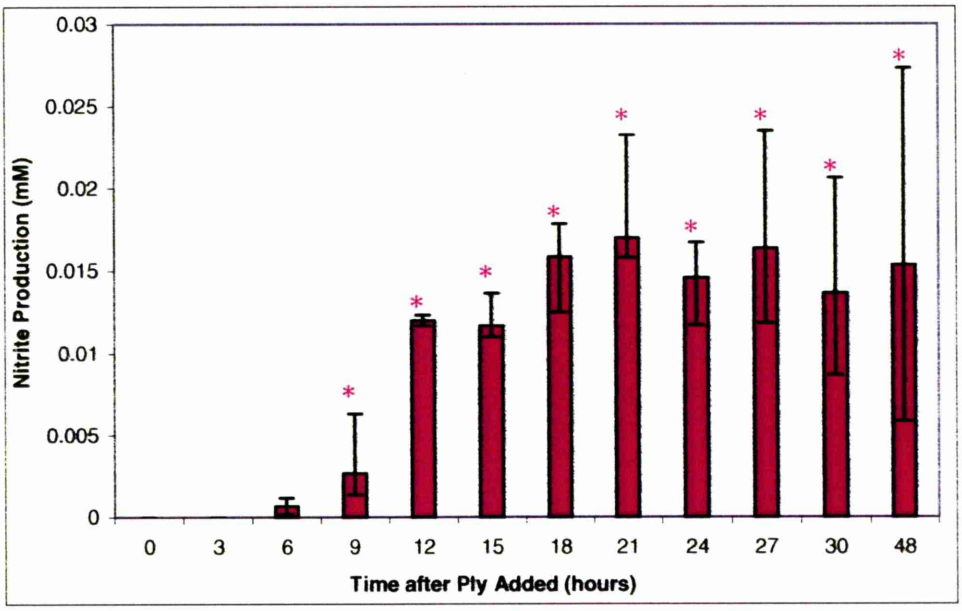


Viability of macrophages incubated with pneumolysin or LPS, was assessed by MTT assay. The cells incubated in media alone were assumed to have a viability of 100% so that different experiments could be compared. Graphs show median values with maximum error bars. *Significantly less viable cells compared with those in media at same time point ($p<0.05$). Data from 3 separate experiments with duplicate samples are shown.

Figure 5.11 (a) – NO Production from Macrophages incubated with Pneumolysin

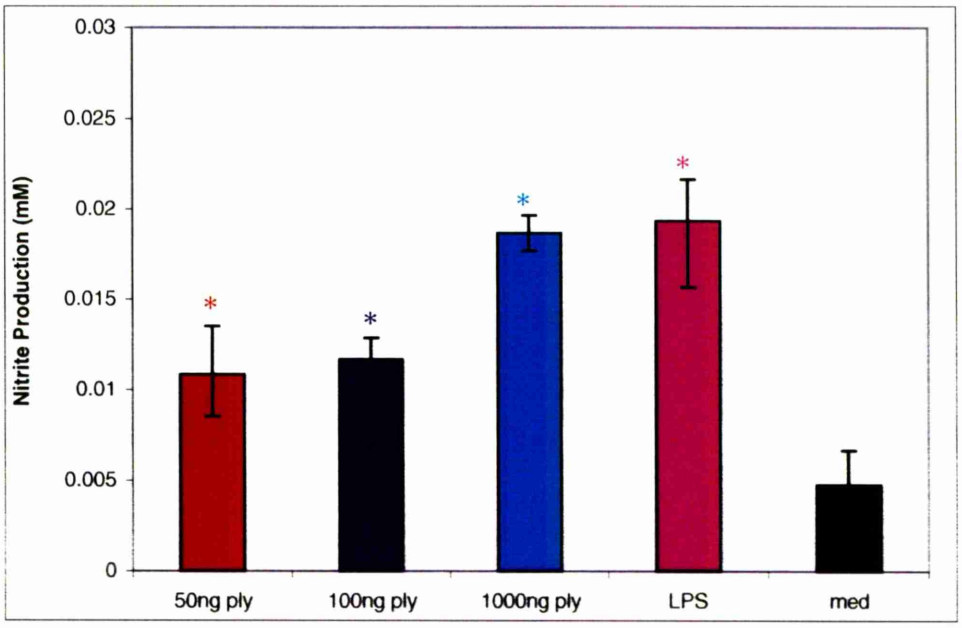


(b) – NO Production from Macrophages incubated with LPS



Cells were primed for four hours with IFNγ before addition of pneumolysin or LPS. In these graphs the average amount of nitrite produced by unstimulated macrophages at each time point (background NO production) has been subtracted from the nitrite produced by cells under experimental conditions. The graph shows medians with maximum and minimum values plotted as error bars. *Significantly more NO produced than cells in media alone ($p<0.05$). Data from 3 separate experiments with duplicate samples are shown.

(c) - NO Production from Macrophages after 24 hours



This graph shows the actual amount of nitrite produced by the cells after 24 hours. Medians with maximum and minimum values are shown. *Significantly more NO produced than cells in media alone ($p<0.05$). Data from 3 separate experiments with duplicate samples are shown.

After incubation with IFNγ, the macrophages spontaneously produce a small amount of NO. From 12 to 48 hours, the NO produced by these unstimulated cells was significantly greater than from unstimulated cells at 0 hours ($p<0.05$). The data in Figure 5.11(a) and (b) shows the nitrite production from the macrophages with the baseline nitrite production subtracted

The cells incubated with 50 ng and 100 ng/ml pneumolysin show a similar pattern of NO production over time. The increase becomes significantly different compared to cells in media alone 12-24 hours after pneumolysin was added. Due to an increase in background nitrite levels, the amount of NO produced was no longer significant at time points later than 24 hours. Most nitrite was detected in the supernatant when 1000 ng/ml pneumolysin was used. At 21 hours and later, the nitrite production is increased compared to the lower concentrations of pneumolysin used. At these times, when most nitrite is seen, the viability of the cells is ~50% less than unstimulated cells. These cells could either store a lot of NO, which is released as they are lysed, or the cells that are still alive are making twice as much NO.

The graph in Figure 5.11(c) shows the nitrite production from the macrophages after 24 hours. Macrophages primed with IFNγ produced a median value of 0.004 mM nitrite. At this

time point, all the concentrations of pneumolysin used produced significantly more NO than macrophages in media alone. The graph suggests that there may be a dose-related effect and at this time point the amount of pneumolysin produced from macrophages stimulated with 1000 ng/ml pneumolysin is significantly higher ($p<0.05$) than from macrophages stimulated with either 50 or 100 ng/ml pneumolysin. In fact the amount of nitrite production from these cells is not significantly different to the amount of nitrite produced by LPS-stimulated cells.

These results show that pneumolysin can stimulate nitric oxide production from macrophages activated with IFNγ in a dose dependent manner.

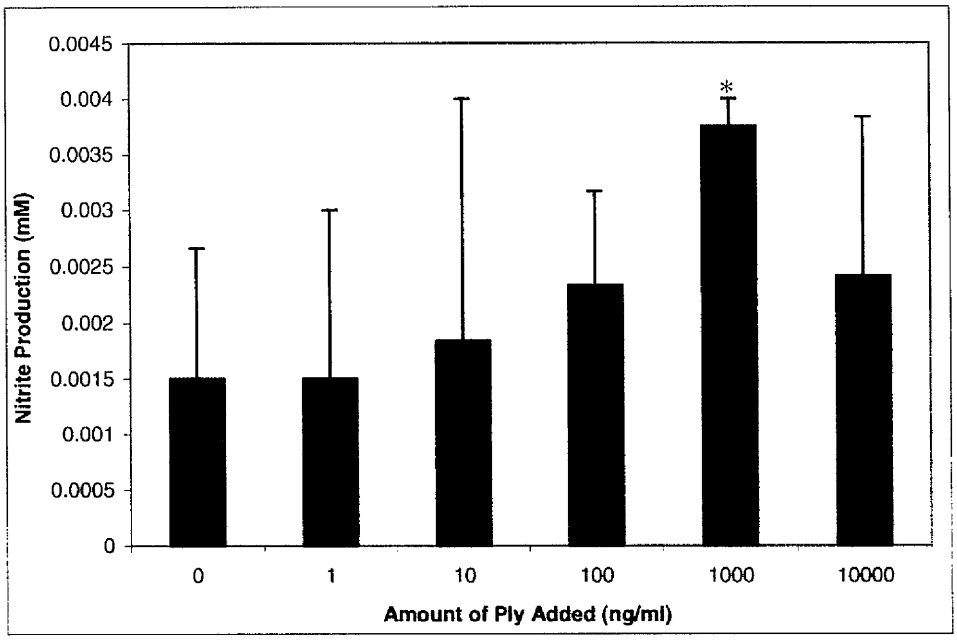
5.3 Nitric Oxide Production from Lung Epithelial Cells

Cells that line the airways have been assigned an active role in the host response to infection. Nitric oxide production has been reported in response to various cytokines and viral infection (Robbins *et al.* 1994; Kao *et al.* 2001). We investigated the possibility that pneumolysin could potentially stimulate these cells to produce nitric oxide.

Previous studies (Robbins *et al.* 1994; Kao *et al.* 2001) have shown that a mixture of cytokines, likely to be present during the inflammatory response in the lung (IL-1, TNF and IFNγ) can stimulate lung epithelial cells to produce nitrite. When we used this mixture no nitrite production was seen.

Pneumolysin was added to the cells for 24 hours. At lower concentrations (<100 ng/ml) no increase in nitrite production was seen from the cells. At 100 ng, the pneumolysin does seem to affect the cells but this result is not significantly different to unstimulated cells. 1000 ng/ml pneumolysin did stimulate significant amounts of NO from the lung epithelial cells (Figure 5.12), but higher concentrations than this did not stimulate a significant amount of nitrite production.

Figure 5.12 – NO Production from Lung Epithelial Cells incubated with Pneumolysin



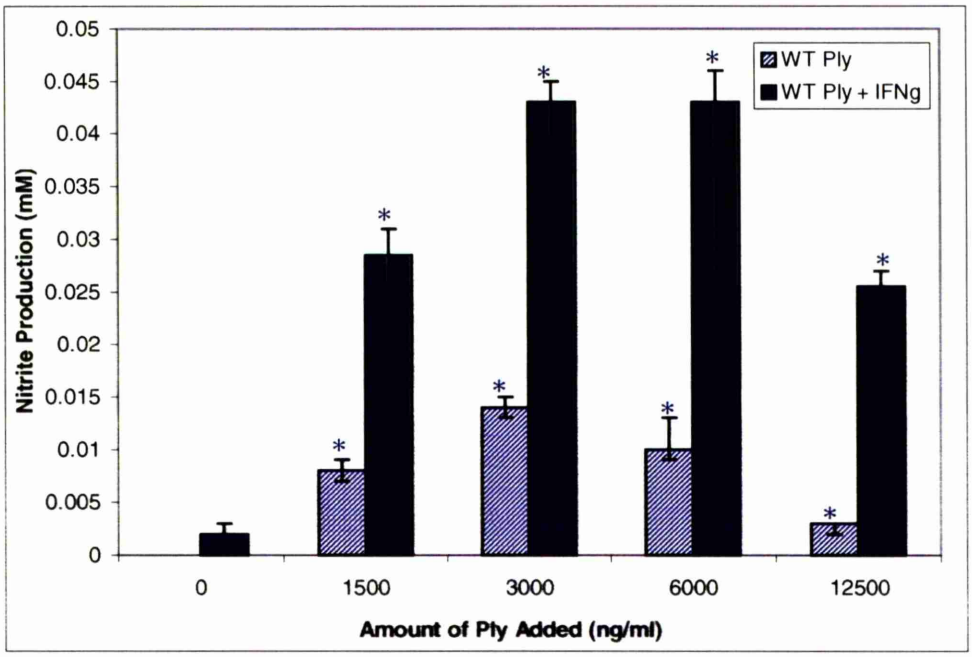
Lung epithelial cells (5 x 10^5 cells) were stimulated with pneumolysin for 24 hours and nitrite levels in the supernatant were measured using the Griess reaction. Graph shows median values with maximum and minimum error bars. *Significantly more NO production than from unstimulated cells. Data from 2 separate experiments with duplicate samples are shown.

The amount of NO production seen from lung epithelial cells is much lower than from macrophages. The maximum amount of NO produced from the lung epithelial cells is 0.0035 mM when 1000 ng/ml pneumolysin is added. When the same amount of pneumolysin is added to macrophages, 0.02 mM nitrite is detected. This is almost a six-fold difference in the NO production from the different cell types and could be due to the utilisation of different nitric oxide synthases.

5.3.1 NO from Macrophages with lytic concentrations of Pneumolysin

As 1000 ng/ml pneumolysin stimulated more NO than sublytic concentrations, we decide to use higher concentrations to see what effect they may have on nitrite production from macrophages (Figure 5.13). As peak nitrite production was seen at 24 hours, only this time point was studied.

Figure 5.13 – NO Production from Macrophages by lytic concentrations of Pneumolysin



Macrophages were incubated with pneumolysin for 24 hours. Cells with IFNγ were primed for four hours before addition of pneumolysin. Nitrite levels in the supernatant were measured. *Significantly more NO production compared to cells in media alone ($p<0.05$). Data from 3 separate experiments with duplicate samples are shown.

The cells produced a significant amount of nitrite without IFNγ priming when 1500 ng/ml of pneumolysin or more was added. Unprimed macrophages produced a maximum of about 0.015 mM nitrite after 24 hours when incubated with 3000 ng/ml pneumolysin. When higher concentrations are used, the amount of NO production decreased. A similar shaped curve is seen when the cells are primed with IFNγ but the amount of nitrite produced is higher. All concentrations of pneumolysin shown in Figure 5.13 stimulated significant nitrite production from the macrophages compared to unstimulated cells. A peak of about 0.4 mM is reached when 3-6 μg/ml of pneumolysin is added to macrophages primed with IFNγ. As shown above in section 5.1, at these concentrations of pneumolysin, more than 50% of the cells are dead. Triton-X was used as a control to lyse cells. The supernatants from these cells were also assayed using the Griess reaction and no nitrite was detected at any time (data not shown). This suggests that NO detection is not purely due to permeabilisation of the cells.

These results concur with the effects seen by Braun *et al.* (1999a) although in their study no measure of cell viability was made. The NO production seems to be related to cell death as although small amounts of pneumolysin stimulate a little nitrite production, most is seen

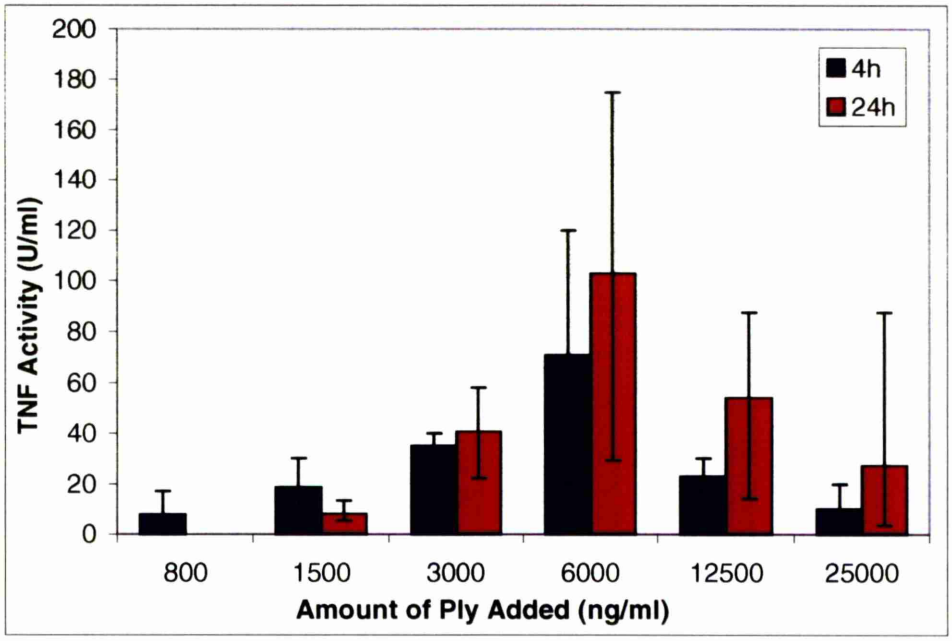
when lytic concentrations of pneumolysin are used. These data raise several questions, which will be discussed later (section 8.2).

5.4 TNF Production from Pneumolysin-stimulated Macrophages.

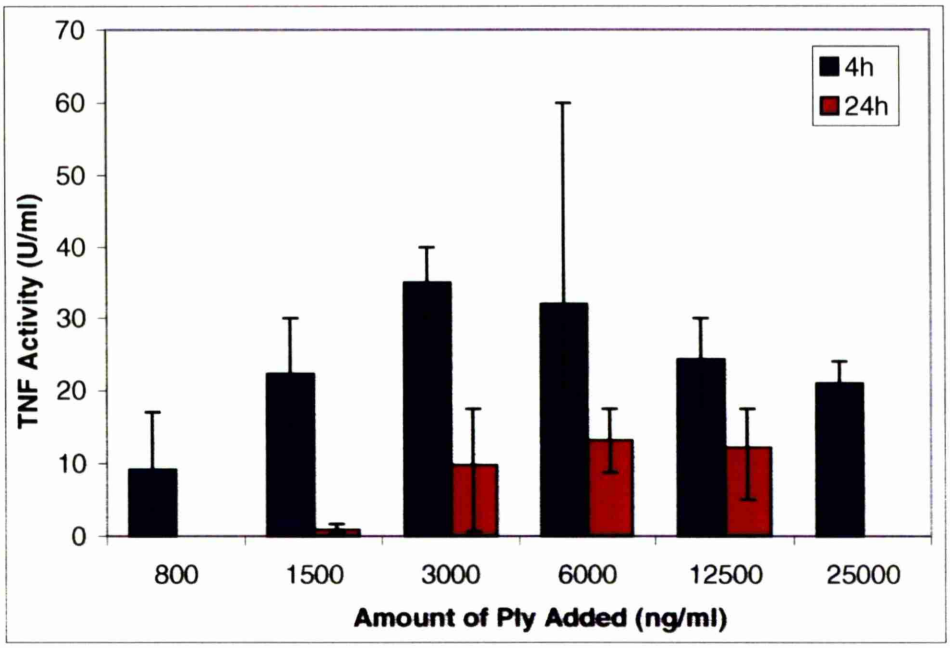
The same supernatants used in the Griess reaction were also used in a TNF bioassay to measure TNF activity. In this assay the TNF activity required to kill 50% of a sensitive cell line (L929 connective tissue cells) is measured. Therefore if a sample does not have enough TNF to kill 50% of the cells it is not detected in this assay. The supernatants showed no haemolytic activity, so any connective tissue cell killing is probably induced by TNF and is not due to residual pneumolysin in the samples. Samples that displayed TNF activity are shown below in Figure 5.14. No TNF activity was detected in the supernatant from unstimulated macrophages.

When the supernatant from the cells incubated with 100 ng/ml pneumolysin was used in this assay, there was a decreased viability of the connective tissue cells. However the TNF activity did not reproducibly kill 50% of the cells - in some experiments the TNF activity fell just below this cut off point. So although it appears there is some TNF produced by macrophages stimulated with sub-lytic concentrations of pneumolysin, this assay was not sensitive enough to measure it. The N142/143 deletion mutant stimulated a similar pattern of TNF production from macrophages (data not shown).

Figure 5.14 (a) – TNF Production from Macrophages stimulated with Pneumolysin



(b) - TNF production from IFNγ-activated Macrophages stimulated with Pneumolysin



Where appropriate, cells were primed with IFNγ for 4 hours and then pneumolysin was added to the cells. After 4 or 24 hours TNF activity was measured in the supernatants. Median values with maximum and minimum error bars are shown. Concentrations of pneumolysin that stimulated TNF activity in four separate experiments are shown. No TNF activity was detected from unstimulated cells. For each graph, data from 2 separate experiments with duplicate samples are shown.

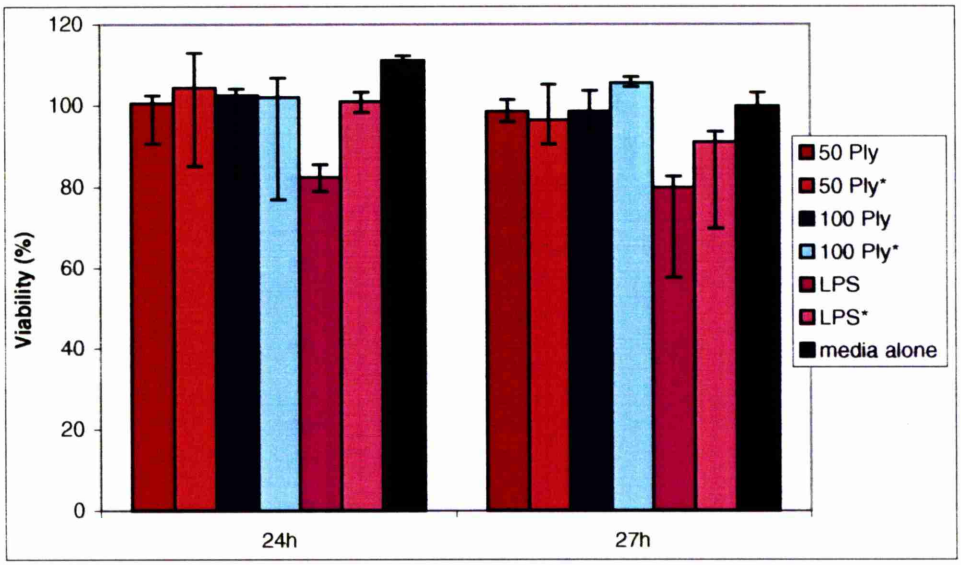
As was seen with the NO production, most TNF is released from macrophages incubated with lytic concentrations of pneumolysin. After four hours, there were no differences in TNF production from the macrophages activated with IFNγ compared to the un-primed macrophages. After 24 hours, more TNF activity was seen from the cells that were not primed with IFNγ. These differences reached significant levels when 1.5, 3 and 6 μg/ml of pneumolysin were used ($p<0.05$). It is possible that when the macrophages are primed with IFNγ the majority of the TNF has been released earlier than 24 hours.

5.5 Testing for the Potential Effects of Contaminants

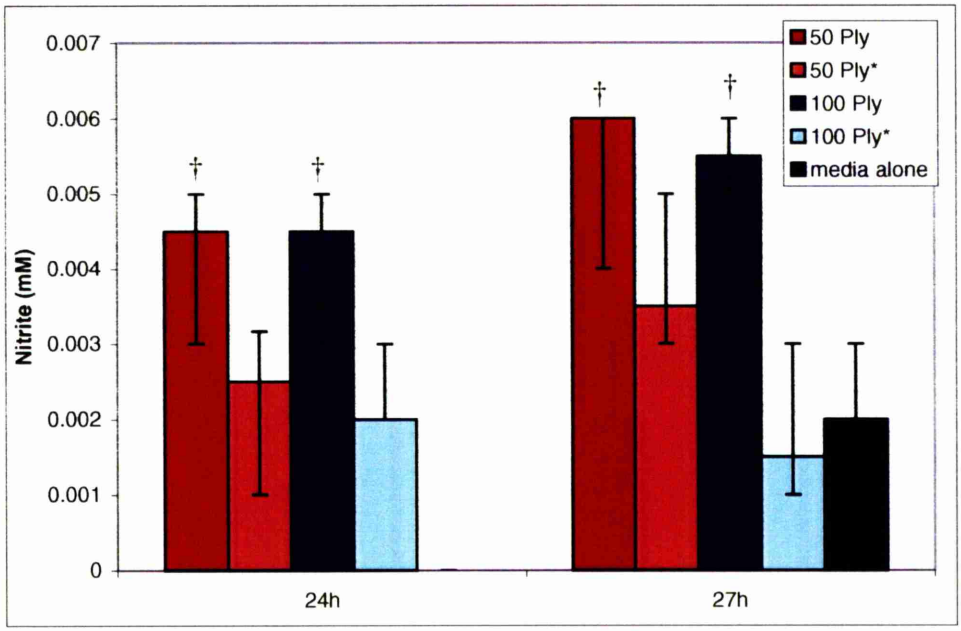
To prove the effects described above in section 5.2 are actually due to pneumolysin and not due to a contaminant in the protein preparation such as LPS, we tested heat-inactivated pneumolysin in these assays. Heating the sample to 56°C for ten minutes inactivates the protein but will not destroy the activity of any lipids etc in the sample such as LPS. The inactivation of the pneumolysin was checked in a haemolytic assay.

The macrophages were incubated with 50 ng and 100 ng of active and heat inactivated pneumolysin. The viability data is shown in Figure 5.15(a). There is no difference between macrophages incubated with active or inactive pneumolysin. At 27 hours there is a significant decrease in the viability of cells incubated with LPS ($p<0.05$) compared to cells in media alone.

Figure 5.15(a) – Viability of Macrophages with Heat-Inactivated Pneumolysin



(b) - NO Production from Macrophages incubated with Heat-Inactivated Pneumolysin



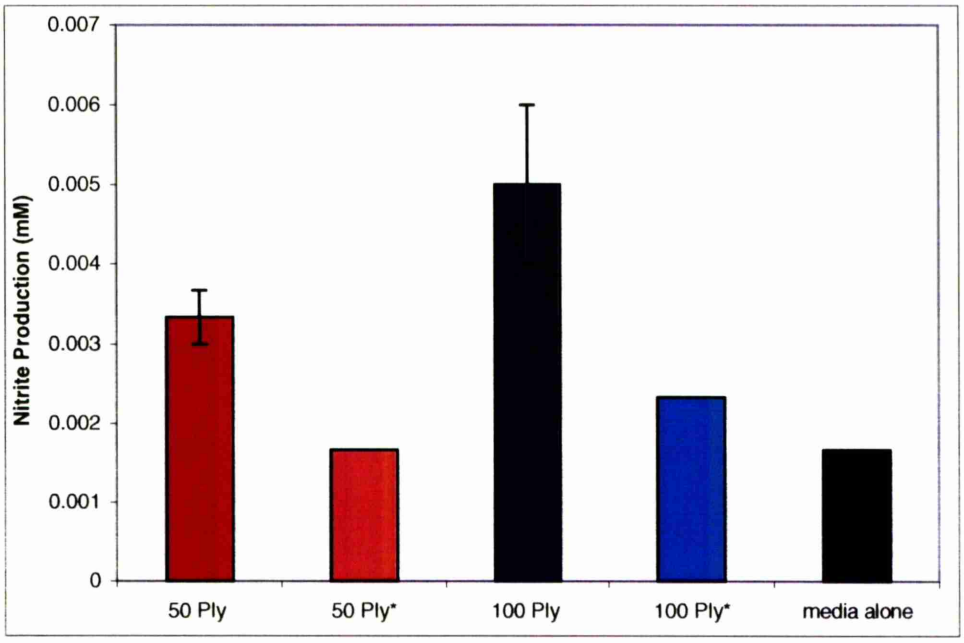
Cells were primed for four hours with IFNγ before addition of appropriate stimuli. Graph shows median values with maximum and minimum error bars. Ply*/LPS* = heated to 56°C for 10 minutes. † = NO Production is significantly higher than from cells incubated with the same concentration of heat-inactivated pneumolysin ($p<0.05$). Data from 2 separate experiments with duplicate samples are shown.

Heating the pneumolysin sample denatures the toxin and abolishes its ability to induce NO production from macrophages. The graph in Figure 5.15(b) shows that heat inactivating the toxin has significantly reduced the amount of nitrite production from the macrophages. There is some residual nitrite in the supernatant of the macrophages stimulated with the heat-inactivated pneumolysin sample. Whether this amount is significant or not depends on the amount of NO produced by unstimulated cells. As mentioned previously, in some experiments unstimulated macrophages produce up to ~0.002 mM nitrite. This effect can be seen in the unstimulated cells from the 27 hour time point. Because of this, the NO production from cells stimulated with heat-inactivated pneumolysin at 27 hours is not significantly different to the NO produced from unstimulated cells. At 24 hours, this spontaneous nitrite production was not seen, so the NO levels from the cells incubated with the heat inactivated pneumolysin samples appear significantly higher than the unstimulated cells.

The same experiment was carried out using the pneumolysin purified using metal chelate chromatography as this had lower endotoxin levels than the HIC purified pneumolysin. The results of this can be seen in Figure 5.16. There were no differences in viability between the pneumolysin-stimulated cells when compared to unstimulated cells. Cells incubated with LPS do show a decrease in viability compared to cells in media alone. This data is not shown, as it is very similar to that shown in Figure 5.15(a).

The graph in Figure 5.16 shows the nitrite production from duplicate samples of macrophages incubated with pneumolysin. The results are very similar to those in Figure 5.15(b) suggesting that the different levels of LPS (see Table 3.3) in the two samples does not affect the stimulation of NO from macrophages. NO production from the cells incubated with heat-inactivated pneumolysin is not above baseline levels. Again in this experiment, after 24 hours there is some spontaneous NO production from macrophages. This suggests that the significance of Figure 5.15(b) at 24 hours may be affected by the lack of NO production by control cells. Therefore, NO production is stimulated by pneumolysin and not by contaminants such as LPS in the protein preparation.

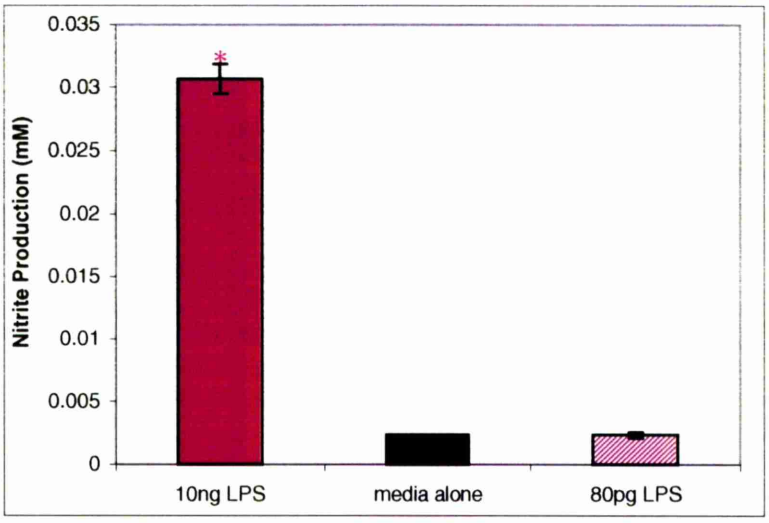
Figure 5.16 – The effect of Heat Inactivating His-Tagged Pneumolysin



Graph shows NO production after 24h from macrophages primed with IFNγ and then incubated with pneumolysin. LPS stimulated an average value of 0.031 +/-0.003 mM nitrite. Heated LPS stimulated an average value of 0.028 +/-0.003 mM nitrite. As this experiment was only carried out twice (in triplicate) statistical analysis cannot be performed. * = Heated to 56°C for 10 minutes.

The pneumolysin purified by metal chelate chromatography has <70 pg LPS per 100 ng, the dose used in most of the experiments. 80 pg/ml of LPS was added to macrophages and there was no increase in NO from these cells compared to unstimulated cells (Figure 5.17).

Figure 5.17 – LPS-stimulated NO Production from Macrophages



Macrophages were incubated with 10 ng or 80 pg/ml LPS. After 24 hours, the supernatants were assayed for the presence of nitrite. *Significantly more NO production than from unstimulated cells ($p<0.05$). There was no significant NO production from cells incubated with 80 pg/ml LPS. Data from 2 separate experiments with duplicate samples are shown.

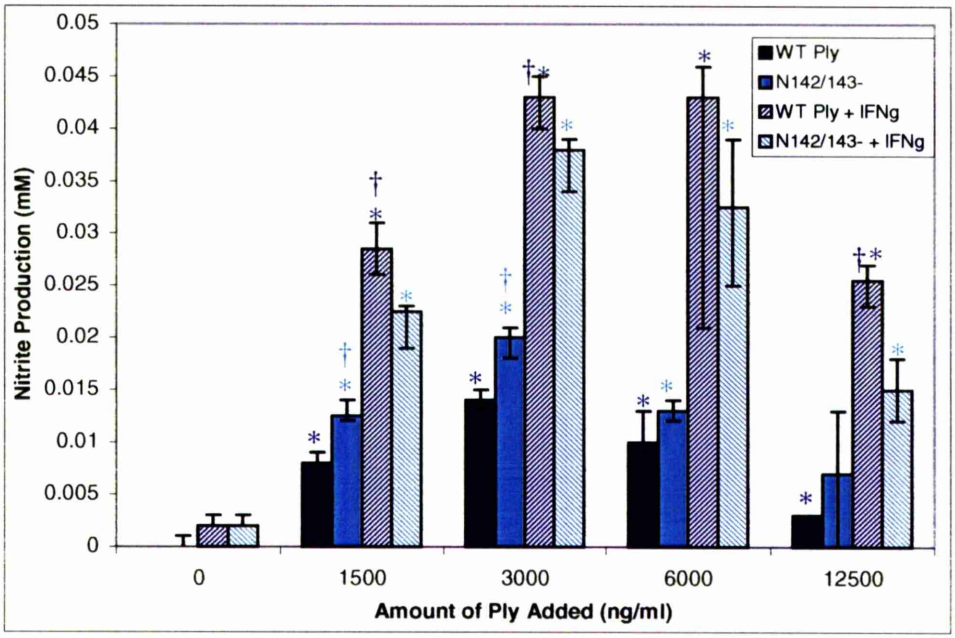
5.6 Comparison of Mutant and Wild Type Toxins

The N142/143 deletion mutant pneumolysin was used to see if the mutation had an effect on its ability to stimulate NO production. Also a pneumolysin mutant with only 0.1% of the haemolytic activity of the wild type toxin was used to see if the cytolytic activity of the toxin is involved in nitrite production.

5.6.1 The N142/143 deletion Pneumolysin Mutant can stimulate NO from Macrophages

The NO production from macrophages stimulated with the N142/143 deletion or wild type pneumolysin preparations is shown below in Figure 5.18.

Figure 5.18 – NO Production by the N142/143 deletion Pneumolysin Mutant



Macrophages were incubated with pneumolysin for 24 hours and the supernatants assayed for the presence of nitrite. Cells with IFNγ (IFNg) were primed for four hours before the addition of pneumolysin. The graph shows median values with maximum and minimum value error bars. *Significantly more NO production than cells in media alone. †Significantly more NO than from cells incubated with mutant/wild type pneumolysin. Data from 2 separate experiments with duplicate samples are shown.

The shape of the curves produced by the wild type and mutant toxins are the same. However, when the macrophages have not been primed with IFNγ, there is always slightly more NO production from the cells stimulated with the N142/143 deletion mutant than those with the wild type. When the cells have been primed with IFNγ, the reverse situation is seen where most nitrite is seen from the cells incubated with the wild type toxin. On closer

examination of the viability data for these concentrations of toxins it appears that the NO production is related to the viability. For example, when 3 μg/ml of either toxin is used significantly more nitrite is produced by cells stimulated with the N142/143 deletion mutant when the cells have not been primed with IFNγ. However under these conditions, the viability of the cells incubated with the N142/143 deletion mutant is also significantly higher than those incubated with the wild type toxin. When the cells have been primed with IFNγ the same situation is apparent but the nitrite production and viability are higher in the cells incubated with the wild type toxin.

These results suggest that there is no difference in the ability of the N142/143 deletion mutant to stimulate NO production from macrophages compared to the wild type toxin. Any differences seen in the assay are due to the differing numbers of viable cells.

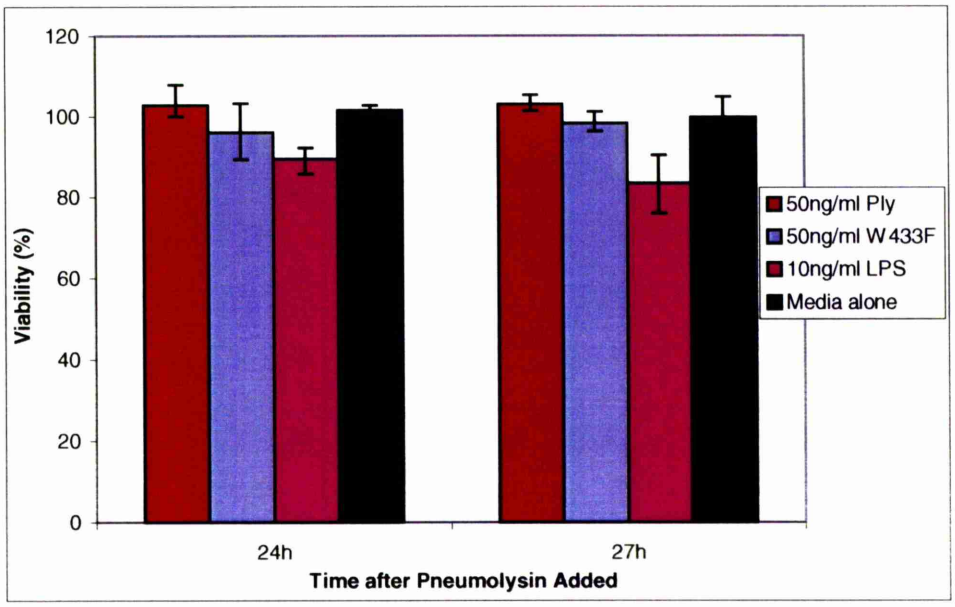
5.6.2 Using the W433F Pneumolysin Mutant

A pneumolysin mutant where the tryptophan at position 433 has been mutated to a phenylalanine (W433F) was purified by Ms F. Seow and has only 0.1% of wild type haemolytic activity (Korchev *et al.* 1998). This mutant was compared with the wild type pneumolysin to see if it had the same ability to induce NO production from macrophages. As lytic concentrations stimulated higher amounts of NO production, we wanted to see if the cytolytic activity of the toxin is involved in NO production. The viability of the cells was not affected by the concentrations used (Figure 5.19(a)).

The nitric oxide production from cells stimulated with IFNγ is shown in Figure 5.19(b). Cells incubated without IFNγ did not show significant NO production.

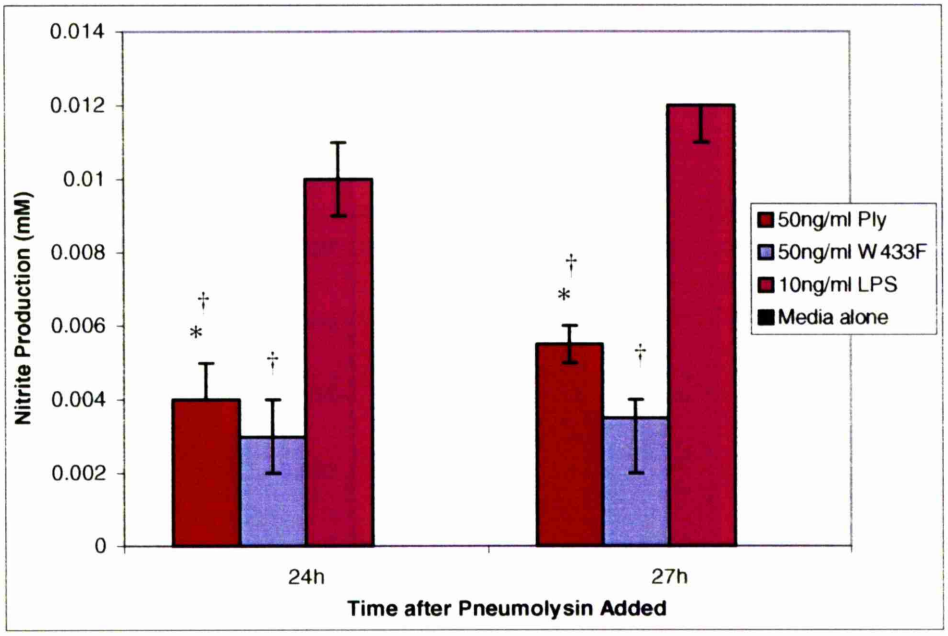
50 ng/ml pneumolysin plus IFNγ produced significantly more nitrite production from macrophages than cells in media plus IFNγ at both 24 and 27 hours ($p<0.01$). This is in keeping with the data presented above in Figure 5.11. The W433F mutant pneumolysin stimulates significantly less nitrite than wild type pneumolysin ($p<0.01$) but significantly more than cells incubated in media alone ($p<0.01$). This suggests that the haemolytic activity of pneumolysin is important in stimulating nitrite production from macrophages but is not the only factor involved.

Figure 5.19(a) – Viability of Macrophages incubated with Pneumolysin



Similar results were seen for cells also incubated with IFNγ.

(b) – NO production by the W433F pneumolysin mutant



Macrophages were primed with IFNγ for four hours and then either wild type of W433F pneumolysin was added. The supernatants were assayed for the presence of nitrite. *=Wild type pneumolysin produces significantly more NO than the W433F mutant ($p<0.05$). †=Wild type and W433F pneumolysin produce significantly more NO than media alone ($p<0.05$). Data from 3 separate experiments with duplicate samples are shown.

5.7 Summary

- The lytic action of pneumolysin mainly occurs over the first four hours after incubation with cells.

- The six-histidine-tagged pneumolysin and non-tagged pneumolysin had similar effects on the viability of macrophages.
- The macrophages and airway epithelial cells display similar sensitivities to pneumolysin after four hours.
- After 24 hours, much lower concentrations of pneumolysin caused a 50% drop in viability. This effect was more marked in the macrophages.
- Priming cells with IFNγ did not protect the cells from pneumolysin-induced death.
- Pneumolysin stimulates NO production from lung epithelial cells and from activated macrophages.
- Most NO production is seen when concentrations used are known to cause cell death.
- NO production from macrophages is partly dependent on the cytolytic activity of toxin.
- Lytic concentrations of pneumolysin can stimulate TNF from macrophages.
- The N142/143 deletion does not abolish the ability of pneumolysin to produce NO or TNF.
- Contamination with LPS has a nominal effect on pneumolysin-induced NO production.

6. EFFECTS OF STREPTOCOCCUS PNEUMONIAE ON CELLS

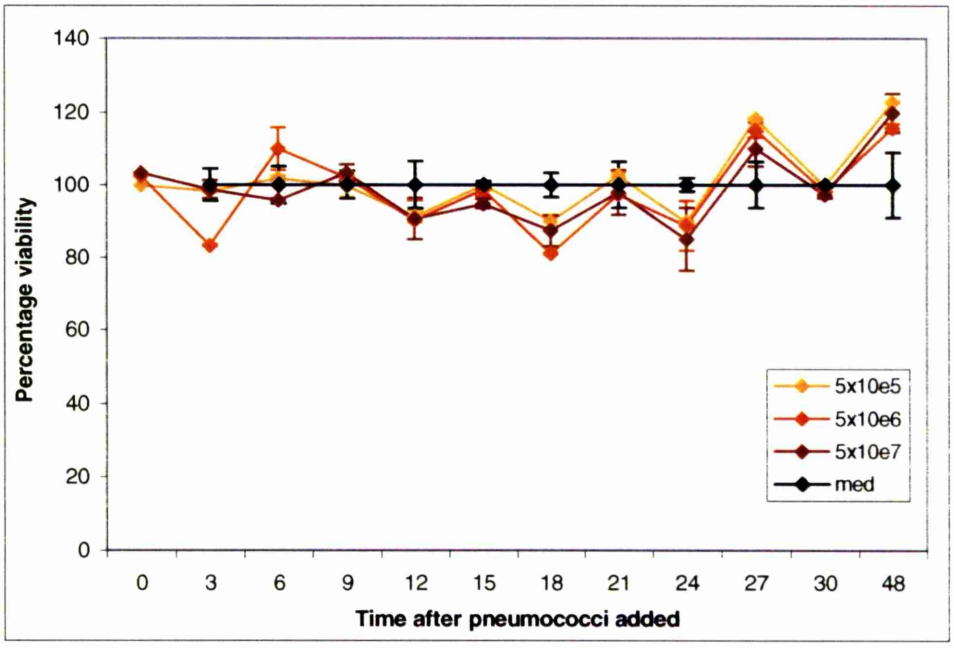
6.1 Effects of *S. pneumoniae* on Macrophages

To put the effects of pneumolysin in the context of the whole organism, experiments using whole bacteria were carried out. The bacteria used were a wild type and a pneumolysin-negative mutant of D39 *S. pneumoniae* made by Ms L. Craven.

6.2 Effects of Heat-Killed Pneumococci on Macrophages

We started by looking at the effect of heat-killed pneumococci, to see if factors such as the cell wall would have an effect. The bacteria were killed by heating at 60°C for ten minutes and spotted onto blood agar plates, which confirmed they were not viable. Macrophages were seeded at 5 x 10^5 cells/ml with or without IFNγ and ten-fold concentrations of heat-killed pneumococci from 5 x 10^1 to 5 x 10^7 were added. The viability of the macrophages was measured by MTT assay and the results are shown in Figure 6.1.

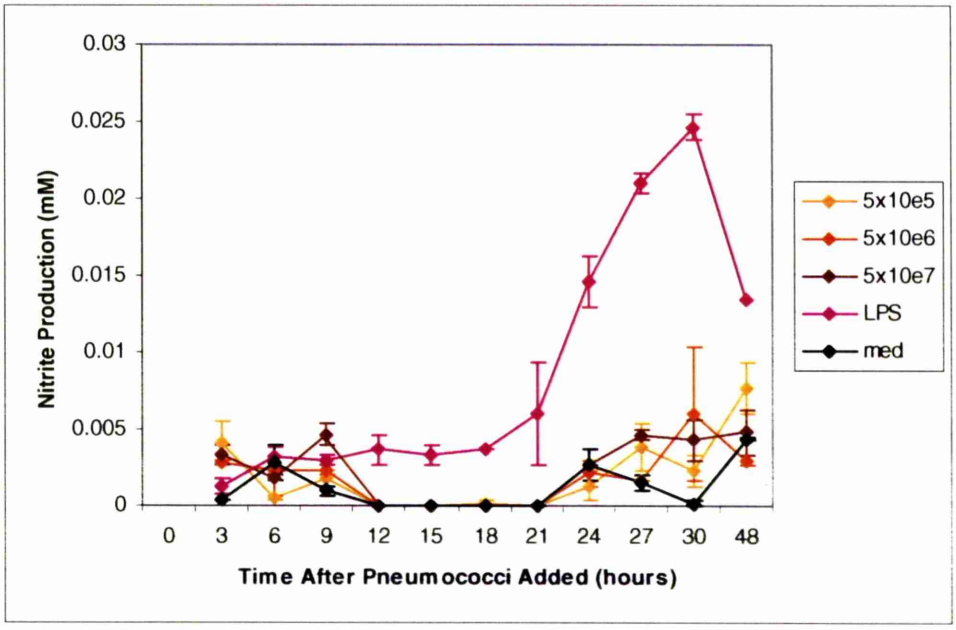
Figure 6.1 – Viability of Macrophages with Heat-killed Pneumococci



Macrophages were incubated with heat-killed pneumococci for various time points. The viability of the macrophages was assessed using MTT assay. The legend shows the amount of pneumococci added to the cells. The graph displays median +/- max/min values. Data from 2 separate experiments with duplicate samples are shown.

There is no significant decrease in the viability of the macrophages incubated with the heat-killed pneumococci with or without IFNγ. The supernatants were used in a Griess reaction to measure nitrite from the macrophages, the results of which are shown in Figure 6.2. Cells stimulated with LPS and IFNγ were used as a positive control.

Figure 6.2 – NO Production stimulated by Heat-killed Pneumococci



Macrophages were primed with 0.5 ng/ml IFNγ before addition of heat-killed bacteria. Supernatants were assessed for the presence of nitrite. The graph shows median with maximum and minimum values. Data from 2 separate experiments with duplicate samples are shown.

No significant differences were found in NO production from macrophages incubated with heat-killed pneumococci compared to those cells in media alone. No significant nitrite production was found from macrophages incubated with heat-killed pneumococci without IFNγ. The supernatants were also used in a TNF assay and no TNF activity was detected. In this system, heat-killed pneumococci do not have any effect on the macrophages.

6.3 Effects of Live *S. pneumoniae* on Macrophages

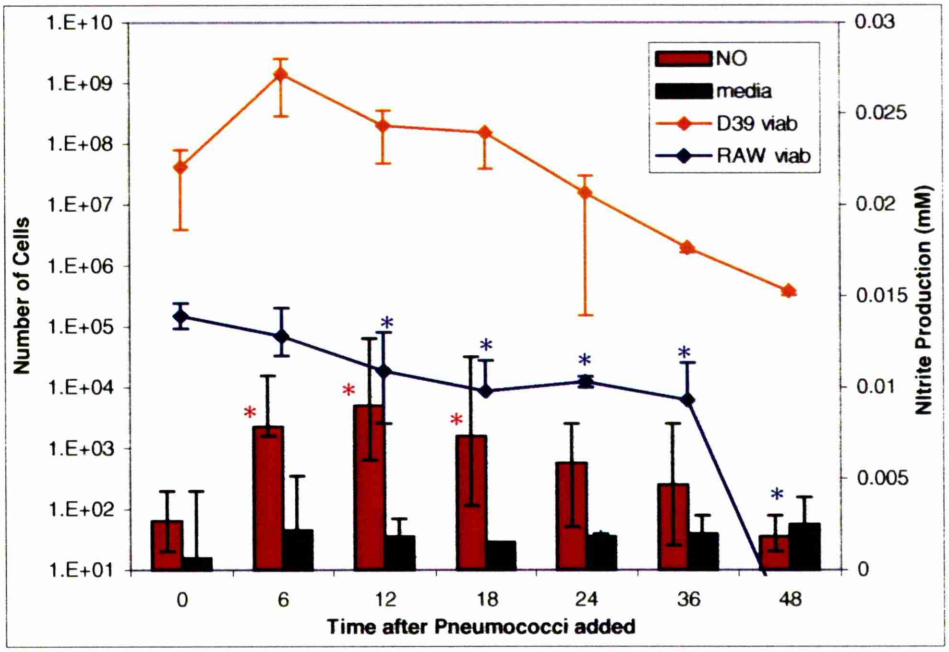
The next experiments looked at the effect that live pneumococci had on the macrophages. Macrophages were seeded as before with or without IFNγ and three different concentrations of live bacteria were added. Samples were taken at various time points over 48 hours. Preliminary experiments showed that live bacteria could uptake the MTT and interfere with the assay. So to calculate the macrophage viability, trypan blue exclusion counts in a haemocytometer were carried out and numbers of live and dead cells were recorded. Viable counts of the bacteria were also carried out. The supernatants were stored at -80°C until used in the Griess reaction and TNF bioassay. The results for these are shown below.

Figure 6.3 shows an example of the data obtained from the time courses carried out using the wild type D39 *S. pneumoniae*. Three concentrations of D39 were used as starting inoculum

for the experiments (5 x 10^5, 5 x 10^6, and 5 x 10^7 cfu/ml) and they showed similar results. The bacterial cells grew rapidly to reach a maximum of 10^9 – 10^{10} cfu/ml between 6-12 hours. After this time the viability dropped steadily, probably due to a lack of nutrients and build up of waste products in the culture media.

The viability of the macrophages dropped steadily and is significantly lower ($p<0.05$) than cells incubated without bacteria after twelve hours for all concentrations of bacteria used. Similar results were seen when the cells were primed with IFNγ.

Figure 6.3 – Macrophages incubated with 5 x 10^7 cfu/ml Pneumococci



Macrophages were incubated with 5 x 10^7 cfu/ml of D39 *S. pneumoniae*. Samples were taken at various times and macrophage and bacterial viability counts were carried out. In this graph, cell viability (viab) is plotted as a line chart. NO measured from the supernatant is shown as a bar chart. The black bars show the background levels of NO measured from the supernatant of unstimulated cells. Median +/- maximum and minimum values are shown. Data from 2 separate experiments with duplicate samples are shown. * = Macrophage (RAW) viability is significantly less than cells in media alone ($p<0.05$). * = NO production is significantly higher than from cells in media alone ($p<0.05$)

When 5 x 10^7 or 5 x 10^6 cfu/ml were used as a staring inoculum, NO production was significantly increased compared to unstimulated cells from 6-18 hours. When 5 x 10^5 cfu/ml was used, significant amounts of NO were seen from 12-18 hours after addition of bacteria. NO production from the macrophages stimulated with LPS occurred at later time points, from 18-48 hours. This indicates the possibility of different mechanisms being involved in NO production. Unstimulated cells showed no significant differences in NO production

throughout all experiments. Priming the cells with IFNγ made no difference to NO production from the macrophages incubated with *S. pneumoniae.*

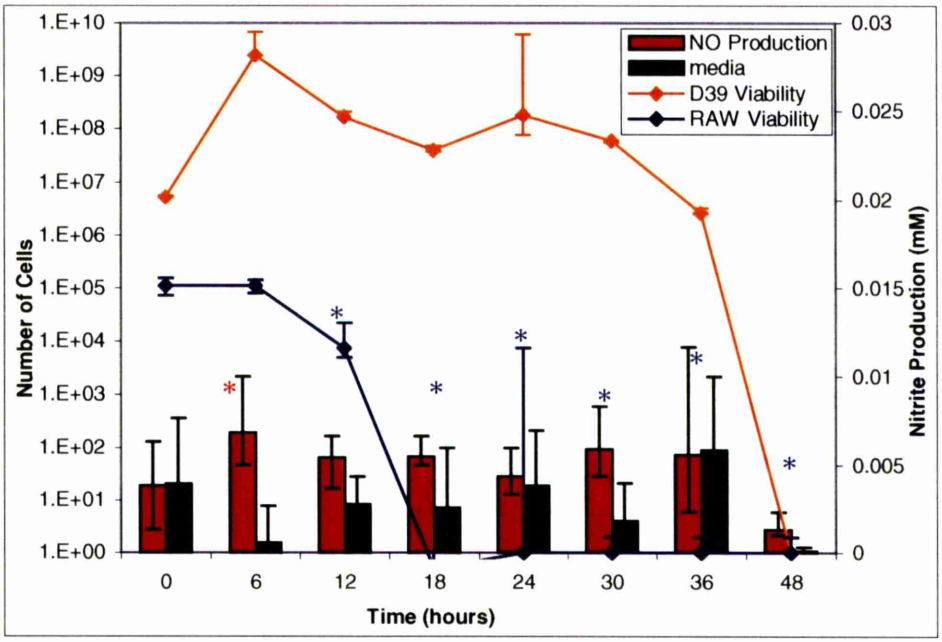
There was no significant TNF production found from any of the samples at any time point, except those stimulated with LPS and IFNγ (not shown). In the TNF bioassay, the TNF activity required to kill 50% of the connective tissue cells is measured. In some experiments, supernatant from the cells incubated with *S. pneumoniae* for 6 hours, caused a decreased viability of the connective tissue cells. In repeat experiments the TNF activity fell just below the 50% kill cut off point. It is likely that there is some TNF produced by the macrophages after six hours in this system; if these samples were measured using a more sensitive assay such as an ELISA they could probably be detected. For the standards used in the TNF bioassay, 1 U is equivalent to 0.5 ng/ml TNF. This means that the bioassay will not detect levels of TNF lower than this.

In our group, TNF activity was detected in bronchus associated lymphoid fluid (BALF) and bronchus associated lymphoid tissue (BALT) in lungs from mice infected with 10^4-10^6 cfu/ml of D39 *S. pneumoniae.* Between 6-36 hours after infection, this TNF production was associated with resistance to infection in BALB/c mice (Kerr *et al.* 2002). The difference in detection could be due to the numbers of cells used as TNF activity was measured per lung, which is likely to contain more than 5 x 10^5 cells - the number of macrophages used in these studies. Cells recovered from the airways and stimulated with the stocks of pneumolysin made for this project produced ~150 pg/ml TNF protein detectable by ELISA. RAW macrophages are derived from BALB/c mice and, in mice at least, the macrophage is the main producer of such cytokines in the lung (Vassalli 1992). *In vivo,* there is of course a much more complex network of cellular and cytokine interactions some of which could act synergistically to induce mediator production.

To test whether the viability effects and NO production seen above were due to pneumolysin, the same experiments were carried out using a mutant of D39 *S. pneumoniae,* which does not contain the pneumolysin gene (see Figure 6.4). For the first six hours the bacteria grow rapidly, when 5 x 10^5 cfu/ml were used at the start of the experiment this growth continues for 12 hours. The numbers then decline steadily except at 24 hours there is a slight increase in numbers again; this was seen in all experiments. At 48 hours the levels have dropped below the level of detection of the viability count i.e. less than 50 cfu/ml. In

the experiments with the wild type pneumococci, the numbers of bacteria did not drop below 10^4 cfu/ml after 48 hours.

Figure 6.4 – Macrophages incubated with $5x10^6$ cfu/ml Pneumolysin-negative Pneumococci



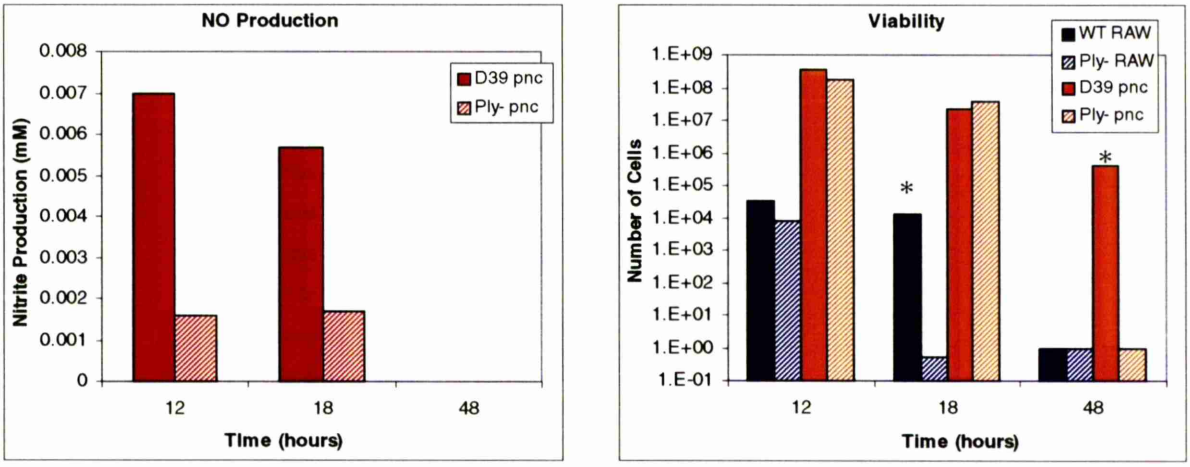
Macrophages were incubated with 5 x 10^6 cfu/ml pneumolysin-negative mutant of *S. pneumoniae*. Samples were taken at various times and macrophage and bacterial viability counts were carried out. In this graph, cell viability is plotted as a line chart. NO measured from the supernatant is shown as a bar chart. The black bars show the background levels of NO measured from the supernatant of unstimulated cells. Graph shows median +/- maximum and minimum values.Data from 2 separate experiments with duplicate samples are shown. * = Macrophage viability is significantly less than cells in media alone ($p<0.05$). * = NO production is significantly higher than from cells in media alone ($p<0.05$)

The viability of the macrophages starts to decline significantly after twelve hours in experiments using either wild type or pneumolysin-negative D39. However after twelve hours, the decrease in macrophage viability is much greater when the pneumolysin-negative pneumococci were used. At 18 hours, the number of cells dropped below the level of detection of the assay and stayed at this level for the rest of the experiment.

After six hours, there is significant NO production from macrophages incubated with the pneumolysin-negative pneumococci. The macrophages stimulated with wild type pneumococci produce significantly more NO than cells stimulated with the pneumolysin-negative pneumococci at six and twelve hours ($p<0.05$). Figure 6.5 compares the NO production from the macrophages stimulated with either wild type or pneumolysin-negative

D39 pneumococci. It also highlights the differences in viability of the macrophages and the bacteria at three time points.

Figure 6.5 – Differences between Experiments with Macrophages incubated with Wild Type or Pneumolysin-negative Pneumococci



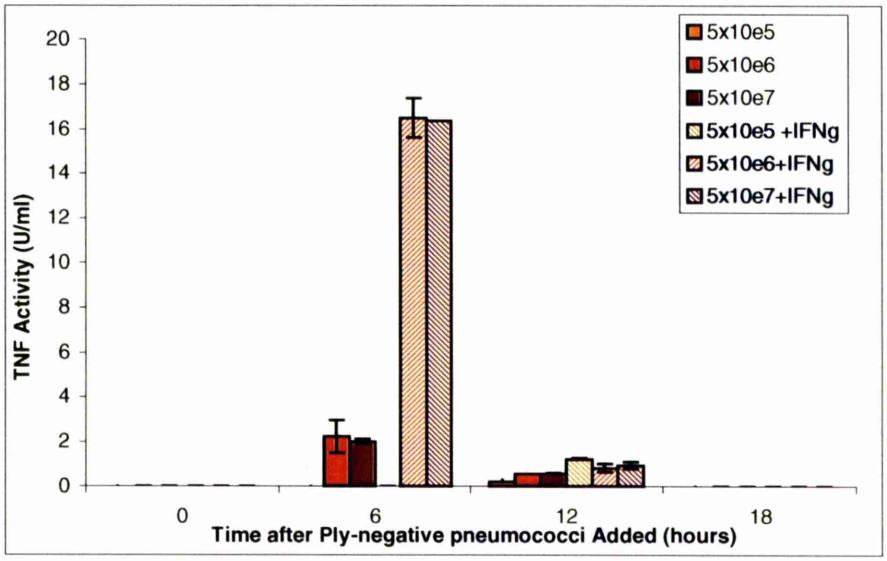
These graphs display the median values from the experiments described above to emphasise the differences between the wild-type and pneumolysin-negative pneumococci at different time points. The bars showing nitric oxide production from the macrophages have the background levels from unstimulated cell subtracted. *Significantly more cells in experiments with D39 bacteria compared to those using pneumolysin-negative bacteria. RAW = macrophages; pnc = pneumococci.

There was some TNF activity detected from the supernatants of these experiments as shown in Figure 6.6. Without IFNγ priming, 2U/ml of TNF was seen after six hours from the two higher concentrations of bacteria used. After twelve hours a small but detectable amount (~0.5U/ml) of TNF was produced from all experimental conditions. In this case priming of the macrophages with IFNγ increased the amount of TNF produced, with peak production at six hours (16 U/ml). The pneumococcus is not as potent a stimulator of TNF as LPS. Cells stimulated with LPS and IFNγ, produced up to 90 U/ml TNF after six hours.

These results suggest that factors other than pneumolysin in the pneumococci are capable of stimulating TNF production from macrophages. In the experiments with the wild type pneumococci, TNF activity was below the level of detection of the bioassay. TNF production from the pneumolysin-negative pneumococci occurs at the maximum bacterial growth (median value 5 x 10^9 cfu/ml). At six hours in the wild type pneumococci, the number of bacteria is slightly lower (1 x 10^9 cfu/ml). It is possible that the maximum bacterial growth and TNF production were just missed in the wild type growth curves. It is likely that the wild type bacteria did stimulate TNF production below the level of detection of the bioassay.

Pneumococcal-induced TNF has been confirmed in other studies (Bergeron *et al.* 1998; Kerr *et al.* 2002).

Figure 6.6 – TNF Production from Macrophages by Pneumolysin-negative Pneumococci



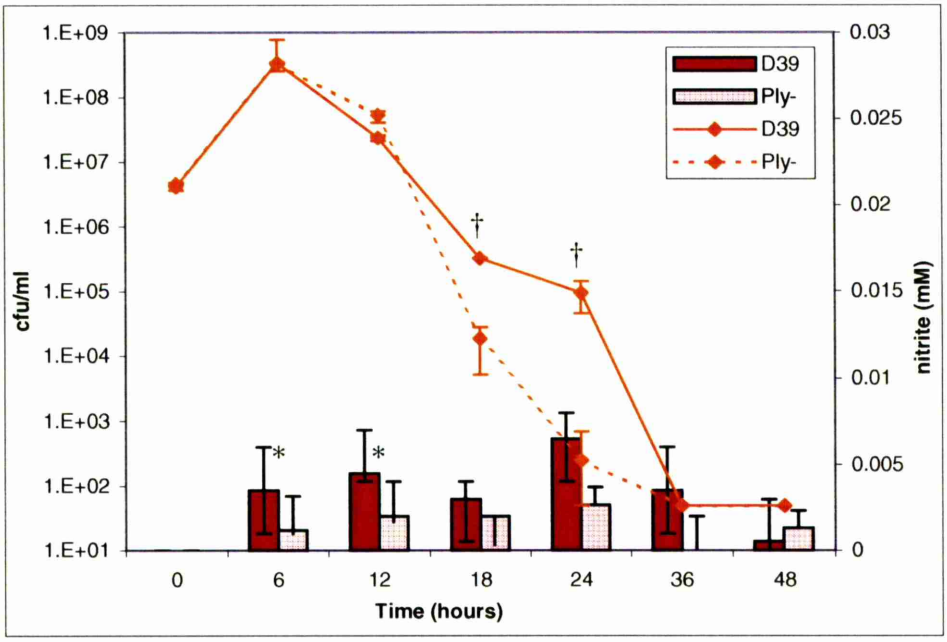
The supernatants from the experiments described in section 6.3 were assayed for the presence of TNF activity. TNF activity was only detected in the cell supernatants incubated with the pneumolysin-negative pneumococci and are shown here. IFNg = Interferon gamma. Graph shows median values +/- maximum and minimum values. Data from 2 separate experiments with duplicate samples are shown.

6.3.1 Viability of *S. pneumoniae* in Macrophage Culture Media

The viability of *S. pneumoniae* and the pneumolysin–negative mutant in the media used to grow the macrophages (DMEM) was observed. Samples from this experiment were used to see if there was any spontaneous NO production from the bacteria. The pattern of viability for each concentration used (5 x 10^5, 5 x 10^6, and 5 x 10^7 cfu/ml) was similar for each. The results for 5 x 10^6 cfu/ml are shown in Figure 6.7. At first, the viability of the wild type and mutant *S. pneumoniae* is very similar. After twelve hours, the viability of the mutant pneumococci drops much more quickly than the wild type bacteria. At 18 and 24 hours the numbers of pneumolysin-negative bacteria are significantly less than the wild type ($p<0.01$). This was also seen when the starting inoculum was 5 x 10^5 cfu/ml. When 5 x 10^7 cfu/ml was used as the starting inoculum, both the wild type and pneumolysin-negative numbers decline more rapidly after six hours. Although there were still significantly less cfu/ml of the pneumolysin-negative bacteria compared to the wild type ($p<0.05$) the difference was not as marked. No differences in the viability of wild type and pneumolysin-negative pneumococci have been observed before.

There seems to be some spontaneous nitrite production from *S. pneumoniae* or the bacteria may be producing something that interferes with the assay. There does seem to be a slight trend for more NO to be produced from the wild type bacteria. At 24 and 36 hours, these differences are significant ($p<0.05$). At 5×10^5 and 5×10^7 concentrations, the amount of NO produced by *S. pneumoniae* alone in DMEM is significantly less ($p<0.05$) than that produced by *S. pneumoniae* and macrophages at six and twelve hours.

Figure 6.7 – Viability of *S. pneumoniae* and Nitrite Production in DMEM



The orange lines show the viability of the wild type (solid line) and pneumolysin-negative (dashed line) bacteria. The red columns show the nitrite levels in the supernatant. The graph shows median values and maximum and minimum error bars. *Significantly less NO production than detected from the supernatant of macrophages incubated with either D39 or pneumolysin-negative bacteria ($p<0.05$). †Significantly more D39 compared to pneumolysin-negative bacteria ($p<0.05$).

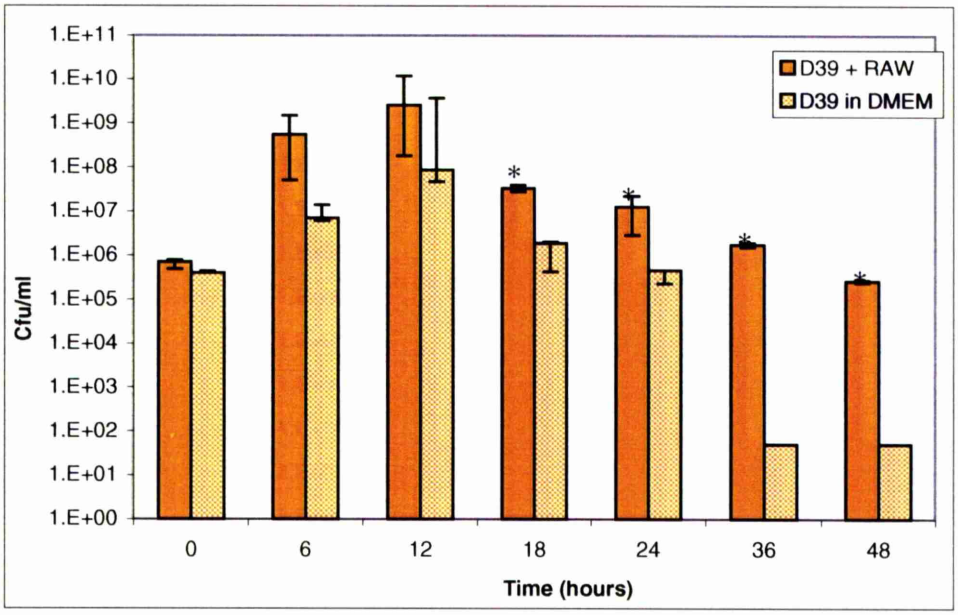
Nitrite-producing bacteria have been isolated from tongues of laboratory rats (Li *et al.* 1997a). In this study, a large proportion of bacteria were found to reduce nitrate to nitrite. The main nitrite-producing genera were *Staphylococcus* (65%), *Pasteurella* (20%) and *Streptococcus* (10%). The increase in nitrite production may be due to increased expression of respiratory nitrate reductase enzymes in conditions of reduced oxygen tension in the deep clefts on the posterior tongue (Li *et al.* 1997a). However, a study of NO from commensal bacteria in the human oral cavity found the major nitrate reducers were species of *Actinomyces* and *Veillonella*. Although *Streptococcus* species were the predominant bacteria isolated, they found none that could reduce nitrate (Smith *et al.* 1999). A search using the

WIT project (http://wit.mcs.anl.gov/WIT2) revealed that several nitrate reductase enzymes are present in the pneumococcal genome. These enzymes are capable of releasing nitrite by catalyzing a reaction between nitrate and an electron donor. Pneumococci therefore have the potential to produce nitrite which could account for the nitrite detected from the cultures grown without macrophages.

A nitrate reductase conversion assay was carried out using the DMEM media to see if there was any nitrate present in the media, which could potentially be converted to nitrite by the bacteria. The levels of both nitrite and nitrate in the media were below the zero standards used in the assay.

It is interesting to note that when grown in DMEM, after 36 hours, the viability of the bacteria was below the level of detection of the assay indicating that there are less than 50 cfu/ml. When the bacteria were grown with the macrophages, the viability did not drop below 10^4 cfu/ml (Figure 6.8).

Figure 6.8 – The effect of Macrophages on Pneumococcal Viability



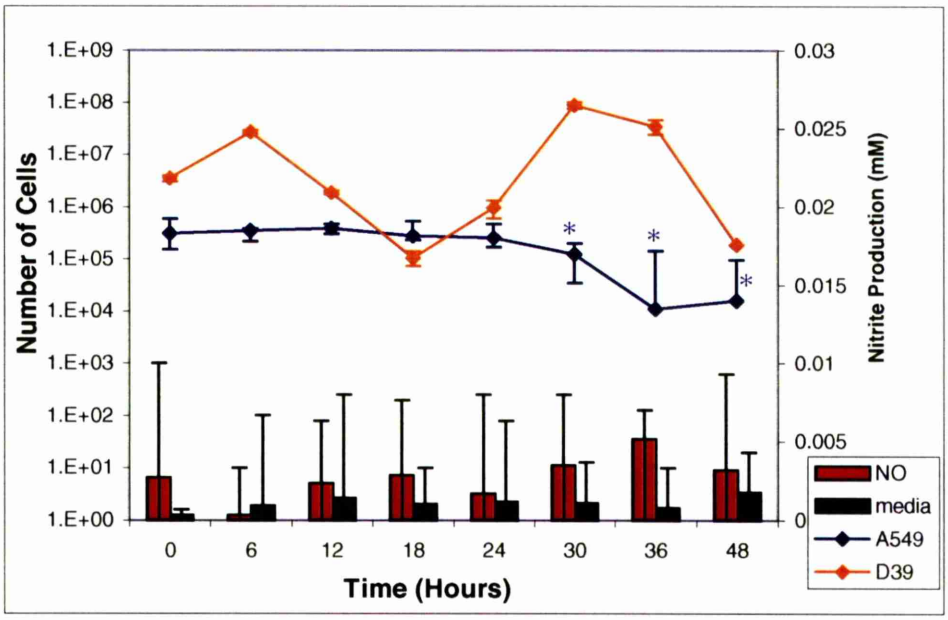
This graph emphasises the differences in bacterial viability in the presence of macrophages compared to in the macrophage growth media alone. The solid bars show the number of bacteria at each time point when grown in culture with macrophages. The checked bars show D39 growth in the macrophage growth media alone. *Significantly more bacteria when grown in culture with macrophages compared to those grown in DMEM ($p<0.05$). Median values with maximum and minimum error bars are shown.

6.4 Effects of *S. pneumoniae* on Lung Epithelial Cells

The same experiments were then carried out using the A549 lung epithelial cell line. The time courses were carried out in the same way as those described earlier using the macrophages (See Section 6.3). The same starting concentrations of D39 *S. pneumoniae* were used and the viability of the bacterial cells and the lung epithelial cells were measured. The results from the experiment that started with 5 x 10^6 cfu/ml are shown in Figure 6.9.

The bacterial growth was very similar regardless of whether the starting inoculum was 5 x 10^5, 5 x 10^6 or 5 x 10^7 cfu/ml. As can be seen in Figure 6.9, the viability of the bacteria drops for the first 18 hours of the experiment. After 18 hours the D39 growth starts to increase until 30 hours when it starts to decrease again. No significant NO production was seen in any of the experiments using the lung epithelial cells. Therefore, unlike macrophages, the lung epithelium does not seem to be a major source of NO production during incubation with live pneumococci.

Figure 6.9 – Lung Epithelial Cells incubated with 5 x 10^6 cfu/ml Pneumococci

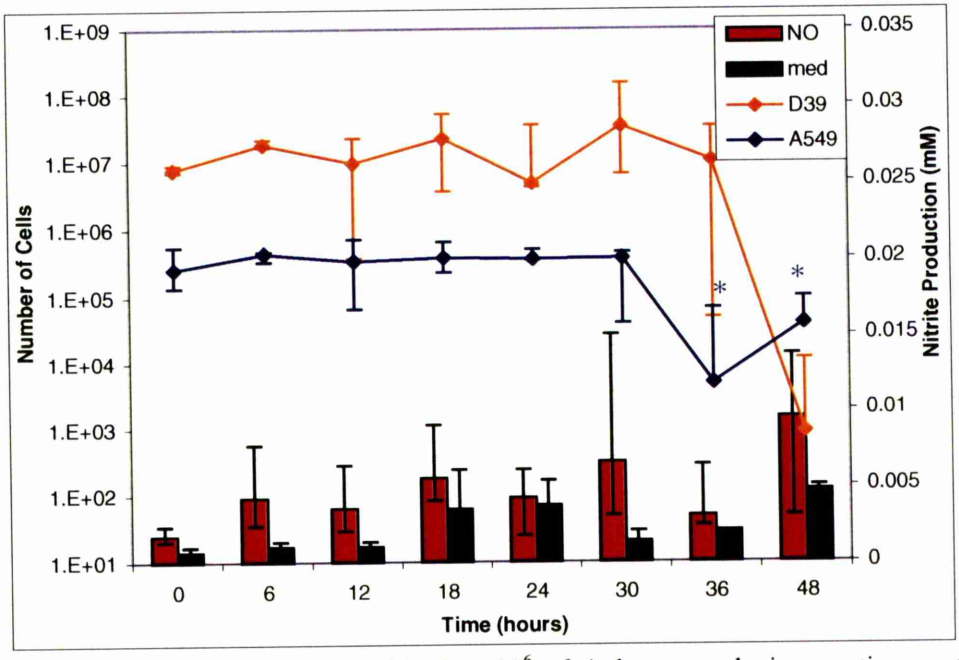


Lung epithelial cells were incubated with 5 x 10^6 cfu/ml D39 *S. pneumoniae*. Samples were taken at various times and epithelial and bacterial cell viability counts were carried out. In this graph, cell viability is plotted as a line chart. NO measured from the supernatant is shown as a bar chart. The black bars show the background levels of NO measured from the supernatant of unstimulated cells. Graph shows median +/- maximum and minimum values. Data from 3 separate experiments with duplicate samples are shown. * = Lung epithelial viability significantly lower than cells in media alone ($p<0.05$)

The lung epithelial cells seem to be more resistant to the bacteria than the macrophages; their numbers do not decrease significantly until after 30 hours incubation. When the highest inoculum was used, the lung epithelial cell numbers start to decrease slightly earlier at 24 hours. Priming the cells with IFNγ made no difference to the bacterial growth or the lung epithelial cell viability.

The experiments were then repeated using the pneumolysin-negative D39 (Figure 6.10). The drop in *S. pneumoniae* viability between 12-24 hours is only seen when wild type bacteria are used. This indicates that pneumolysin is involved in this effect. It could be stimulating the lung epithelial cells to produce something that is harmful but this would not explain why the viability increases again after 18 hours. Alternatively, the bacteria could be invading the cells. This would happen *in vivo* if the bacteria were to invade the bloodstream. This effect will be discussed in more detail in section 8.3.

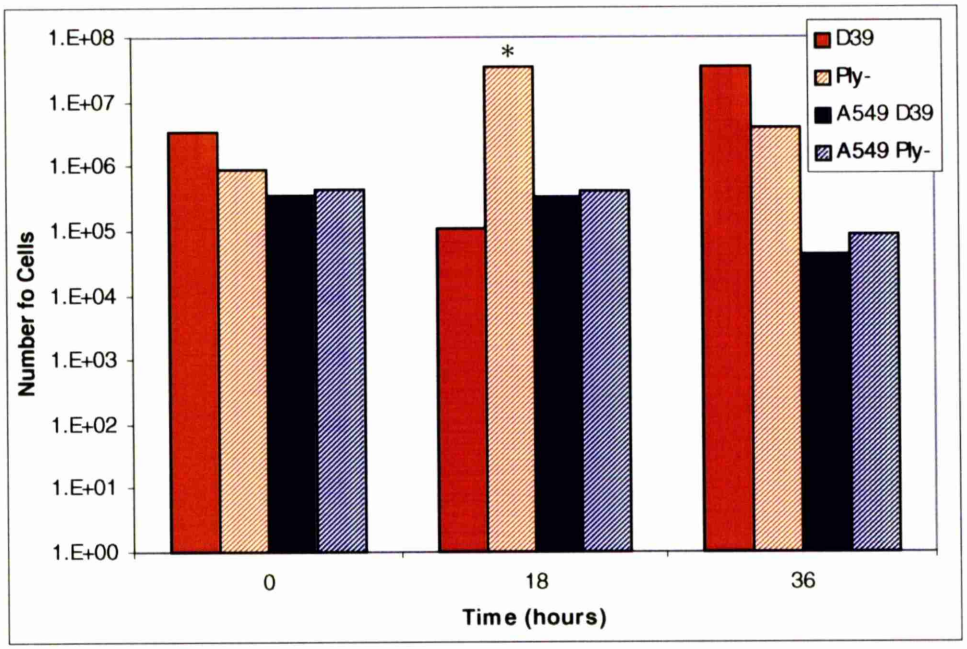
Figure 6.10 – Lung Epithelial Cells incubated with 5 x 10^6 cfu/ml Pneumolysin-negative Pneumococci



Lung epithelial cells were incubated with 5 x 10^6 cfu/ml pneumolysin-negative mutant of *S. pneumoniae*. Samples were taken at various times and epithelial cell and bacterial viability counts were carried out. In this graph, cell viability is plotted as a line chart. NO measured from the supernatant is shown as a bar chart. The black bars show the background levels of NO measured from the supernatant of unstimulated cells. Graph shows median +/- maximum and minimum values. Data from 2 separate experiments with duplicate samples are shown. * = Lung epithelial viability significantly lower than cells in media alone ($p<0.05$)

Comparisons between the growth of the wild type and pneumolysin-negative bacteria with lung epithelial cells are shown in Figure 6.11. The growth of the lung epithelial cells is not affected by the presence of pneumolysin in the bacteria. The time points displayed in the graph accentuate the differences in growth of the wild type and pneumolysin-negative *S. pneumoniae*.

Figure 6.11 – Cell Viability with Wild Type or Pneumolysin-negative Pneumococci



This graph emphasises the differences in bacterial and macrophage viability between the experiments with wild type and pneumolysin-negative pneumococci. The viability of bacteria (orange bars) and lung epithelial cells (blue bars) from experiments with D39 and pneumolysin-negative bacteria are compared in this graph. Median values are displayed. *Significantly more cells in experiments with pneumolysin-negative bacteria compared to those using D39 bacteria.

6.4.1 Growth of *S. pneumoniae* incubated with Lung Epithelial Cells

During the first time course using the lung epithelial cells, it was noted that the growth of the bacteria plated out for viable counts was very strange. The colonies were very tiny and difficult to count. It looked as though the lung epithelial cells may be producing a substance that was harmful to the bacteria. In order to investigate this 5 x 10^5 cfu/ml of wild type *S. pneumoniae* were incubated with F12k media, 5 x 10^5 lung epithelial cells/ml or supernatant from a flask of confluent lung epithelial cells. Samples were plated out twice for viable counts, one plate was incubated at 37°C as normal and one plate was incubated at 37°C in an anaerobic environment. Over two hours, there was no difference in the growth of the bacteria incubated in media, with the cells or with supernatant from the cells. However the colonies

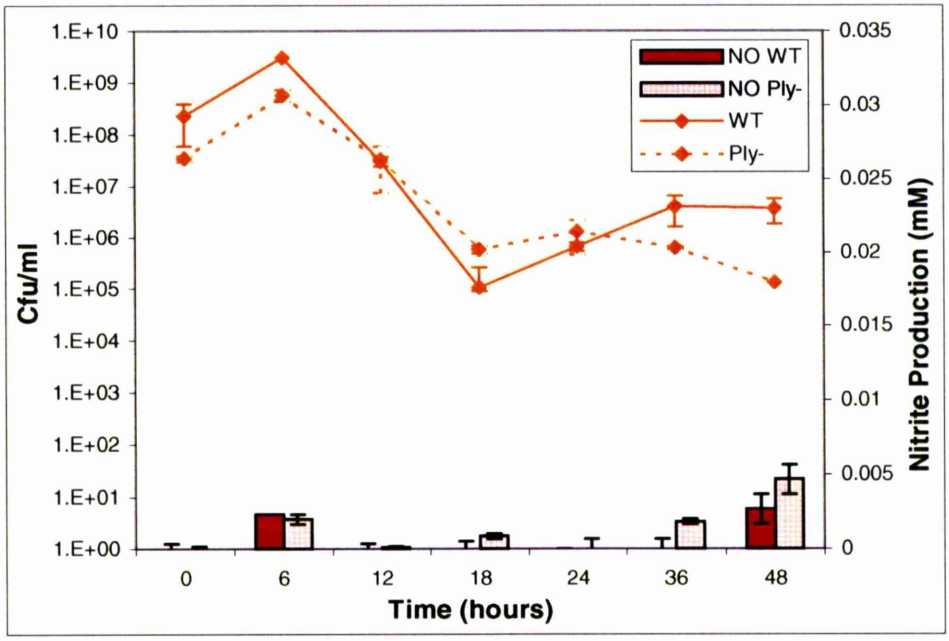
that were not grown in an anaerobic environment were impossible to count, as they were tiny. These colonies did not grow any larger after further incubation.

In future experiments the agar plates containing bacterial viable counts were incubated in an anaerobic atmosphere to enable more accurate counts to be made. The different colony morphology was not seen when the bacteria were incubated with the macrophages and suggests that something in the growth conditions during experiments with lung epithelial cells is altering the phenotype of the bacteria to one with a preference to grow anaerobically.

6.4.2 Growth of *S. pneumoniae* in Lung Epithelial Cell Culture Media

The growth of the bacteria in the media used to culture the macrophages was observed and the cultures were measured in the Griess reaction to see if there was any NO production (Figure 6.12). There were no differences in the growth of the pneumolysin-negative and wild type bacteria growth in F12k media.

Figure 6.12 – Viability of *S. pneumoniae* and Nitrite Production in F12k Medium



The orange lines show the viability of the wild type (solid line) and pneumolysin-negative (dashed line) bacteria. The red columns show the nitrite levels in the supernatant. Graph shows median values and maximum and minimum error bars. Data from 2 separate experiments with duplicate samples are shown.

A nitrate reductase conversion assay was carried out using the F12k media to see if there was any nitrate present in the media, which could potentially be converted to nitrite by the

bacteria. The levels of both nitrite and nitrate in the media were below the zero standard used in the assay.

6.5 Summary

- Heat-killed pneumococci have no effect on the macrophages in the assays used.
- Live *S. pneumoniae* kill macrophages and promote NO production.
- Pneumolysin-negative pneumococci cause more macrophage cell death, but less NO production compared to wild type bacteria.
- Wild type pneumococci survive longer than pneumolysin-negative pneumococci when incubated with macrophages.
- The presence of macrophages promotes pneumococcal survival compared to its growth in media alone.
- Pneumolysin-negative pneumococci survive less well than wild type bacteria in DMEM medium.
- Lung epithelial cells are more resistant to pneumococci than macrophages.
- When incubated with lung epithelial cells, a drop in bacterial viability between 12 and 24 hours was seen, but only when wild type pneumococci were used.
- Growth with the lung epithelial cells caused a change in the phenotype of the bacteria to one which had a preference to grow anaerobically.

7. GENE REGULATION BY PNEUMOLYSIN

7.1 Introduction

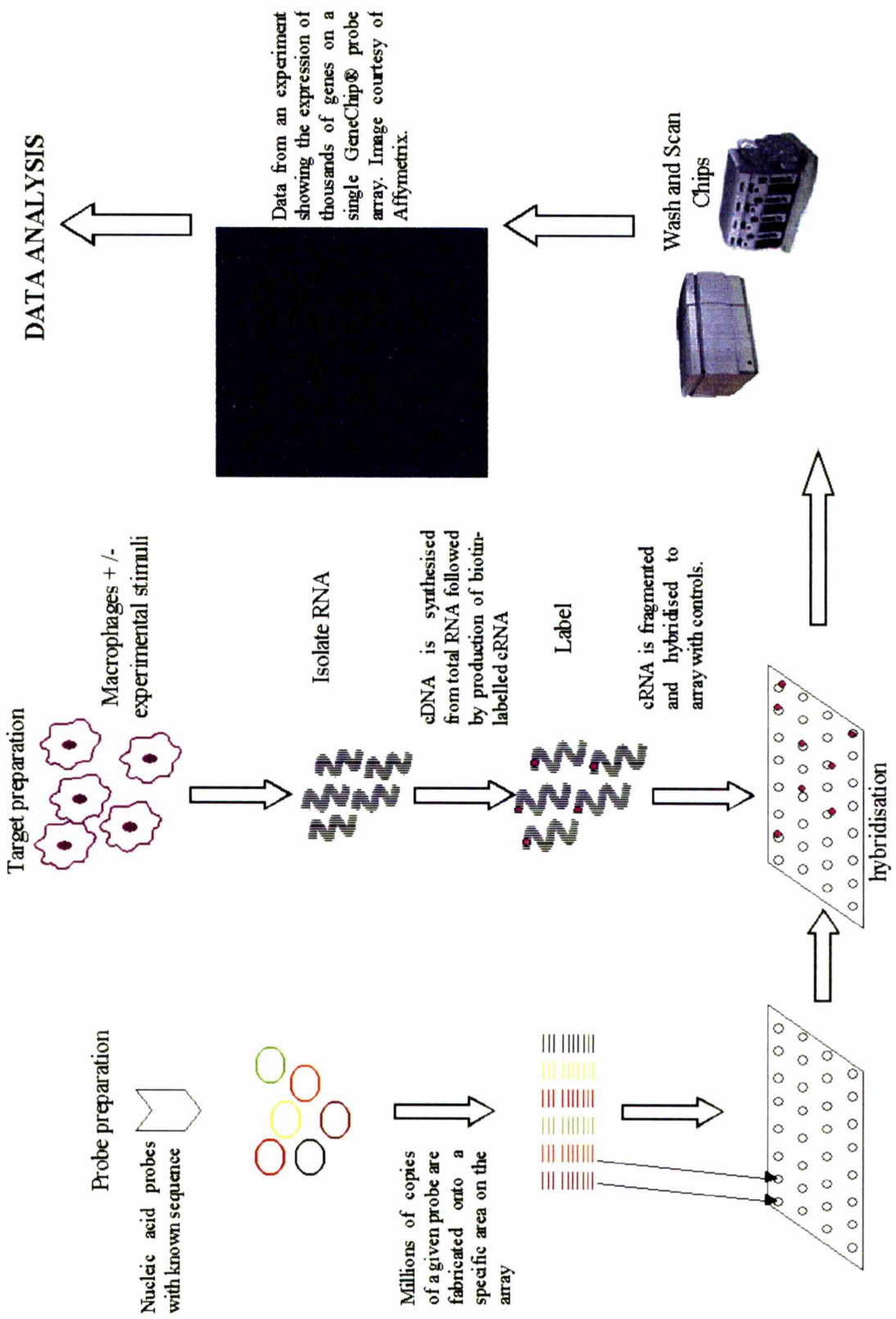
In chapter 5, pneumolysin was shown to up-regulate NO and TNF production in macrophages. As these mediators form part of a complex inflammatory response we were interested in looking at other mediators regulated by pneumolysin. Microarray technology enables monitoring of thousands of genes on DNA chips. Towards the end of this project, the opportunity to use this new technology became available. DNA microarray, or DNA chips are fabricated by high-speed robotics on glass or nylon substrates. Nucleic acid probes with known sequences are tethered to the chip and complementary binding of the target nucleic acid in the sample is detected. This technology allows a whole genome to be monitored on a single chip, allowing studies on gene expression, gene discovery and the interactions among thousands of genes to be carried out.

The GeneChip™ products developed by Affymetrix Inc. were used in this project. Probe sets of 16-20 probe pairs, containing a perfect match and a mismatch of each gene are synthesised throughout the array and these are fabricated onto silicon arrays. Each oligonucleotide is located in a specific area called a probe cell. Each probe cell contains millions of copies of a given oligonucleotide or probe. Biotin-labelled RNA is hybridised to the probe array and stained. The arrays are scanned at a wavelength of 570 nm and the light emitted is proportional to the amount of bound target at each location on the probe array. Figure 7.1 gives an overview of the microarray procedure.

Chips containing the murine genome (MG)-U74 set were used in this experiment. This set contains a comprehensive coverage of the mouse genome. It can be used to measure the temporal and spatial expression levels of greater than 36,000 mouse genes and expressed sequence tags (ESTs).

As described in Section 2.12 RNA was isolated from two experiments carried out on different occasions. The RNA from each experiment was hybridised with the probes on different days leading to two datasets labelled experiment 1 and experiment 2.

Figure 7.1 – An Overview of DNA Microarray



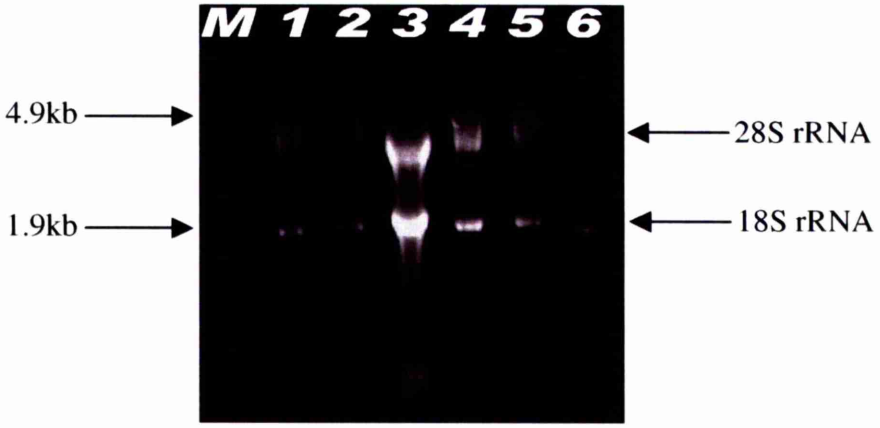
This figure shows the major steps involved in generating microarray data. The signal intensities from each cell on the probe array are computed for each chip using Affymetrix GeneChip® software.

7.2 RNA Isolation and Preparation

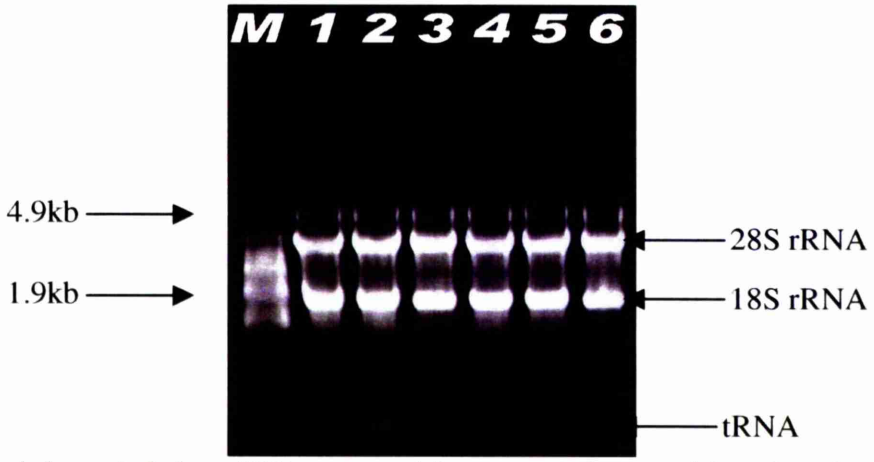
As described in section 2.12, 100 ng of pneumolysin +/- IFNγ and heat-inactivated controls were added to macrophages for 24 hours. The experiment was carried out twice on separate occasions to give duplicate samples for each condition. Each RNA sample was ethanol precipitated and resuspended in 12µl RNase free water. 1µl was used to quantify the RNA and 1µl of each sample was run on a formaldehyde agarose (FA) gel (See Figure 7.2). The remaining 10µl of the samples contained 20µg of RNA and was sent to MBSU for the rest of the protocol. All the A_{260}/A_{280} ratios were between 1.6 and 2.1.

Figure 7.2 – RNA samples for the GeneChip® Microarray

(a) – Experiment 1



(b) – Experiment 2



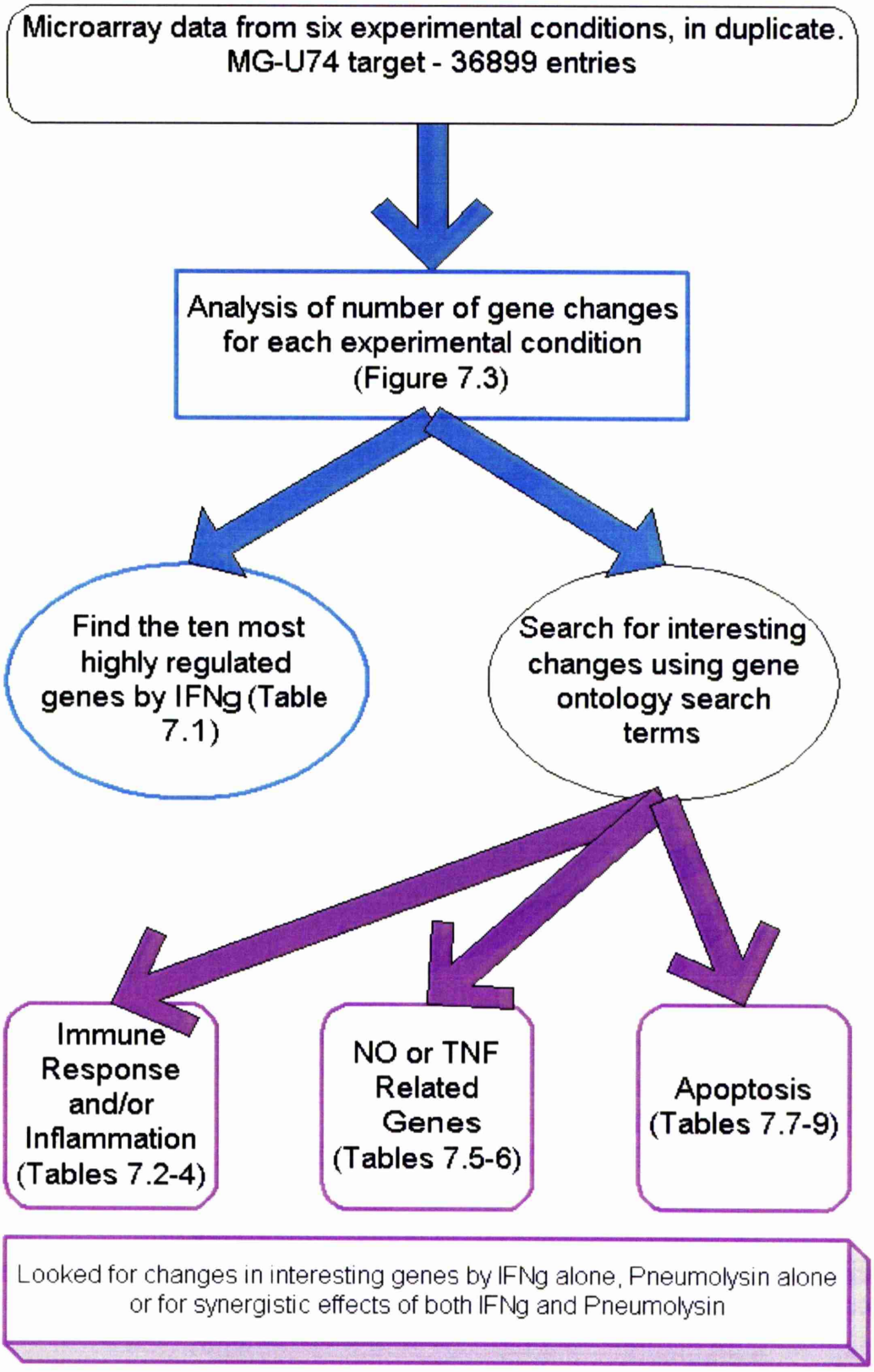
On each gel, lanes 1-6 show RNA extracted from macrophages subjected to the following experimental conditions: 1) Media Alone, 2) 100 ng pneumolysin, 3) 100 ng pneumolysin plus 0.5 ng/ml IFNγ, 4) 100 ng pneumolysin – heat inactivated, 5) 100 ng pneumolysin – heat inactivated plus 0.5 ng/ml IFNγ, 6) 0.5 ng/ml IFNγ. The experiment was carried out twice and the RNA isolated from each experiment is shown. Most of the RNA is in the ribosomal fractions (the two brightest bands in each lane) and in the second gel the transfer RNA is visible at the bottom of the lanes. M= 0.28-6.6kb RNA molecular weight marker (Sigma). rRNA = ribosomal RNA, tRNA = transfer RNA. The size of ribosomal RNA from mouse cells is as follows: 18S =1.9kb, 28S = 4.7kb

7.3 Basic Analysis of Data

An absolute analysis of the data was carried out using Affymetrix® Microarray Suite 5.0 software. This scales all the chips to a hypothetical baseline which is referred to as the target intensity. This was set at 100, so comparisons could be made between chips. Using this data, the presence of housekeeping genes (β-Actin or GAPDH) was checked. The 3' and 5' ratios of the signal intensity from these housekeeping genes were checked to ensure that full-length transcripts of the genes were present. The presence of the spiked controls was also checked.

Using one array as a baseline file and comparing it with another array, comparative analysis files were made. Every array was compared to all arrays in both experiments. Using the reports generated, the scaling factors and background "noise" were checked and found to be acceptable i.e. a scaling factor between chips of less than 3-fold and similar levels of background signal in all chips. The amount of data for each comparison was reduced by removing all probe sets that were absent in both chips or had a signal log ratio of less than 1 (i.e. less than a 2-fold change). Figure 7.3 shows the overall approach then used to analyse the data. The analysis presented here is preliminary and further statistical analysis is necessary before any firm conclusions can be drawn from these data.

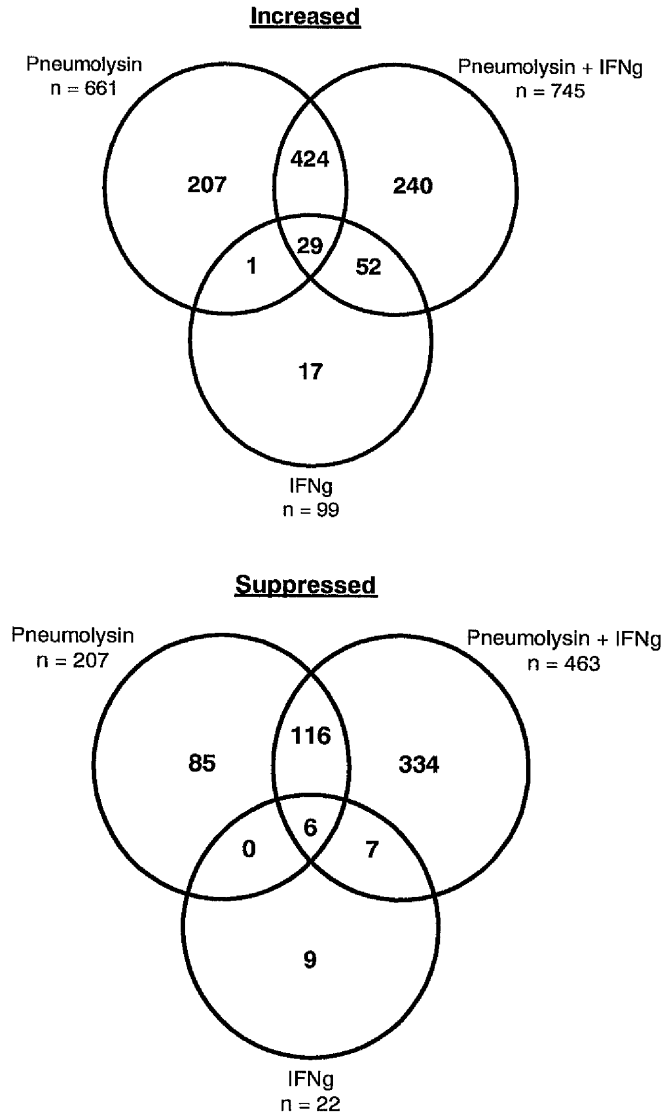
Figure 7.3 - Analysis of Microarray Data



7.4 Searching for changes in Gene Expression

As shown in Figure 7.4 the sets of genes regulated individually by pneumolysin and IFNγ overlapped. However some genes regulated by IFNγ were distinct from those regulated by pneumolysin. The greatest number of genes was regulated by the combination of pneumolysin and IFNγ, demonstrating an extent of synergy between the cytokine and the toxin.

Figure 7.4 – Genes changed by greater than a Signal Log Ratio of 1



This diagram represents the overlap of gene expression from macrophages subjected to the different stimuli. The values shown are the numbers of genes showing a greater than 2-fold change compared to unstimulated macrophages from the chips used in experiment 1. Data from experiment two is not shown, although a similar pattern of gene regulation was observed.

7.4.1 Genes up-regulated by IFNγ

As IFNγ is known to have effects on macrophage gene expression, one chip in each experiment was used as a control for RNA from macrophages incubated with 0.5 ng/ml IFNγ alone. The ten most highly regulated genes are shown in Table 7.1.

Table 7.1 – Most highly regulated genes by IFNγ

GENBANK ACCESSION NO.	PROBE SET NO.	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
M55544	95974	8	Guanylate nucleotide binding protein 1 (GBP-1)	Binds GTP, GDP and GMP
L38444	102906	7.8	T-cell specific GTPase	Binds GTP
AJ007971	96764	7.8	Interferon-inducible GTPase	Binds GTP
AJ007970	104597	7.1	Guanylate nucleotide binding protein 2	Binds GTP
X04653	93078	5.6	Lymphocyte Antigen 6 complex	T-cell activating protein
AA914345	103963	5.2	Interferon-inducible GTPase	Binds GTP
AB019505	92689	5.2	IFNγ inducing factor binding protein	Unknown
AA816121	100880	5.0	EST	Weakly similar to B chain B, crystal structure of the human acyl protein thioesterase1
AW047476	103202	4.9	Guanylate nucleotide binding protein 3	Binds GTP
M63630	104750	3.7	IFNγ-inducing protein (Ifi47)	Binds GTP

The signal log ratio shown is the average value from the two chips from macrophages incubated with IFNγ alone for 24 hours. An EST (expressed sequence tag) is a unique stretch of DNA within a coding region of a gene that is useful for identifying full-length genes and serves as a landmark for mapping.

In both experiments, seven of the genes most up-regulated by IFNγ encode GTP-binding proteins. GTP-binding proteins act as ubiquitous cellular regulators and are involved in many different processes. They constitute a large family of proteins and are also called GTPases

because of the GTP hydrolysis that they catalyse. They contain a similar GTP-binding globular domain that binds and hydrolyses GTP to GDP, inactivating the protein.

Ehrt *et al.* (2001) used high-density oligonucleotide arrays to analyse gene expression from primary macrophages that encountered IFNγ, live *Mycobacterium tuberculosis*, killed *Mycobacterium tuberculosis*, inert polystyrene beads or a combination of these stimuli for 24 hours. In this experiment, this group found many more genes to be regulated by IFNγ than we did. They also found that more genes were suppressed by IFNγ than were induced (660 compared to 615). In the ten genes most up-regulated by 100 U/ml IFNγ, they identified MHC class II-related genes, RANTES and a GTP-binding protein. They also found that *Mycobacterium tuberculosis* often mimicked or synergised with IFNγ for example in its effects on the genes encoding iNOS, and RANTES. These differences may be due to the amounts of IFNγ and cell types used. Presti *et al.* (2001) found that IFNγ has cell type-specific effects against murine CMV (cytomegalovirus) infection *in vitro*.

7.5 Sorting by Gene Ontology

7.5.1 Genes involved in the Immune Response and Inflammation

The Gene Ontology (GO) consortium provides controlled vocabularies for the description of the molecular function, biological process and cellular component of gene products. Probe Sets were sorted using the GO Biological Process classifications Inflammatory Response (GO:6954) and Immune Response (GO:6955). For every chip comparison file, a group of probe sets matching these criteria were obtained using the netaffx website. The tables and graphs below show examples of the macrophage genes that displayed altered regulation (in both experiments) in response to incubation with IFNγ, pneumolysin or pneumolysin plus IFNγ, but were not altered to the same extent in the control chips containing heat-inactivated pneumolysin. Some examples in the table did not show a greater than two-fold change but examination of the gene expression in the two experiments, clearly showed a change in gene regulation e.g. the H2-D gene (Figure 7.5(c)).

Genes that were regulated by IFNγ were evident by a change in all three conditions where IFNγ was added to the cells (Table 7.2 and Figure 7.5). Three of these genes are involved in the up-regulation of class I major histocompatability complex (MHC) genes. Class I MHC genes encode proteins that present antigen to $CD8^+$ T-cells. Two genes for chemotactic proteins are also up-regulated; MCP5 is chemotactic for eosinophils, monocytes and

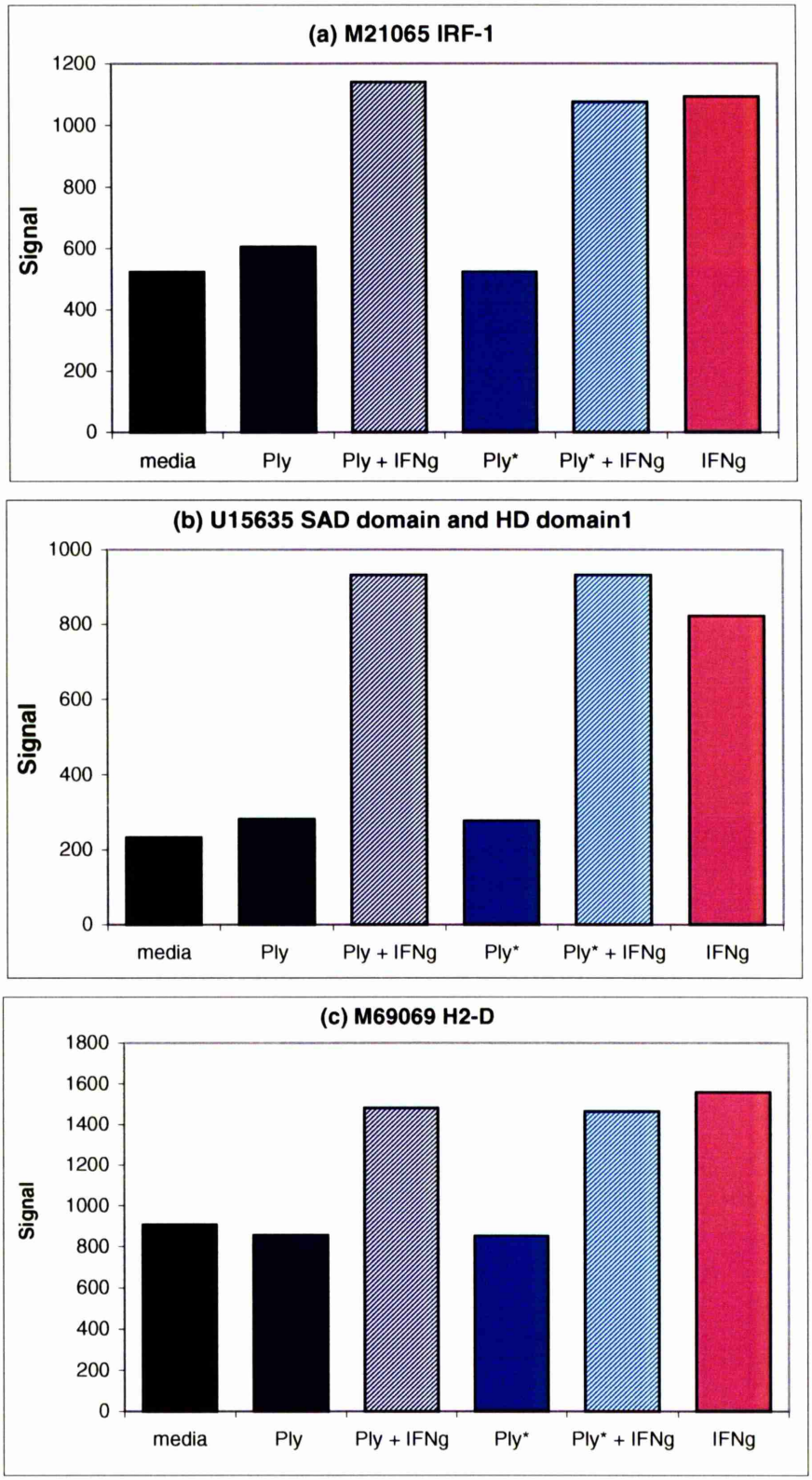
lymphocytes but not neutrophils. It signals through CCR (CC chemokine receptor) 2. Small inducible cytokine B subfamily (CXC) member 10 is chemotactic for T-cells and signals through CXCR3 (CXC chemokine receptor 3). The other genes appear to be GTPases as described above or are involved in gene regulation. The IFN-activated gene 202A binds to and inhibits the transcriptional activity of several transcription factors including NFκB, p50, p53, p65 and ap-1. IRF-1 physically interacts with NFκB subunits, and may be important in the cytokine-induced regulation of multiple promoters (Baldwin 1996).

Table 7.2 – Genes involved in the Inflammatory and/or Immune Response regulated by IFNγ

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
M55544	95974	8	Guanylate nucleotide binding protein 1 (GBP-1)	Binds GTP, GDP and GMP
M21065	102401	4.1	IRF-1	Binds IFN-inducible enhancer element of MHC class I genes
M33266	93858	3.0	Small inducible cytokine B subfamily (CXC) member 10	Chemotactic for T-cells.
U50712	93717	2.0	MCP 5	Chemotactic agent
M31418	94774	1.8	IFN-activated gene 202A	Binds to and inhibits activity of several transcription factors
U15635	103080	1.7	SAD domain and HD domain I	IFNγ-inducible putative GTP-binding protein
M31419	98465,	1.5	IFN-activated gene 204	May inhibit specific DNA binding of rRNA specific ubf1 transcription factor
M32489	98002	1.2	IFN consensus sequence binding protein (IRF-8)	Binds IFN-inducible enhancer element of MHC class I genes
M69069	97540	□1	H2-D1	Antigen presentation

The average signal log ratio for the two experiments is given. □, less than or equal to signal log ratio. MCP = Monocyte chemotactic protein.

Figure 7.5 – Examples of Gene up-regulation by IFNγ



These graphs show the up-regulation of three genes by IFNγ. The average signal from both experiments is shown for each condition. These graphs show clearly that gene up-regulation is seen to the same extent in all conditions containing IFNγ. Ply* = heated to 56°C for 10 minutes.

Some genes that were regulated by pneumolysin or synergistically by pneumolysin and IFNγ are shown below (Table 7.3, Table 7.4 and Figure 7.6).

Table 7.3 – Genes involved in the Inflammatory and/or Immune Response regulated by Pneumolysin

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
X53798	101160	1.2	MIP-2	Chemotactic for neutrophils

Only one gene was clearly up-regulated by pneumolysin alone using these search terms and it encodes the chemokine MIP-2. This mouse gene is analogous to IL-8 in humans and is chemotactic for neutrophils.

Pneumolysin and IFNγ synergistically increase expression of all but one of the genes involved in enhancing the inflammatory response (Table 7.4). The one gene that has been suppressed is prothymosin alpha, which is thought to mediate immune function by conferring resistance to certain opportunistic infections. A decrease in prothymosin alpha has been correlated with staphylococcal enterotoxin B induced immunosuppression (Palvimo and Linnala-Kankkunen 1990).

Two of the genes in the table, which display enhanced expression in response to pneumolysin, have also been reported to show increased mediator activity in response to pneumolysin. These are phospholipase A_2 (Rubins *et al.* 1994; Cockeran *et al.* 2001) and IL-1β (Houldsworth *et al.* 1994).

Phospholipase A_2 hydrolyses fatty acyl bonds generating free fatty acids and lysophosphatides. Phospholipases are involved in "housekeeping" tasks such as regulation of phospholipid synthesis and turnover. They have also been implicated in a variety of other vital cellular functions, principally by the generation of various phospholipid-derived mediators. Phospholipase A2 group VII (Platelet-activating factor (PAF) acetylhydrolase) is involved in the inflammatory response and lipid metabolism. It modulates the action of PAF by hydrolysing the sn-2 ester bond to yield the biologically inactive lyso-PAF. It has specificity for substrates with a short residue at the sn-2 position. It is inactive against long-chain phospholipids. The up-regulation of the phospholipase A_2 gene in this microarray

experiment agrees with other studies where pneumolysin has been shown to activate phospholipase A in pulmonary artery endothelial cells and neutrophils (Rubins *et al.* 1994; Cockeran *et al.* 2001). Phospholipase A_2 activation by pneumolysin may release cytotoxic products, such as free fatty acids and lysophosphatides that in turn magnify the lung injury produced by pneumococcal pneumonia.

Table 7.4 – Genes involved in the Inflammatory and/or Immune Response increased synergistically by Pneumolysin and IFNγ

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
AF065947	98406	5.2	RANTES	Chemotactic for macrophages
M15131	103486	5.0	Interleukin-1β	Inflammatory cytokine
K02782	93497	2.4	Complement component C3	Central role in complement system. Proteolytic degradation yields C3a anaphylatoxin, a mediator of local inflammation
X56602	98822	2.4	Interferon-stimulated protein (15-kDa)	Contains 2 ubiquitin domains
U34277	101923	1.7	Phospholipase A_2 group VII (Platelet-activating factor acetylhydrolase)	Hydrolyses PAF to yield biologically inactive product
U43085	103639	1.4	Interferon-induced protein (Ifit2)/Gluco-corticoid attenuated response gene	Encodes protein containing 11 tetratricopeptide repeat domains
X56135	100718	-1.1	Prothymosin alpha	May mediate immune function
L13832	96562	□1	Nramp1	Iron metabolism and host resistance
D49949	102802	□1	Interleukin-18	Regulator of innate and acquired immune responses

A minus sign indicates that gene expression was suppressed compared to unstimulated cells.
□, less than or equal to signal log ratio

Complement component 3 (C3) plays a central role in the activation of the complement system. Its processing by C3 convertase is the central reaction in both classical and alternative complement pathways. After activation, C3b can bind covalently to cell surface

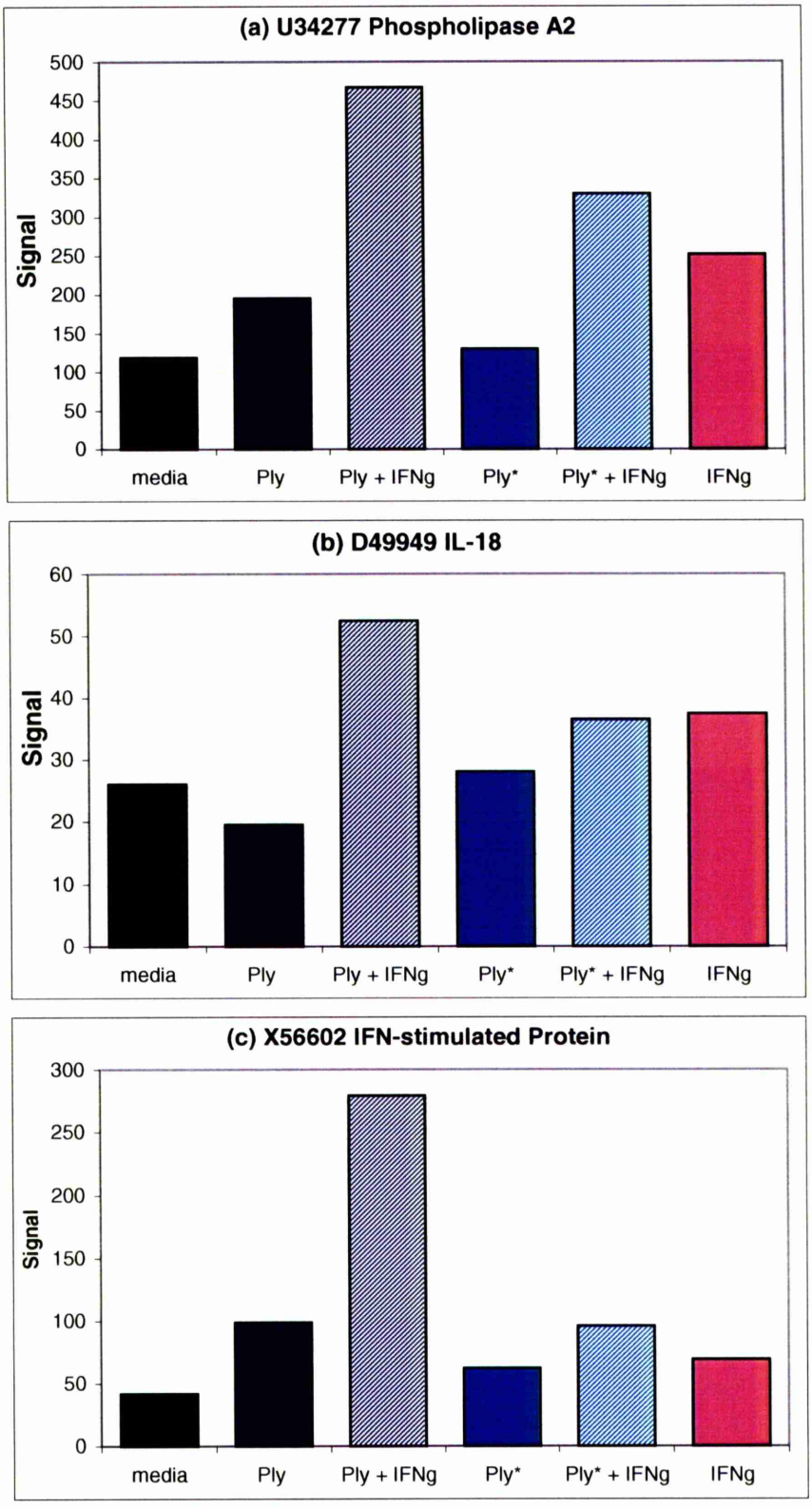
carbohydrates or immune aggregates. C3a anaphylatoxin is a mediator of the local inflammatory process derived from proteolytic degradation of complement C3. It induces the contraction of smooth muscle, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes. Cockeran *et al.* (2001) showed pneumolysin caused a dose dependent up-regulation of CR3 (complement receptor 3 also known as CD11b) expression on neutrophils. CR3 expression is of potential importance in the recruitment and activation of neutrophils and other types of inflammatory cells during *S. pneumoniae* infection. This receptor also binds iC3b and has an important role in opsonic phagocytosis and bacterial killing.

Three other genes encoding proteins known to play an important role in host defence are also up-regulated by pneumolysin; RANTES, Nramp1 and IL-18. We are not aware that induction of these genes by pneumolysin has been described previously. RANTES induces rapid expression of transcripts for the CXC chemokine MIP-2, the CC chemokines MIP-1β and MIP-1α, and the cytokines TNF and IL-6.

Pro-IL-18 is cleaved by ICE to yield the active protein IL-18 (Gu *et al.* 1997). IL-18 is a member of the IL-1 cytokine family and was initially defined as an IFNγ-inducing factor (Ushio *et al.* 1996). The widespread expression of IL-18 receptor indicates that multiple cell types might be responsive to this cytokine (McInnes *et al.* 2000). IL-18-deficient mice and neutraliasation of IL-18 impairs host defence against several infectious species (Kawakami *et al.* 1997; Bohn *et al.* 1998; Takeda *et al.* 1998; Wei *et al.* 1999). It has also been shown to induce TNF, GM-CSF and IFNγ production in cells isolated from patients with inflammatory disease (Gracie *et al.* 1999).

The Tetratrico peptide repeat (TPR) is a degenerate 34-amino acid repeated motif that is widespread in evolution. There appears to be no common biochemical function connecting TPR-containing proteins. Processes involving TPR proteins include cell-cycle control, transcription repression, stress response, protein kinase inhibition, mitochondrial and peroxisomal protein transport and neurogenesis (Goebl and Yanagida 1991).

Figure 7.6 – Examples of Genes synergistically up-regulated by Pneumolysin and IFNγ



These graphs show the up-regulation of three genes by pneumolysin and IFNγ. The average signal from both experiments is shown for each condition. The regulation of these genes is up-regulated a small amount by either pneumolysin or IFNγ alone but the signal is increased when both stimuli are used. Ply* = heated to 56°C for 10 minutes.

It is surprising that the IFN-stimulated protein is up-regulated by pneumolysin and IFNγ but not by IFNγ alone. It is possible that as this experiment was only carried out at one time point, this gene is maximally regulated by IFNγ at a different time point. Perhaps the presence of pneumolysin maintains the expression of this gene whereas in the situation with IFNγ alone, gene expression has been switched off.

7.5.2 Genes involved in regulating NO and TNF

In chapter 5, pneumolysin was shown to stimulate NO and TNF production from macrophages. As no genes directly related to nitric oxide or TNF production came up using the search terms described above, a separate search for these genes was carried out (Table 7.5, Table 7.6 and Figure 7.7).

Table 7.5 – Genes related to TNF or Nitric Oxide regulated by Pneumolysin

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
AF010600	92345	2.6	Expressed sequence	TNF-response element binding protein. Potential transcription factor
AW061307	95152	2.4	RIKEN cDNA 2810052MO2 gene	Unknown
AI842724	160098	1.7	Gene identical to crystalline alpha B	Heat shock protein. Accumulates as function of v-mos and Ha-ras expression
U94331	102887	1.7	TNFR superfamily member 11b	Osteoprotegrin precursor. Regulates bone resorption
AF19046	101632	1.3	TNFR superfamily member 11a (or RANK)	Receptor activator of NFκB
AW228036	93538	-1.1	TRAF and TNFR associated protein (TTRAP)	Inhibits NFκB activation
AA798611	103509	□1	Expressed sequence	Unknown
U43428	104420	□1	NOS2 (iNOS)	Catalyses reaction to produce NO

TNFR = TNF Receptor; TRAF, TNF receptor associated factor. □, less than or equal to signal log ratio

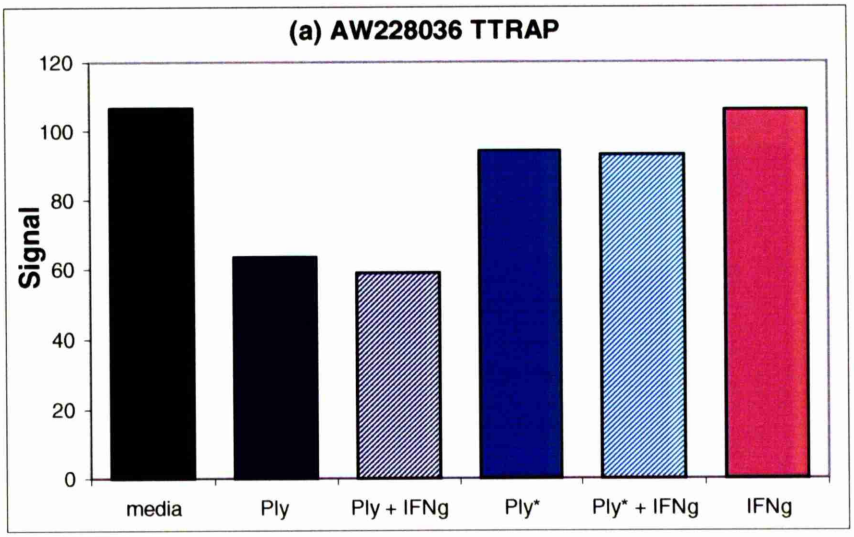
The molecule CD40 is found on many antigen-presenting cells, although it was initially thought to be only on B-cells. CD40 is also required for T-cell function, in other words, the T-cell talks back to the B-cell via CD40. The CD40-ligand interaction was originally thought to be a T-cell-B-cell requirement exclusively, but it is also required for a T-cell-macrophage interaction (Grewal and Flavell 1996). Macrophages are activated through CD40 on their surface via CD40 ligand on the T-cell surface (Stout *et al.* 1996). CD40 belongs to the TNF receptor family. Signalling involves recruitment of TRAFs to its cytoplasmic domain and leads to NFκB activation.

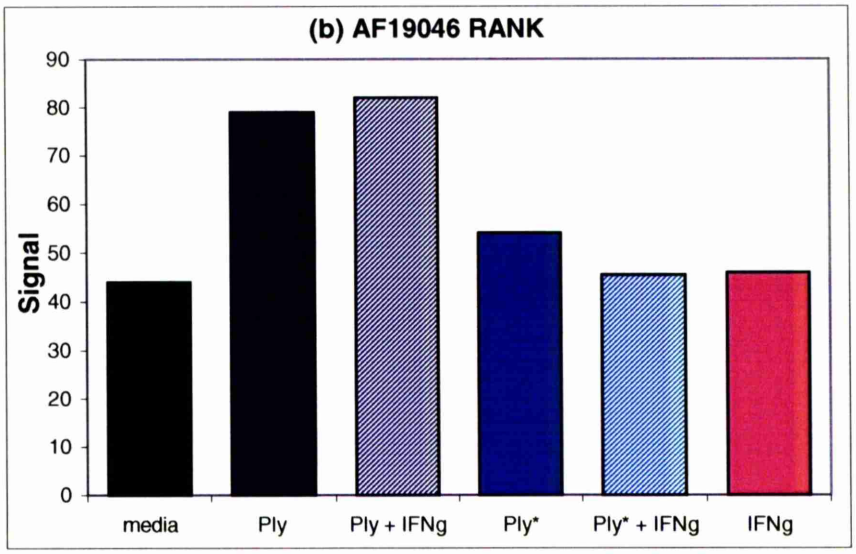
Table 7.6 – Genes related to TNF or Nitric Oxide regulated synergistically by Pneumolysin and IFNγ

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
AA798611	103509	3.9	Expressed sequence	Ortholog to human TNFR superfamily member 9. May induce apoptosis
M83312	92962	1.3	TNFR superfamily member 5 (CD40)	CD40 type ii isoform. Involved in TNF signalling.
U21050	95010	≤-1	TRAF 3	Signal transducer

≤, less than or equal to signal log ratio

Figure 7.7 – Genes related to NO or TNF regulated by Pneumolysin





These graphs show the up-regulation of genes by pneumolysin. The average signal from both experiments is shown for each condition. These graphs show that gene regulation is altered to the same extent in both conditions containing active pneumolysin compared to those without pneumolysin. Ply* = heated to 56°C for 10 minutes.

7.5.3 Regulation of Genes involved in Apoptosis

Previous studies have shown *S. pneumoniae* and pneumolysin to induce apoptosis in some cell types (Braun *et al.* 2000; Zysk *et al.* 2000; Braun *et al.* 2001; Dockrell *et al.* 2001; Braun *et al.* 2002). The search term GO: 6915 apoptosis was used to pick out genes from the microarray data, which are involved in apoptosis pathways. This search term also picked up genes assigned the following descriptions: GO: 6917 induction of apoptosis and GO: 6916 anti-apoptosis. Table 7.7, Table 7.8, Table 7.9 and Figure 7.8 show genes regulated by pneumolysin and/or IFNγ involved in apoptosis.

Table 7.7 – Genes involved in Apoptosis regulated by IFNγ

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
L28095	102064	1.3	Caspase 1 (ICE)	Involved in apoptosis caspase cascade
Y13089	102905	1.1	Caspase 11	Involved in apoptosis caspase cascade
M83649	102921,	≤1	Fas antigen	Cell surface transmembrane protein (35 kD) that mediates apoptosis

ICE = Interleukin-1β converting enzyme. ≤, less than or equal to signal log ratio

Caspases are a set of cysteine proteases activated specifically in apoptotic cells. They selectively cleave a restricted set of target proteins, at a small number of sites. In most cases, they inactivate the target protein but can also activate proteins directly by cleaving off a negative regulatory domain or indirectly by inactivating a regulatory subunit.

Caspase 1, also known as interleukin converting enzyme (ICE) cleaves IL-1β releasing the mature cytokine and is also involved in the caspase cascade leading to apoptosis. Efflux of K^+ from monocytes leads to activation of ICE, so that the cells rapidly process and export IL-1β (Walev *et al.* 1995). ICE is also involved in regulating programmed cell death. Transfection of ICE into cells can induce apoptosis (Miller *et al.* 1997).

The transcription factor p53 is an important tumour suppressor and is involved in the stress response (Matsumoto *et al.* 1994; Hall *et al.* 1996; Ohnishi *et al.* 1996). Up-regulation of p53 causes growth arrest by blocking the G_1/S transition in the cell cycle giving time for DNA repair; however p53 will induce apoptosis if DNA damage is extreme (Levine 1997). In both cases, p53 functions as a transcription factor, increasing the expression of proteins involved in DNA repair or apoptosis (Levine 1997; Polyak *et al.* 1997). In addition, p53 may interact directly with other proteins, leading to apoptosis in a manner independent of its transcriptional action (Levine 1997). p53-targeted gene products include Bcl-2, Bcl-Xl, Bax and Fas/APO-1 and these may be involved in a p53-mediated apoptosis. Many kinds of stress induce the down-regulation of *bcl-2* (Haldar *et al.* 1994) and up-regulation of *Bax Fas/APO-1* and *bcl-Xl* expression due to activation of the p53 pathway (Selvakumaran *et al.* 1994; Zhan *et al.* 1994; Zhan *et al.* 1996) and lead to apoptosis.

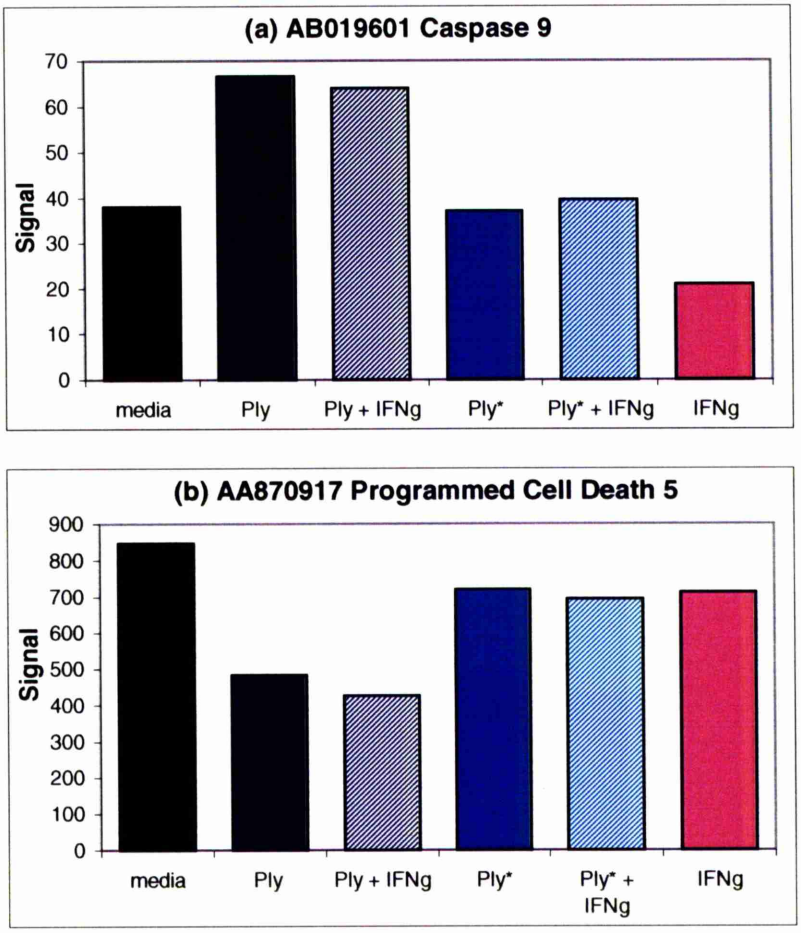
One key function of the bcl-2 family is to regulate the release of pro-apoptotic factors, like cytochrome c, from the mitochondrial inter-membrane compartment into the cytosol. Some bcl-2 members are pro-apoptotic, whilst others are anti-apoptotic. The Bcl-2 homology (bh3) domain is shared and required by bik, bid, bak, bad and bax for their pro-apoptotic activity and for their interaction with anti-apoptotic members of the Bcl-2 family.

Table 7.8 – Genes involved in Apoptosis regulated by Pneumolysin

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
AB019601	100368	3.8	Caspase 9 (Apaf3)	Activates Caspase-3 and subsequent apoptosis via caspase cascade
Y13231	104710	2.1	BCL2-antagonist/killer1 (Bak1)	Apoptosis regulator binds to and antagonises the repressor BCL2
AB021961	104275	2	p53	Transcription factor involved in stress response
L22472	93536	1.9	BCL2-associated X protein (Bax)	Heterodimerises with BCL2 and accelerates apoptosis (p53 target)
L37296	99670	1.7	BCL-associated death promoter (Bad)	Heterodimeric partner for BCL-XL. Accelerates apoptosis
L21973	102963	1.3	E2F transcription factor 1 (E2F1)	Shown to induce p53-independent apoptosis*
AF055638	101979	1.2	Gadd45	Negative growth control (p53 target)
AI837308	97800	1.1	Fas-activated ser/thr kinase (fastk)	Phosphorylates TIA-1 as part of a cell death program mediated by Fas
AI854293	104608	□1	Beta-amyloid binding protein precursor	Putative apoptotic component
AF041054	93836	□1	BCLA2/adenovirus E1B 19-kDa-interacting protein 1 (Bnip3)	Pro-apoptotic mitochondrial protein. Binds to BCL2
AA870917	93968	□-1	Programmed Cell Death 5	General role in apoptotic process

Gadd45 = Growth arrest and DNA-damage inducible 45 gamma. Apaf = apoptotic protease activating factors, TIA-1 is an RNA-binding protein implicated in signaling cascades regulating stress-induced apoptosis. □, less than or equal to signal log ratio. *Holmberg *et al.* (1998).

Figure 7.8 – Apoptotic Gene Expression Induced by Pneumolysin



These graphs show the up-regulation of genes by pneumolysin. The average signal from both experiments is shown for each condition. These graphs show that gene regulation is altered to the same extent in both conditions containing active pneumolysin compared to those without pneumolysin. Ply* = heated to 56°C for 10 minutes.

Table 7.9 – Genes involved in Apoptosis regulated by Pneumolysin and IFNγ

GENBANK ACCESSION NO.	PROBE SET	SIGNAL LOG RATIO	GENE NAME OR PRODUCT	FUNCTION
AB013819	101521	-2.3	Baculoviral IAP repeat containing 5 (Birc5)	Homologue of apoptotic inhibitor, related to cell proliferation*
AF032459	99418	≤1	BCL2-like 11 (Bim)	Binds to BCL2 and accelerates apoptosis

*Kobayashi *et al.* (1999). ≤, less than or equal to signal log ratio

7.6 Induction of Apoptosis by Pneumolysin

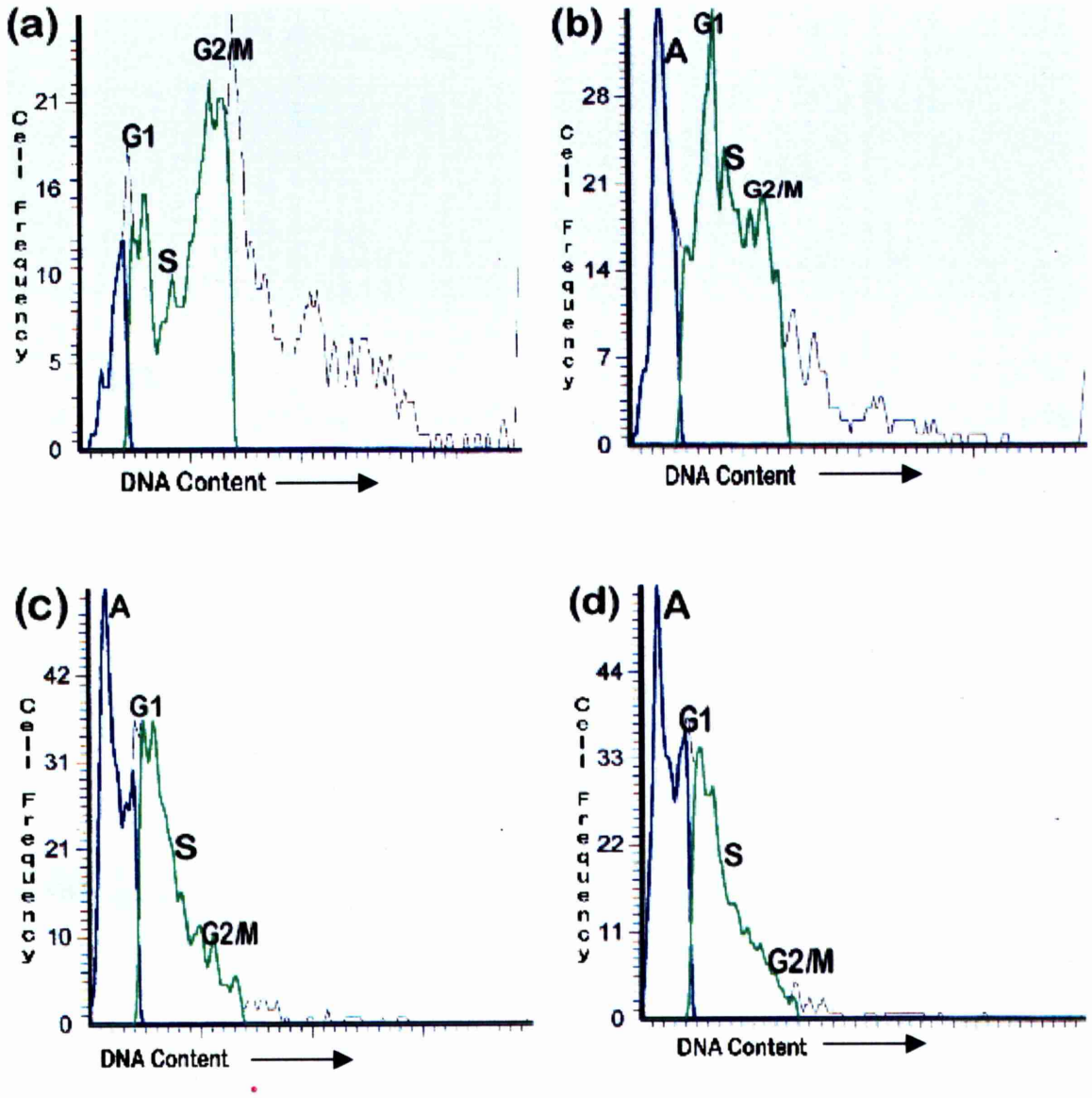
The microarray data suggest that pneumolysin can induce apoptosis in the macrophages as many genes involved in apoptosis are up-regulated. To see if pneumolysin could induce

apoptosis in macrophages laser scanning cytometry of propidium iodide (PI) stained cells was carried out.

A laser scanning cytometer (LSC) is a microscope-based cytofluorometer that offers the combined advantages of flow cytometry and image analysis and can be used to detect apoptotic cells in several ways. Measurement of total cellular fluorescence is performed by LSC via the integration of light intensity of individual pixels over the area of the cytoplasm (Kamentsky *et al.* 1997). Because of the high degree of chromatin condensation in apoptotic cells, DNA stains with greater intensity per unit of the projected nuclear area of these cells. The maximal pixel value of the DNA-associated fluorescence measured in the chromatin of apoptotic cells, therefore, is greater than in non-apoptotic nuclei (Furuya *et al.* 1997; Bedner *et al.* 1999). Apoptosis-associated changes in the gross physical attributes of cells, such as cell size and granularity, can be detected by analysis of laser light scattered by the cell in forward and side directions (Swat *et al.* 1991; Ormerod *et al.* 1995). The detection of DNA fragmentation provides another marker of apoptosis. Apoptotic cells can be recognised by their fractional ("subdiploid," "sub-G1") DNA content due to extraction of low molecular weight DNA from the cell (Umansky *et al.* 1981; Nicoletti *et al.* 1991).

Macrophages were incubated with pneumolysin for 24 hours and then stained with PI and analysed by LSC with the help of Ms A. Morton (Figure 7.9). Camptothecin was used as a positive control. It binds irreversibly to the DNA-topoisomerase I complex (Fan *et al.* 1998), and induces apoptosis in a large number of cell lines (Kaufmann 1998). Apoptotic cells have decreased PI fluorescence and diminished forward light scatter relative to cells in the main peak (G_1). Optimally the "sub-G_1 peak" representing apoptotic cells should be separated from the G_1 peak of the non-apoptotic cell population with little or no overlap between the two (Darzynkiewicz *et al.* 2001).

Figure 7.9 – Induction of Apoptosis by Pneumolysin



This figure shows histograms of integrated red fluorescence for 1500 cells, this is proportional to DNA content. G_1, S, G_2 and M refer to stages of the cell cycle. G=gap phase, S=synthesis, M=mitosis. A = apoptotic cells. The subpopulation of apoptotic cells (in blue) with fractional "subdiploid" DNA content can be seen in the positive control 5 µM camptothecin and in the cells incubated with pneumolysin. (a) Unstimulated cells in media alone, (b) Cells plus 5 µM camptothecin, (c) and (d) Cells plus 10 and 100 ng/ml pneumolysin respectively.

Macrophages incubated in media alone (Figure 7.9a) show few cells in the sub-G1 peak. When the cells are incubated with camptothecin or pneumolysin (Figure 7.9 b, c and d), an increase in this population is seen. An increased number of cells in S-phase are also seen as has been previously reported (Darzynkiewicz *et al.* 2001). This preliminary experiment concurs with other studies that suggest pneumolysin can induce apoptosis (Dockrell *et al.* 2001; Braun *et al.* 2002).

Taken together with the genes shown in Table 7.8, it appears that pneumolysin induced apoptosis at least partly, via a p53 pathway. p53 itself is up-regulated as are several p53 targets. Also, up-regulation of p53 causes growth arrest by blocking the G_1/S transition. In Figure 7.9 (c) and (d) the G_1 peak is higher than in the unstimulated cells.

7.7 Conclusions

- IFNγ up-regulates many genes. The most highly regulated genes are mostly GTP-binding proteins.
- Pneumolysin and IFNγ display synergism in the ability to up-regulate many genes, including those involved in the inflammatory response.
- Pneumolysin and IFNγ induce the expression of several genes involved in cell recruitment and enhancement of the inflammatory response.
- Pneumolysin alone is capable of increasing the expression of the chemokine MIP-2 and ten genes involved in directing the cell towards apoptosis.
- A preliminary experiment provides more evidence that pneumolysin can induce apoptosis in macrophages.

8. DISCUSSION

8.1 Structure and Function of Pneumolysin

A pneumolysin stock was created and several quality control experiments ensured the purity and stability of the protein. The histidine tag does not affect the haemolytic or cytotoxic activity of the protein. Other groups have used a His-tagged version of the toxin and have not reported any problems (For example, Braun *et al.* 1999a). One of the aims of this project was to characterise the role of oligomerisation in the induction of pro-inflammatory mediator production by pneumolysin. Initial experiments focused on the construction and function of pneumolysin mutants that according to monoclonal antibody experiments, (De Los Toyos *et al.* 1996) were not expected to oligomerise.

One way of looking at pneumolysin oligomerisation is to study changes in fluorescence when the toxin self interacts. There are measurable fluorescence changes in an extrinsic fluor, ANS, bound to pneumolysin when the toxin interacts with sodium deoxycholate. These changes were not seen in a chemically modified form of pneumolysin that cannot self-interact, which implies the assay is measuring oligomerisation. We used this assay to look at the behaviour of the pneumolysin N142/143-deletion mutant. In the presence of sodium deoxycholate the mutant pneumolysin behaved in the same way as the wild type toxin. This mutant has the same haemolytic activity as the wild type toxin, displays similar cytotoxic effects on macrophages and airway cells and shows no significant differences in its ability to stimulate NO and TNF production from macrophages. All this evidence strongly suggests that this mutant is still able to oligomerise and form functional pores in cell membranes. Reasons why this mutant still acts in the same way as the wild type toxin will now be discussed.

These residues were thought to be involved in oligomerisation as a monoclonal antibody (PLY4) that binds the pneumolysin molecule at residues 142/143 neutralises the haemolytic activity of pneumolysin but does not affect cell binding (De Los Toyos *et al.* 1996) (see section 1.4.4.3). It is possible that in the De Los Toyos *et al.* (1996) study, oligomerisation was prevented by the presence of the antibody. In the homology model of pneumolysin proposed by Rossjohn *et al.* (1998), residues 142 and 143 are located on a solvent-exposed surface at the end of a helix and in a region that is proposed to be involved in numerous oligomerisation contacts. Binding of a bulky monoclonal antibody to this region could sterically prevent proper oligomerisation. This situation is similar to that proposed for cell binding of the C-terminal end of pneumolysin to the cell membrane. Here, it is the packing

of the residues as a whole rather than the characteristics of individual amino acids that are important for the toxin's ability to bind and penetrate cell membranes (see section 1.7).

One of the other monoclonal antibodies, PLY7 in the De Los Toyos *et al.* (1996) study, recognised residue 419 and inhibited cell binding. The authors proposed that this epitope may be neutralising or alternatively that binding to this region altered another epitope on the molecule. A candidate for this epitope would be the one recognised by PLY5, as PLY7 had a positive modulation effect on the binding of PLY5. A similar possibility could also be the case for PLY4 although it had no effect on the binding of other antibodies in the experiment. However all of the other antibodies recognised epitopes at the C-terminal end of the protein. Maybe the binding of PLY4 alters another epitope in the N-terminal part of the molecule and it is this region that is involved in oligomerisation.

Perhaps deleting the two residues at positions 142/143 was not enough to alter the structure sufficiently to inhibit oligomerisation. It could be worth carrying out further studies on the N142D pneumolysin mutant although it had the same haemolytic activity as the deletion mutant and wild type toxin. Alteration of the polar asparagine residue to a non-polar or more bulky aromatic residue may alter the conformation of this region to an extent where it is unable to oligomerise.

Electron microscopy showed that pre-incubation with PLY4 led to a reduction in the number of pores seen in liposomes compared to the wild type toxin (De Los Toyos *et al.* 1996). It could be that the pneumolysin 142/143 deletion mutant does have a reduced capacity to form pores but that one or two pores are still sufficient to lyse cells (Bhakdi *et al.* 1984) and induce mediator production. The use of electron microscopy would be a useful tool to study pore formation of the mutant and wild type toxins.

The next set of experiments were carried out in order to determine sub-lytic and lytic concentrations of pneumolysin to use later in the project. Knowing whether or not the concentrations of pneumolysin used were lytic or not could help in deciphering what action the toxin may be having and whether release of cell contents could be playing a role in mediator production. The RAW 264 cell line was established from BALB/c tumours induced with Abelson leukaemia virus and expresses properties of macrophages (Ralph and Nakoinz 1977; Raschke *et al.* 1978). We decided to use this cell line, as macrophages are

one of the first cells likely to come into contact with *S. pneumoniae* and pneumolysin during infection and play a major role in determining the outcome of the host response. Airway epithelial cells were also used as they play an important role during pneumococcal pneumonia both as a barrier to bacterial invasion and as a source of inflammatory mediators. Experiments with primary isolates of human small airway epithelial cells did not display different sensitivities to pneumolysin compared to a transformed A549 lung epithelial cell line. As the A549 cells were cheaper and easier to maintain, they were used for the majority of experiments.

Preliminary experiments showed that the lytic action of pneumolysin mainly occurs over the first four hours after incubation with cells. After four hours, the viability of macrophages begins to drop when more than 1.5 μg/ml of pneumolysin is added to the medium. There is a 50% decrease in cell viability when 7 μg of pneumolysin is added to the cells for four hours. When the cells are incubated for 24 hours with pneumolysin, less toxin (<2 μg) is required to cause a 50% reduction in cell viability. In contrast to previous studies we found that priming the macrophages with IFNγ made them more sensitive to pneumolysin-induced cell death. The airway epithelial cells showed a similar sensitivity to the toxin as the macrophages after four hours but did not show as great an increase in sensitivity after 24 hours. As with the macrophages, IFNγ increased the sensitivity of the airway cells to the toxin.

Hirst *et al.* (2002) measured 50% kill values from different cell types incubated with pneumolysin for twenty minutes using an LDH (lactate dehydrogenase) release assay. They found U937 monocytic cells to be significantly more resistant to pneumolysin than cells from the monocytic line THP1. Pneumolysin was equipotent for two lung epithelial cell lines. Differentiation of U937 cells with IFNγ significantly increased their resistance to pneumolysin but had no effect on viability of THP1 cells. Differentiation of the lung epithelial cells had no effect on susceptibility to pneumolysin. They hypothesised that the difference in pneumolysin sensitivity between different cell types results from differences in the structure and dynamics of the plasma membrane. In membranes the pore-forming toxins aerolysin and staphylococcal alpha-toxin associate with cholesterol-glycolipid rafts (Abrami *et al.* 1998). These differences have been proposed to be the reason for different sensitivities between rabbit and human erythrocytes to alpha-toxin (Valeva 2000). If a similar event

occurred with pneumolysin then the abundance and composition of cholesterol-glycolipid rafts in the each cell type may change the dynamic properties of the plasma membrane.

The results of the viability curves in section 5.1 were compared with two publications in which the toxic effect of pneumolysin was studied (Saunders *et al.* 1989; Hirst *et al.* 2002). The comparisons can be seen in Table 8.1 below. There is a great deal of variation in the concentrations of pneumolysin required to kill 50% of the cells between experiments. There are also differences between different cell types.

Table 8.1 – Sensitivity of different Cell Types to Pneumolysin

REFERENCE	CELL TYPE	TIME	HU PNEUMOLYSIN	
				Activated Cells
Saunders *et al.* 1989	Freshly isolated human PMN	15 minutes	120	Not done
Hirst *et al.* 2002	Airway Epithelial Cell (A549)	20 minutes	7.6	5.8
"	Airway Epithelial Cell (L132)	"	9.5	7
"	Human Monocyte (U937)	"	26.3	67.6
"	Human Monocyte (THP1)	"	7.5	3.2
This study	Mouse Macrophage (RAW 264.7)	4 hours 24 hours	1460 260	960 280
"	Airway Epithelial Cell (A549)	4 hours 24 hours	1580 920	500 140
"	Small Airway Epithelial Cells	4 hours	1240	1400

The numbers in the table refer to haemolytic units (HU) of pneumolysin required to kill 50% of 10^6 cells/ml. The times listed indicate the length of time the cells were incubated with pneumolysin. The specific activities of the toxins for each study were: Saunders *et al.* – 1.5 x 10^6 HU/mg, Hirst *et al.* – 1.5 x 10^5 HU/mg, this study – 1 x 10^5 HU/mg. PMN, polymorphonuclear leukocyte.

After four hours, our results show the cells to be less sensitive to pneumolysin than those used in the other studies. The cells seem to display varying degrees of sensitivity to pneumolysin over time. Another difference between the viability studies is the assays used; in this project we used the MTT assay, Hirst *et al.* (2002) used an LDH release assay

whereas Saunders *et al.* (1989) used a trypan blue exclusion assay. It is possible that at the time points studied by the other groups, what is being measured is immediate membrane disruption by the toxin. The assays used would measure disruption if the membrane (trypan blue exclusion) and release of LDH from the cells that could occur through pneumolysin-induced pores. This does not necessarily mean the cells cannot recover from this. At the later time points we have used, the cells have had time to recover from this initial membrane perturbation and the death seen is real. Also, although Hirst *et* al. (2002) found a significant decrease in sensitivity of U937 cells primed with IFNγ, the other cell types all show a slight increase in sensitivity when primed. It could be that activated cells have to focus on other activities and do not have as much energy and resources available to combat pore formation.

There are several factors that may play a part in these differences. Firstly, different cell types and even different cell lines display different sensitivities to pneumolysin. Different macrophage cell lines can exhibit distinct patterns of gene expression in response to IFNγ (Lucas *et al.* 1998). Streptolysin-O had differential effects on murine macrophage cell lines and differentiation increased sensitivity (Tanigawa *et al.* 1996). However one of the cell types used in the Hirst study was the same as one used here (A549 lung epithelial cells) and there was still a big difference in sensitivity. The stage of the cell cycle and culture conditions probably all play a role in the fitness of the cells and their susceptibility to the toxin. Also the pneumolysin preparations will be produced and purified in a slightly different way between groups. For example some groups purify pneumolysin from inclusion bodies whereas others (including us) purify it from the soluble fraction of disrupted cell preparations. As mentioned previously, pneumolysin has a tendency to aggregate in solution and form inactive species as measured by haemolytic assay (Gilbert *et al.* 1998). However the effect that these aggregates may have on other cell types is not known.

Another study measured pneumolysin cytotoxicity by measuring ATP levels and LDH release from neutrophils (Cockeran *et al.* 2001). They found that in Ca^{2+}-replete buffer, a significant decrease in viability compared to unstimulated cells was observed only at the highest concentration of pneumolysin used – 250 HU (per 10^6 cells). However when they used Ca^{2+}-free media, much less toxin was required to cause a significant reduction in cell viability, 10-50 HU depending on the assay used.

It has been suggested that cells may recover from low doses of pore-forming toxin by shedding of the toxin (Walev *et al.* 1994) or by repair of lesions by fusion of cytoplasmic vesicles with the plasma membrane (Walev *et al.* 2001). Transmission electron micrographs demonstrated numerous intracellular vesicles that may be due to increased cell membrane turnover following treatment with pneumolysin (Hirst *et al.* 2002). In the other studies in Table 8.1, the time frame used is much shorter i.e. minutes rather than hours. We also saw an immediate decrease in cell viability after a high concentration of pneumolysin was added but most cell death occurred after four hours when high concentrations of pneumolysin were added, which is why we chose this time to find 50% kill. Our 50% kill values are higher than the other studies mentioned. It could be that after this time at lower concentrations, the cells have time to recover. This would have the effect of pushing the curve to the right and result in a higher concentration of pneumolysin necessary to kill 50% of the cells.

8.2 Effects of purified Pneumolysin on cultured Cell lines

As described in Table 1.4, pneumolysin has been shown to cause a wide range of effects on various cell types. There are some discrepancies between studies, for example in a recent study, Cockeran *et al.* (2001) reported a pneumolysin-induced increase in the respiratory burst in neutrophils. In previous studies, pneumolysin has been shown to decrease the respiratory burst in cells (Paton and Ferrante 1983; Nandoskar *et al.* 1986). These differences may be due to minor variations in the experiments such as the Ca^{2+} concentration of the media and the stimulus used to activate the cells (Cockeran *et al.* 2001).

The inducible activation of NFκB stimulates the transcription of the iNOS gene (Xie *et al.* 1994), leading to an increase in NO production. Braun and co-workers (1999a) showed that minimal requirements for NO production by pneumolysin included NFκB activation. NFκB is also implicated in the transcriptional regulation of several cytokine and growth factor genes including IL-2, IL-6, IL-8 and G-CSF (Baldwin 1996). Pneumolysin seems to act as a general activator of macrophages possibly through activation of NFκB. We have shown in the microarray experiment (section 7.5.1) that genes involved in NFκB activation tended to be up-regulated and those that inhibit NFκB activity suppressed by pneumolysin.

In this study, data has been presented that shows pneumolysin can stimulate NO production from macrophages and to a lesser extent from airway epithelial cells (section 5.2). NO production from macrophages induced by pneumolysin has also been reported by Braun *et*

al. (1999a). The results from that study compare well with the results presented here. When the macrophages were primed with IFNγ, they found a maximum of 35 μM nitrite release when 2.6 μg of pneumolysin was used. We found a maximum of 43 μM nitrite when 3-6 μg pneumolysin was used. However Braun *et al.* did observe NO production (10 μM nitrite) when as little as 2.6 ng pneumolysin was used to stimulate IFNγ-primed macrophages. We could not detect NO production when 25 ng or less pneumolysin was used.

10 pg of pneumolysin is equivalent to 10^3 cfu/ml *S. pneumoniae* (Houldsworth *et al.* 1994). During sepsis, concentrations $\geq 10^5$ cfu/ml are reached in humans (La Scolea and Dryja 1984; Shenep *et al.* 1988) and 10^9-10^{10} cfu/ml have been found in mice (Benton *et al.* 1995). The concentrations of pneumolysin used to stimulate maximum NO production in this project therefore correlate to 6×10^8 cfu/ml, which could be present during severe pneumococcal infections. The large amount of NO produced in this situation could be very detrimental to the host.

IFNγ is required for macrophages to produce NO in response to pneumolysin as IFNγ receptor$^{-/-}$ mice cannot produce NO (Braun *et al.* 1999a). Therefore, un-primed macrophages must be able to produce some IFNγ in response to large amounts of pneumolysin in order to produce NO. Baba *et al.* (2002) showed that pneumolysin could induce IFNγ production from murine spleen cells However their experiments indicated that IFNγ and NO production was only induced by pneumolysin when its cytolytic activity was abolished. These results appear to contradict our data and that of Braun *et al.* where maximum NO (and presumably IFNγ) production is associated with lytic concentrations of pneumolysin. Furthermore, when we used the W433F pneumolysin mutant, which has 0.1% of wild type cytolytic capability, we found it displayed reduced capacity to induce NO production from macrophages. However this mutant did still have some ability to induce NO production from macrophages suggesting that although the cytolytic activity does play a role, another part of the toxin is also involved in NO production.

In the study by Baba *et al.* (2002) very high concentrations of pneumolysin were used to induce IFNγ and NO production from the cells. No IFNγ production was seen from two toxins with the C-terminal 34 or 45 amino acids removed unless more than 200 nM (11 μg/ml) or 800 nM (42 μg/ml) respectively was added to spleen cells. With the wild type

toxin (pre-treated with cholesterol), peak IFNγ production was seen when 25 nM (1.3 μg/ml) of pneumolysin was used. This publication does not include a control experiment to show that cholesterol alone does not stimulate mediator production from these cells. During infection, IFNγ production may be stimulated directly by pneumolysin. Alternatively, pathogens such as GBS have been shown to induce IL-12 and TNF production from macrophages, which then promoted IFNγ secretion from natural killer cells. These activities led to an enhancement of innate phagocyte resistance mechanisms (Derrico and Goodrum 1996).

Maximum NO production from macrophages appears to be related to cell death. Although small amounts of NO were seen when sub-lytic concentrations of pneumolysin were used, most NO was seen when concentrations of pneumolysin known to have cytotoxic effects were used. These data raise questions about the mechanism of NO production and release, and how it is related to cell death. Triton-X was used as a control to lyse cells. The supernatants from these cells were also assayed using the Griess reaction and no nitrite was detected at any time (data not shown). This suggests that NO detection is not purely due to nitrite release from lysed cells. Another interesting observation came about when nitrite production stimulated by the N142/143 deletion pneumolysin mutant was compared to the wild type toxin. It became apparent that the numbers of cells present had an effect on NO production, higher levels of NO stimulation correlated with the numbers of macrophages present. Perhaps NO is released as a stress response just before the cells die. Alternatively, when the cells are lysed, they could release something that stimulates the remaining live cells to produce NO.

What could be causing the large amount of NO release at high pneumolysin concentrations? At normal intracellular calcium levels NO production by iNOS is limited only by the amount of enzyme, substrate or cofactor present (Xie and Nathan 1994; Gross and Wolin 1995). In contrast, the constitutive NOS enzymes, eNOS and nNOS are inactive at normal calcium concentrations, producing "puffs" of NO in response to transient increases in cytosolic calcium, but chronic calcium elevation will cause persistent NO production. Pneumolysin could not only stimulate iNOS production but could also lead to calcium influx through pore formation causing NO production from cNOS. Whole pneumococci, pneumolysin and pneumococcal H_2O_2 have all been shown to induce a Ca^{2+} influx in neurons (Braun *et al.* 2002). It has been shown that iNOS localises to membranous actin in activated macrophages

(Webb *et al.* 2001). This could lead to an abrupt increase in NO release when pneumolysin pores are formed in the cell membrane.

Unregulated NO production can cause cell death through oxidative stress, disrupted energy metabolism, DNA damage, activation of poly(ADP-ribose) polymerase (PARP), or dysregulation of cytosolic calcium; such disturbances can lead to either necrotic or apoptotic cell death (Murphy 1999). NO may reversibly regulate mitochondrial function *in vivo* by blocking respiration and ATP synthesis, and by causing the release of mitochondrial calcium (Richter *et al.* 1994; Brown 1995). The tumour suppressor protein p53 is also up-regulated in response to DNA damage caused by NO (Messmer *et al.* 1994; Messmer and Brune 1996). The production of peroxynitrite can damage DNA leading to either necrosis or apoptosis (Bonfoco *et al.* 1995).

In our experiments, a small amount of NO production was observed in unstimulated macrophages. Braun *et al.* (1999a) did not observe any NO from the same cell type without stimulus but other studies have reported NO from unstimulated cells. A small amount of nitrite was seen from naïve unstimulated rat alveolar macrophages and eNOS was detected in the cells by western blot (Miles *et al.* 1998). In the publication by Baba *et al.* (2002) a small amount of nitrite production is evident from control spleen cells incubated with PBS. It is possible that the variations between groups could depend on the culture conditions of the cells for example levels of calcium in the growth media.

In chapter 5, it was speculated that peak TNF production by pneumolysin from macrophages primed with IFNγ may have been missed at both 4 hours and 24 hours. The results with whole organisms from chapter 6 indicate that maximum TNF production occurs some time around six hours.

In our group, TNF activity was detected in bronchus associated lymphoid fluid (BALF) and bronchus associated lymphoid tissue (BALT) in lungs from mice infected with 10^4-10^6 cfu/ml of D39 *S. pneumoniae*. Peak TNF production was seen at 6-12 hours and was associated with resistance to infection in BALB/c mice (Kerr *et al.* 2002). Cells recovered from the airways of these mice and stimulated with the stocks of pneumolysin made for this project produced ~150 pg/ml TNF protein detectable by ELISA. In the TNF bioassay, 1 U of TNF activity is equivalent to 0.5 ng/ml TNF. The bioassay will generally not detect levels of

TNF lower than this. There may also be differences between primary cell isolates and transformed cell lines in their abilities to produce TNF. *In vivo* there is, of course, a much more complex network of cellular and cytokine interactions some of which could act synergistically to induce mediator production.

As discussed in section 1.11, TNF is associated with resistance to *S. pneumoniae* infection when production is mostly within the lungs. Susceptible mice showed TNF production in association with the tissues. Airway cells from mice susceptible to pneumococcal infection (CBA/Ca) release less TNF upon *in vitro* stimulation compared to cells from the resistant mouse strain BALB/c. 20 ng of pneumolysin induced detectable TNF protein with BALB/c cells releasing significantly more TNF (146 pg/ml) than CBA/Ca cells (55 pg/ml). The combination of 10^6 cfu of heat-killed bacteria and 0.2 ng pneumolysin released significantly higher concentrations of TNF from BALB/c cells than from CBA/Ca cells (32 pg/ml compared to 0 pg/ml); these levels were higher than either stimulant alone at these concentrations, suggesting that heat-killed bacteria and pneumolysin act in synergy to stimulate TNF production (Kerr *et al.* 2002). Previous studies have also shown that pneumococcal factors other than pneumolysin can stimulate TNF production (Freyer *et al.* 1996; Freyer *et al.* 1999; O'Brien *et al.* 1999; Um *et al.* 2000).

8.3 Effect of whole *S. pneumoniae* organisms on cultured cell lines

In order to study the more complex situation of pneumolysin in the context of the whole organism, experiments were carried out using wild type pneumococci and pneumolysin-negative pneumococci mutants. Firstly heat-killed bacteria were used to look at the effect of the cell wall and other bacterial components on the mammalian cells.

It has been shown previously that oxacillin-killed pneumococcus or purified pneumococcal cell wall induced iNOS protein, NO and TNF production from RAW 264.7 macrophages (Orman *et al.* 1998). However in this study, we found that heat-killed pneumococci induced no NO or TNF from these cells. This could be partly due to the fact that by heating the bacteria to 65°C the pneumolysin protein will also be destroyed. A study carried out by Orman and English (2000) may support this theory. A murine macrophage cell line was stimulated with antibiotic-killed pneumococci. Two types of antibiotics were used to kill the pneumococci; the first i.e. oxacillin, caused cell wall lysis, whereas the second type i.e. clindamycin, inhibited protein synthesis. They found that the pneumococci exposed to

antibiotics that caused cell lysis stimulated greater iNOS protein and TNF production than pneumococci exposed to antibiotics that inhibited protein synthesis. Presumably if the pneumococci were killed by cell lysis, the contents of the cell, including pneumolysin would be available to interact with the macrophages. Conversely the pneumococci killed by protein synthesis inhibition might not have as much pneumolysin available to interact with the macrophages. In this publication it was suggested that the increase in inflammation might be due to a greater amount of cell wall release.

Other groups have found that heat-killed pneumococci or cell wall components are capable of inducing NO production (Freyer *et al.* 1996; Orman *et al.* 1998). These effects may depend on the cell type and the culture conditions. In meningeal inflammation the cell wall plays an important part. However, this may not be important in all situations of pneumococcal infection (Tuomanen *et al.* 1985).

In our system, heat-killed pneumococci had no effect on the production of mediators assayed and we moved on to study the effects of live pneumococci. We found that live, wild type pneumococci stimulated significantly more NO production from macrophages than the pneumolysin-negative mutant. In keeping with our results, Braun *et al.* (1999a) detected a maximum of 12 μM nitrite after 18 hours, when a starting inoculum of between 10^1-10^6 cfu/ml live pneumococci were used, they also detected iNOS protein when 10^6 cfu/ml were used. PLN-A produced low or undetectable levels of NO or iNOS. NO production from macrophages stimulated with live bacteria displays a different pattern i.e. earlier nitrite release than when stimulated with pneumolysin alone suggesting different mechanisms and therefore other factors are also involved. The bacteria stimulated maximum NO production between 6-18 hours, whereas with pneumolysin, peak NO production was detected between 24-27 hours. As pneumolysin is not thought to be released from pneumococci until late in the growth phase, it is unlikely that there would be much toxin present in the supernatant at these early time points. However pneumolysin is involved in some way in influencing the activity of the bacteria and tissue culture cells in this system.

When live *S. pneumoniae* were added to DMEM in the absence of macrophages small amounts of nitrite were detectable in the Griess reaction. There is no reported nitrite production by *S. pneumoniae* in the literature, although a search of the genome sequence revealed presence of several nitrate reductase enzymes. The amount of nitrite produced was

significantly less than that produced by pneumococci-stimulated macrophages at six and twelve hours. However there is a possibility that the bacteria themselves may be contributing towards the nitrite detected in these assays.

Pneumolysin-negative D39 caused increased killing of macrophages compared to wild type bacteria. Dockrell *et al.* (2001) demonstrated that pneumolysin-negative *S. pneumoniae* resulted in lower levels of detectable internalised bacteria at four hours in contrast to wild type pneumococci. Apoptosis of macrophages was associated with internalisation and killing of pneumococci and may be an appropriate and successful host response against *S. pneumoniae*, which could be mediated by pneumolysin. It is possible that pneumolysin in wild type bacteria induces more NO production which has been shown to have a bacteriostatic effect on some bacteria. This could enable macrophages to successfully phagocytose and kill bacteria and may be associated with macrophage apoptosis. When pneumolysin-negative bacteria used, less NO production is seen – perhaps there is also a less effective respiratory burst – there is currently some controversy in the literature as to whether pneumolysin increases or decreases this activity.

Culture of bacteria with mammalian cells is a two way process and the mammalian cells must have some effects on the bacteria. The cells must produce many factors in response to the bacteria, some of which are likely to be beneficial to the bacteria and others potentially harmful. Compared to bacterial growth in tissue culture media, it appears that the cells are producing something that is keeping the bacteria alive. This is especially evident in the experiments with the pneumolysin-negative mutant as after the macrophages die; the numbers of *S. pneumoniae* also drop and end up much lower than the wild type bacteria. It is possible that after 48 hours, as the macrophages have been killed, the number of wild type bacteria would also start to decline. Alternatively pneumolysin could induce macrophages to make something that benefits the bacteria, which could also make macrophages less effective at microbial killing. In the experiments where live pneumococci were added to cultures of airway epithelial cells, a drop in the viability of the bacteria was observed between 6-24 hours. This suggested that the lung epithelial cells might be producing something that was toxic to the bacteria. There was no significant production of TNF or NO during the experiment, so some other toxic mediator must be released. However, this would not explain why the numbers of pneumococci increased again after 24 hours before there was a significant drop in the viability of the lung epithelial cells.

There are several pieces of evidence suggesting that pneumococci can invade host cells. Pneumococci have been shown to traverse epithelial and endothelial cells in a vacuole without intracellular replication (Ring *et al.* 1998). *In vitro* the process requires nine to eleven hours to complete and is more efficient for the transparent phenotype; the few opaque variants that enter cells appear to be killed (Tuomanen and Masure 2000). The interaction between the bacteria and host cell leading to cell translocation is thought to involve the platelet activating factor (PAF) receptor (Cundell *et al.* 1995). Translocation of pneumococci across A549 lung epithelial cells was attenuated in the presence of the PAF receptor antagonist. (Tuomanen and Masure 2000). It could be envisioned that, *in vivo*, the bacteria would invade the cells in order to cross the epithelial barrier to invade the blood stream. Therefore after invasion they would need to be released from the cells. If this was happening during the experiments presented in section 6.4.1, when the bacteria were inside the cells they would not be counted during the viability assay, after "translocation" they would again be free in the media and be counted in the viability assay. This could explain the apparent decrease and subsequent increase in bacterial viability during these experiments. Furthermore, the timing of this effect is similar to that observed in other studies (Ring *et al.* 1998; Tuomanen and Masure 2000).

If the bacteria were invading the lung epithelial cells this might help to explain the strange appearance of the growth of *S. pneumoniae* after incubation with them. Transparent colony variants have an increased phenotype for cell transmigration and opaque variants are more readily killed, although differences between colony morphology of transparent and opaque variants have not been reported for growth on blood agar. However the transparent variant does produce less capsule (Weiser 1998), which presumably could lead to a smaller colony size. Also transparent variants grow better in an anaerobic environment (Weiser 1998) and the bacteria recovered from the lung epithelial cells grew better under anaerobic conditions. This effect was totally absent from the pneumolysin-negative pneumococci experiments suggesting that this ability to invade the cells is driven by pneumolysin. It is possible that pneumolysin could activate the cells and cause an increase in receptor expression, which leads to cell invasion by the bacteria.

Further experiments need to be carried out to support this theory. We would need to observe the presence of bacteria in the epithelial cells incubated with wild type or pneumolysin-negative pneumococci. Phenotyping of organisms would enable us to see if the transparent

variant is more common during time points where invasion is expected to occur. It would also be interesting to establish if the bacterial phenotype is related to the strange growth observed on the blood agar plates when pneumococci were incubated with lung epithelial cells. We would also need evidence that the PAF receptor is involved in this process and whether pneumolysin can directly affect translocation of the bacteria.

In order to support bacterial translocation, the cells need to be activated (Cundell *et al.* 1995). The adherence of *S. pneumoniae* to chinchilla tracheal epithelium was increased by the addition of either TNF or IL-1α (Tong *et al.* 1999). Cundell *et al.* (1995) found that TNF caused increased pneumococcal adherence to endothelial cells. However we found no evidence of TNF production from lung epithelial cells. The presence of IFNγ in these experiments also had no effect. Pneumolysin could be involved in directly activating the cells allowing translocation to occur and/or up-regulating PAF receptor expression. It could indirectly lead to cell invasion by causing mediator release, which acts to activate the cells and/or induces phenotype change in the bacteria. Interestingly, the transparent phenotype is more susceptible to autolysis (Weiser 1998) suggesting that a phenotype change to the transparent variant could potentially lead to increased pneumolysin release.

After the first time course with lung epithelial cells, when the strange growth of the bacteria was observed, agar plates containing the bacterial viable counts were incubated in a candle jar to promote a more anaerobic environment. Therefore, we do not know if the different growth phenotype was also present in the pneumolysin-negative bacteria. It would be interesting to repeat the experiment described in section 6.4.1. This experiment was carried out for two hours – only long enough to determine that growing the bacteria in a candle jar was sufficient to be able to count the colonies. If the experiment was repeated, it could be carried out over a larger time span and with the pneumolysin-negative pneumococci. Microscopy techniques could be used to study phase variation (Weiser 1998).

In a previous study, A549 lung epithelial cells incubated with Group B *Streptococci* (GBS) neither secreted detectable NO nor expressed iNOS mRNA. GBS interaction with human mononuclear cells, however, stimulated release of soluble factors that readily induced iNOS mRNA expression and NO secretion by A549 cells (Goodrum and Poulson-Dunlap 2002). This was an experiment that we had planned to do using the supernatant from the

macrophages after incubation with pneumolysin. Unfortunately, due to time constraints it was not carried out.

8.4 Induction of Apoptosis by Pneumolysin

Severe cell damage can lead to the activation of PARP, depletion of cellular ATP levels, and mitochondrial damage (Murphy 1999). These events lead to cell necrosis by disrupting ion gradients, cell swelling and rupturing of the plasma membrane (Kerr *et al.* 1972; Farber *et al.* 1981). In contrast, if the damage is insufficient to cause necrosis, up-regulation of p53 stops cell division and provides the opportunity for damaged DNA to be repaired (Hale *et al.* 1996; Levine 1997). Beyond a certain damage threshold the cell will undergo apoptosis, but how the cell makes the decision to die is unknown. The apoptotic machinery is constitutively expressed within the cytoplasm so cells can commit to apoptosis without inducing new gene expression or protein synthesis (Kroemer *et al.* 1997). However, changes in transcription can also initiate apoptosis (Levine 1997).

Many apoptotic stimuli induce release of cytochrome c from mitochondria into the cytosol. Release of cytochrome c from the mitochondria is prevented by the anti-apoptotic proteins Bcl-2 and Bcl-X_L and activated by the pro-apototic protein Bax (Jurgensmeier *et al.* 1998). Once in the cytoplasm, cytochrome c binds to Apaf 1, which induces binding to pro-caspase 9, leading to the sequential activation of caspase 9. Active caspase 9 then directly cleaves to and activates pro-caspase 3, initiating a cascade of additional caspase activation that culminates in apoptosis (Li *et al.* 1997b).

The immune response of macrophages to *S. pneumoniae* includes a novel form of apoptosis that is associated with successful phagocytosis and bacterial killing (Dockrell *et al.* 2001). This response *in vivo* may regulate the inflammatory response to infection during a successful host response against *S. pneumoniae*. Induction of apoptosis could be beneficial to the host during bacterial infection by limiting the release of pro-inflammatory mediators and ROSs. Apoptotic cells in tissues are usually cleared rapidly and unlike necrotic cells, are not associated with increased inflammation. Apoptotic macrophages represent an effective source of antigen for presentation by dendritic cells (Yrlid and Wick 2000).

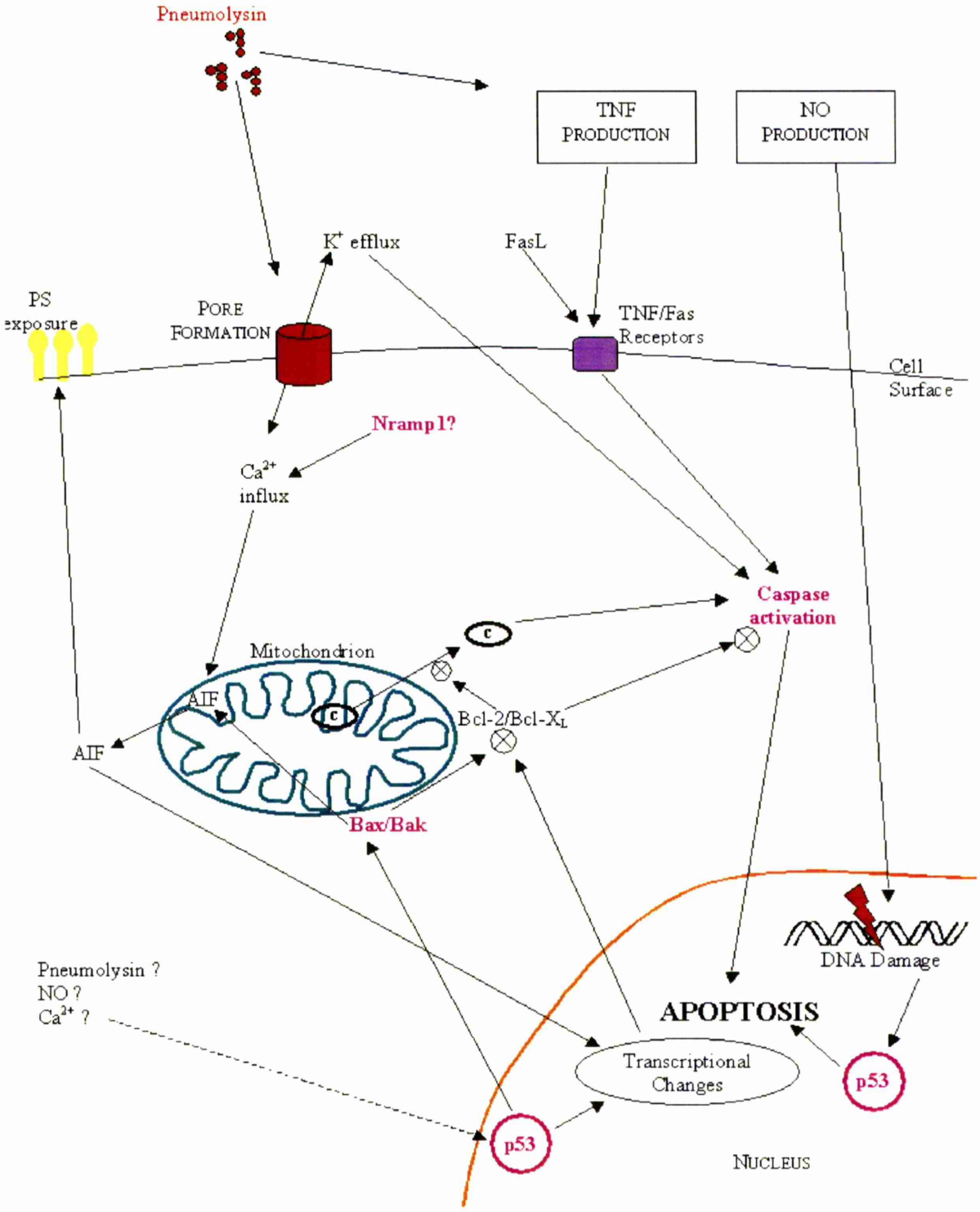
There is some controversy about the mechanisms involved in apoptosis caused by *S. pneumoniae*. In one publication, apoptosis caused by *S. pneumoniae* was not associated with caspase activation and not inhibited by the caspase inhibitor z-VAD-fmk. It was found to be due to rapid increases of intracellular ROS and Ca^{2+}, which damage mitochondria and

released apoptosis inducing factor (AIF) (Braun *et al.* 2001). This group however, also published data that suggest pneumococcal-induced apoptosis does involve a caspase pathway. They found that hippocampal neuronal death was due to apoptosis and could be blocked by a broad-spectrum caspase inhibitor (Braun *et al.* 1999b). Another study (Dockrell *et al.* 2001) found that apoptosis of human monocyte-derived macrophages caused by opsonised *Streptococcus pneumoniae* was associated with phagocytosis of bacteria and intracellular killing that was blocked by a caspase inhibitor but not by Fas-blocking antibody. Apoptosis was also induced in uninfected macrophages by fixed infected macrophages and this process was blocked by anti-Fas antibody.

Possible pathways that pneumolysin could induce to cause apoptosis are shown in Figure 8.1. Pore formation by pneumolysin could directly induce apoptosis by causing an imbalance in intracellular divalent cations. Calcium influx was shown to be necessary to induce AIF resulting from pneumolysin-stimulation (Braun *et al.* 2001). Intracellular K^+ seems to be a key inhibitor of cell death by preventing caspase activation (Warny and Kelly 1999). Cockeran *et al.* (2001) demonstrated that pneumolysin could cause efflux of K^+ from neutrophils. Our results from chapter 7 suggest that a caspase pathway is initiated and p53 is likely to be involved in pneumolysin-induced apoptosis. Reactive oxygen species appear to play a critical role in regulating p53 function, and p53 controls the expression of a large number of redox regulatory proteins. Recent work has shown that NO controls p53 activation, and that p53 can regulate inducible NO synthase (iNOS)/NOS-2 promoter activity. Given the unique role of oxygen in the lung microenvironment it is likely that p53 plays a critical role in the maintenance of normal lung physiology (Davis *et al.* 2000).

An experiment utilising laser scanning cytometry of propidium iodide (PI) stained cells was carried out using cells that had been incubated with or without pneumolysin. Apoptotic cells have decreased PI fluorescence and diminished forward light scatter relative to cells in the main peak (G_1). Optimally the "sub-G_1 peak" representing apoptotic cells should be separated from the G_1 peak of the non-apoptotic cell population with little or no overlap between the two (Darzynkiewicz *et al.* 2001). Macrophages incubated in media alone show few cells in the sub-G1 peak. When the cells are incubated with 10 or 100 ng/ml of pneumolysin an increase in this population is seen. Induction of apoptosis by these sublytic concentrations could help to explain why macrophages are susceptible to lower concentrations of pneumolysin after 24 hours.

Figure 8.1 – Possible Mechanisms involved in Pneumolysin-induced Apoptosis



Pneumolysin could cause apoptosis directly, through cell changes as a result of pore formation or indirectly via NO or TNF production. Proteins in **pink** were shown to be up-regulated in response to pneumolysin in chapter 7. Bcl-2 and Bcl-X_L are anti-apoptotic factors so some pro-apoptotic gene products act to suppress their activity. Abbreviations: TNF/Fas, receptor pathways of caspase activation; c, cytochrome c; PS, phosphatidylserine; AIF, apoptosis inducing factor; FasL, Fas ligand.

8.5 Signal Transduction

Calcium has been implicated in many pneumolysin activities. Calcium levels in the cytoplasm and nucleus of cells can be kept very low due to efficiently operating calcium extrusion mechanisms. However upon exposure of cells to environmental stimuli, calcium concentrations can increase sharply. The calcium transients are used by cells to turn many processes on or off. Transient increases in cytosolic calcium are a pre-requisite for activation of many of the pro-inflammatory activities of neutrophils, including adhesion to vascular endothelium, production of reactive oxidants, degranulation and activation of cytosolic phospholipase A_2 (Lew *et al.* 1986; Thelen *et al.* 1993; Pettit and Hallett 1996). The transients can be small or large, short-lasting or sustained. Calcium regulates the immune response, secretion, cell division, meiosis and likely to be involved in cell degeneration (Bading 2000).

Cockeran *et al.* (2001) showed that pneumolysin resulted in a dose related increase in cytosolic calcium, apparently as a result of an influx of extracellular cations rather than its mobilisation from intra-cellular stores. They found that activity of the plasma membrane, calcium efflux pump of neutrophils was not inhibited by pneumolysin, and the calcium influx was dependent on the membrane modifying actions of the toxin, as mutant toxin (W433F) was ineffective. The authors therefore hypothesised that the calcium influx was due to the pore forming ability of the toxin. Divalent cations, particularly Ca^{2+} and Zn^{2+} have been found to counteract cellular damage inflicted by pneumolysin and other pore-forming toxins (Harshman and Sugg 1985; Korchev *et al.* 1992; Korchev *et al.* 1998). These protective effects were attributed to voltage-dependent closure of channels rather than to a blocking mechanism (Korchev *et al.* 1992; Korchev *et al.* 1998). Extracellular Ca^{2+} concentrations at sites of pneumococcal infection may determine the action of pneumolysin – that is whether it is pro-inflammatory or cytolytic. In either case the outcome could be detrimental to the surrounding host tissue.

Pneumolysin stimulation of phospholipase A and stimulation of apoptosis inducing factor (AIF) are calcium-dependent (Cockeran *et al.* 2001; Braun *et al.* 2002). The cytotoxic potential of pneumolysin was enhanced when neutrophils were suspended in calcium free HBSS (Cockeran *et al.* 2001). This could mean that the host cell response to a calcium influx is appropriate and protective.

As mentioned above, Baba *et al.* (2002) showed that IFNγ and NO production were stimulated by pneumolysin that lacked pore-forming activity. This study also raises questions about the membrane receptor used by pneumolysin to exert these effects. In this study, full length and truncated toxins were used to stimulate murine spleen cells. They found that the full-length toxin was not capable of inducing IFNγ production even at sublytic concentrations. However truncated pneumolysin mutants with deletions of the C-terminal 34 or 45 amino acids, displayed no cytotoxic activity and could induce IFNγ production. Furthermore the full-length toxin could only induce IFNγ production when it was treated with cholesterol to inhibit its cytolytic activity. The truncated versions of pneumolysin also stimulated more nitrite production than the pre-treated full-length toxin. These data all point towards a receptor, other than cholesterol, that pneumolysin can bind to on the cell surface, assuming that pneumolysin does bind to the cell in order to induce IFNγ and NO production. However it is possible that pneumolysin could enter the cells and interact directly with the nitric oxide synthase enzyme to stimulate its activity. This mechanism has been proposed for the activation of phospholipase A and is dependent on the formation of functional pores (Rubins *et al.* 1994).

It has been suggested before that membrane components other than cholesterol may play a role in pore formation (Palmer 2001). Individual toxins in the TAT family display varying preferences for target erythrocytes from different mammalian species (Smyth and Duncan 1978) for example, intermedilysin is largely specific for human cells (Nagamune *et al.* 1996). Another study where haemolytic and complement-activating activity were abolished, suggested that there could be another site on pneumolysin capable of binding the Fc portion of IgG (Berry *et al.* 1999).

Another pathway that may be activated during *S. pneumoniae* infection and pneumolysin-induced inflammatory mediator activation could involve CD14 signalling. CD14 signalling is thought to occur through toll-like receptor (TLR) 2 and NFκB activation (van Furth *et al.* 1999; Yoshimura *et al.* 1999). Interaction with CD14, which is found mainly on monocytes and macrophages, can lead to production of TNF, IL-1 and IL-6. Gram-positive bacteria (micrococci) and peptidoglycan were shown to induce TLR2-dependent activation of the gene for IL-8 and this activation requires myeloid differentiation protein (MyD88), IL-1

receptor-associated kinase (IRAK), NFκB-inducing kinase (NIK), IκB kinase (IKK) and NFκB (Wang *et al.* 2001).

NFκB regulates the expression of many cytokines and iNOS, and is likely to be involved in pneumolysin-induced inflammatory effects. NFκB molecules are inactive in the cytosol, masked by inhibitory κB (IκB) proteins. Cytokines such as TNF, CD40 ligand, and IL-1 are able to induce IκB protein phosphorylation. This activates NFκB so it can travel to the nucleus. The activation of NFκB may play a role in the development of septic shock due to over stimulation by microbial products of various inflammatory cytokines (Baldwin 1996). In the microarray, there was no evidence that NFκB itself displayed altered gene expression. However, one gene which activates NFκB (RANK) was up-regulated and a gene which inactivates NFκB (TTRAP) was down-regulated by pneumolysin (See section 7.5).

GBS as well as two other gram-positive bacterial species, *Staphylococcus aureus* and *Staphylococcus epidermidis*, have been shown to stimulate NO production in murine macrophages in the presence of IFNγ (Goodrum *et al.* 1994). Phagocytosis-induced TNF production was responsible for this GBS-induced NO production in IFNγ-treated macrophages (Goodrum *et al.* 1995), and phagocytosis was dependent on complement receptor type 3 (CR3). Goodrum *et al.* (1994) found that ligated CR3 and IFNγ act synergistically to induce NO production, and CR3 mediates the GBS-induced signal for NO production in IFNγ-treated macrophages. We found that pneumolysin induced NO production from macrophages at later time points than the whole bacteria. Pneumolysin-stimulated NO production may therefore be independent of TNF as it occurs so late and other factors may be involved such as TLR-2 (Pers Comm. Dr. R. Read, Sheffield University)

As *S. pneumoniae* is a Gram-positive bacterium it does not contain LPS. In this respect it is important to abolish it from our *in vitro* systems as it would not be present in the bacteria. We have shown in section 5.5, by heat-inactivating the toxin that LPS does not contribute to pneumolysin-induced NO production. During infection, it would be plausible to expect a small amount of endotoxin to be present from Gram-negative commensal organisms. It would be interesting to look at the effect that small amounts of LPS and pneumolysin have

on cell activation. Maybe they act synergistically together and utilise the CD14 surface receptor.

8.6 Gene Regulation by Pneumolysin

During pneumococcal infection, the regulation of certain genes may be important in causing damage to host cells. Cell detachment in an *in vitro* BBB model induced by live pneumococci or pneumolysin was shown to depend on *de novo* protein synthesis and required activity of caspases and tyrosine kinases (Zysk *et al.* 2001). When human meningeal epithelial cells were exposed to *Neisseria meningitidis* for four hours, 32 genes were found to be up-regulated and 4 down-regulated. Those increased included the cytokines IL-6 and TNF, chemokines including IL-8 and MCP1 and apoptosis-related genes such as caspase 10 and IRF-1 (Wells *et al.* 2001).

The results of the microarray experiment shown in chapter 7 gave us an interesting snap-shot of gene regulation by pneumolysin. Gene chips were hybridised with cDNA from macrophages incubated with pneumolysin, IFNγ and both pneumolysin and IFNγ. Although we were interested mainly in the effects of pneumolysin, during infection other cytokines and inflammatory mediators will be present such as IFNγ which is why we also looked at this combination. We have shown in section 5.2 that NO production stimulated by pneumolysin is increased in the presence of IFNγ so we thought it would be interesting to see if these two inflammatory mediators could have other synergistic effects on gene regulation.

We found that seven of the genes most up-regulated by IFNγ encode GTP-binding proteins. Ehrt *et al.* (2001) identified MHC class II-related genes, RANTES and a GTP-binding protein in the ten genes most up-regulated by 100 U/ml IFNγ. They also found that more genes were suppressed by IFNγ than were induced. However they used 100 times more IFNγ than we did which may account for these differences.

When the gene ontology search terms immune response or inflammation were used only one gene was clearly up-regulated by pneumolysin alone. This gene, which encodes MIP-2 is chemotactic for neutrophils. Inoculation of mice with pneumolysin-negative pneumococci leads to a slower and less intense influx of neutrophils into the lung compared to wild type pneumococci (Kadioglu *et al.* 2000). Pneumolysin could therefore initiate an influx of neutrophils by increasing MIP-2 expression and activity. In fact it has just been shown that

pneumolysin can up-regulate IL-8 production from human neutrophils (Cockeran *et al.*, in press)

Many genes involved in the inflammatory response were up-regulated synergistically by pneumolysin and IFNγ. Some of the up-regulated genes encode products that have been shown to be induced by pneumolysin *in vitro* in previous publications. Others such as RANTES, Nramp1 and IL-18 have not been previously reported to be induced by pneumolysin.

The most striking observation in the microarray experiment was the number of genes involved in apoptosis pathways that were regulated by pneumolysin. Many of the up-regulated genes encoded p53 targets and expression of p53 itself was induced. Also caspase 9, which is at the start of the caspase cascade leading to apoptosis, was up-regulated. Whether pneumolysin itself can directly cause apoptosis or whether it is stimulated indirectly by pneumolysin-induced NO is not clear.

Nramp1 is not ubiquitously expressed but is found in high levels in phagocytic cells and is located in the lysosomal compartment of the phagocyte (Gruenheid *et al.* 1995). Identification of the highly similar Nramp2 which is a divalent cation transporter led to the hypothesis that Nramp1 is also involved in the transport of divalent cations such as Fe^{2+}, Mg^{2+}, Zn^{2+} or Ca^{2+} (Fleming *et al.* 1997). Is it possible that Nramp could play a role in the Ca^{2+} influx that may lead to apoptosis?

Surprisingly, there was no significant increase in the expression of iNOS in the macrophages stimulated with pneumolysin or with pneumolysin and IFNγ. Peak RNA expression may not correlate directly with peak mediator production. The 24 hour time point was used for the microarray as this is when most nitrite (0.012mM) was detected from the supernatant of stimulated cells. Perhaps at 24 hours, when peak nitrite is detected from the macrophages, the iNOS gene has been switched off. The microarray experiment was carried out towards the end of the project so unfortunately there was not time to back up these findings with RT-PCR experiments to detect mRNA expression.

Further microarray experiments over a range of time points would no doubt provide more interesting data and perhaps start to answer many of the questions raised in these experiments. Investigation of interactions of pneumolysin with the lung epithelial cells could

tell us if these cells produce any microbicidal agents. Interactions of whole pneumococci and the pneumolysin-negative mutants with host cells could also be studied by carrying out both bacterial and mammalian cell microarray experiments. At the moment these types of experiments are restricted by the cost of the chips, equipment and software required to process and analyse the data. Also mRNA expression does not necessarily mean that protein expression is also increased. However, protein arrays are also becoming available and these no doubt, will provide more insights in to the complexity of host-pathogen interactions. It is important to remember that each gene chip gives us a snap-shot of gene regulation and needs to be backed up by other experiments. The regulation of genes will vary enormously depending on the gene type, stimulus and culture conditions of the cells.

8.7 Conclusions

The concentrations of pneumolysin present during an infection may play a role in the extent of local tissue damage and the overall outcome (Figure 8.2). High concentrations of pneumolysin stimulate a large amount of NO production from macrophages that could be detrimental to the host and cause tissue damage and septic shock. Lower concentrations of pneumolysin stimulate smaller amounts of NO that could form part of an appropriate host response to infection. Unfortunately we have not been able to resolve the effect that oligomerisation has on these activities as site directed mutagenesis of the asparagine residues at positions 142 and 143 failed to block oligomerisation. The data obtained using the W433F pneumolysin mutant suggests that lytic activity is important. The fact that lytic concentrations of pneumolysin stimulate greater amounts of NO also point towards the involvement of lytic mechanisms.

Data presented here using whole organisms also confirm the importance of pneumolysin in the ability of *S. pneumoniae* to induce NO production. These experiments have also highlighted some other interesting interactions between the bacteria and mammalian cells that involve the toxin. Pneumolysin may also play a role in the invasion of airway epithelial cells, possibly by associating with or up-regulating expression of surface receptors such as the PAF receptor. However more work needs to be done to support this observation.

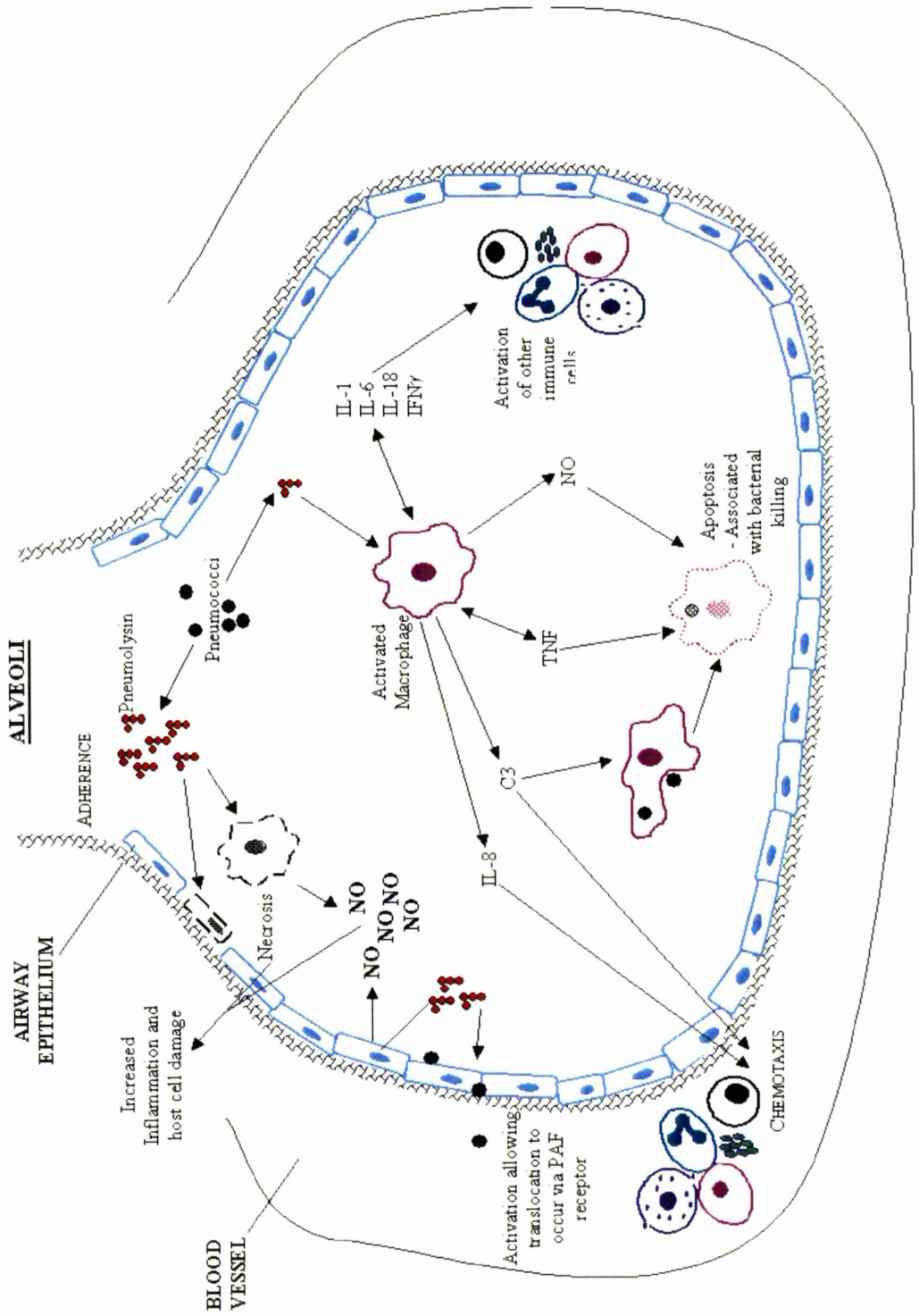
The overall picture of mediator stimulation and what effect this may have in patients with pneumococcal disease is very complex. Most cytokines have multiple and diverse biological functions. They are produced by more that one cell type and act on a variety of target cells at

different stages of cellular proliferation and differentiation. Data from the microarray confirms the up-regulation of some previously reported mediators such as IL-1β and phospholipase A_2 but not others such as TNF. This highlights the complexity of this method and the need for more experiments to be carried out over different time points.

Results from this project and other studies have shown that pneumolysin is capable of inducing apoptosis in some cell types (Braun *et al.* 2000; Zysk *et al.* 2000; Dockrell *et al.* 2001). This may be an appropriate immune response associated with bacterial killing and could also help to limit the inflammatory response.

There is increasing evidence that Ca^{2+} influxes, and possibly K^+ effluxes play an important role in many of the signalling pathways involved. Further investigation of these mechanisms could provide information that could be used to block the inflammatory responses induced by high concentrations of pneumolysin which lead to host damage and sepsis.

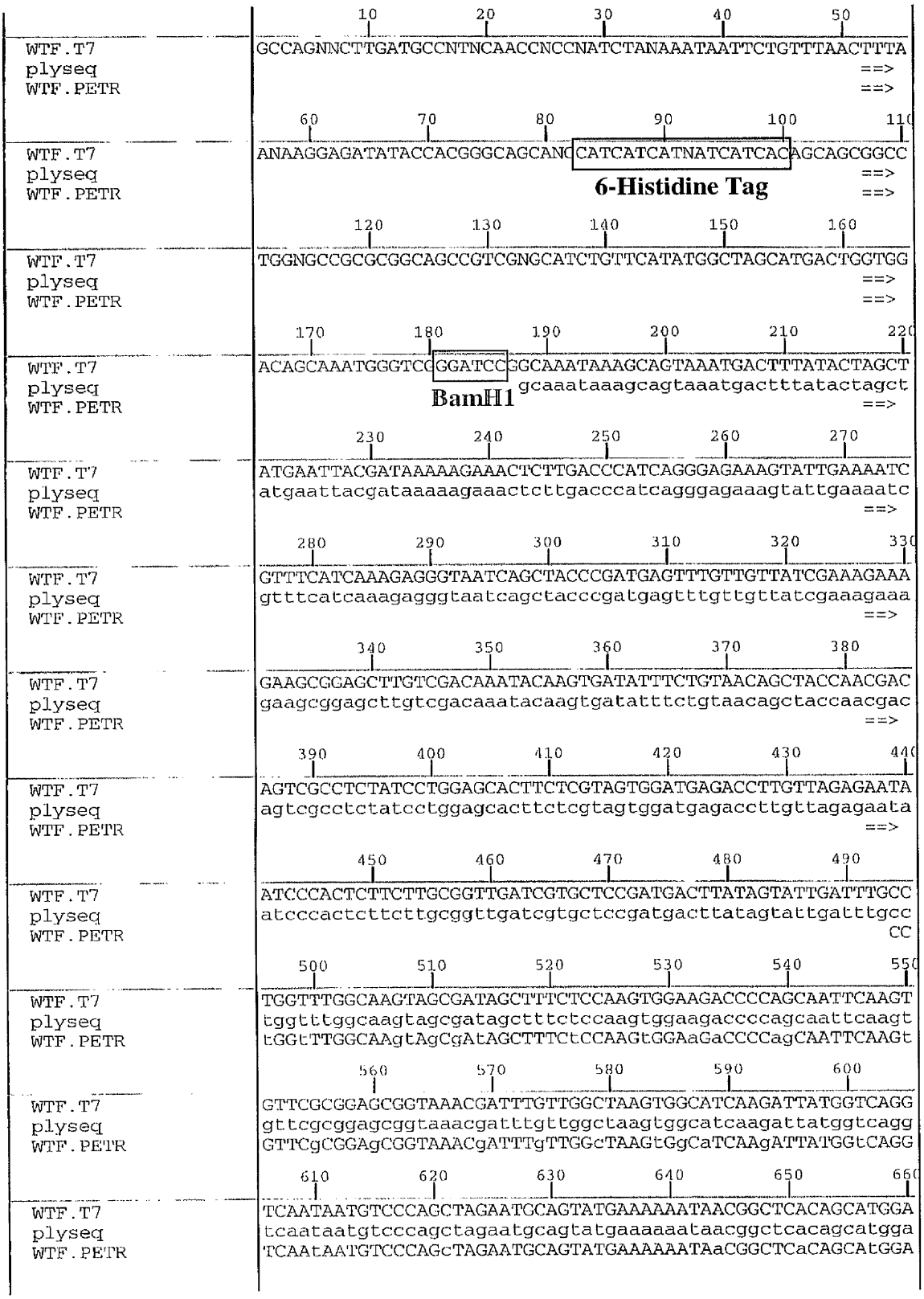
Figure 8.2 – Effects of Pneumolysin during Pneumococcal Infection

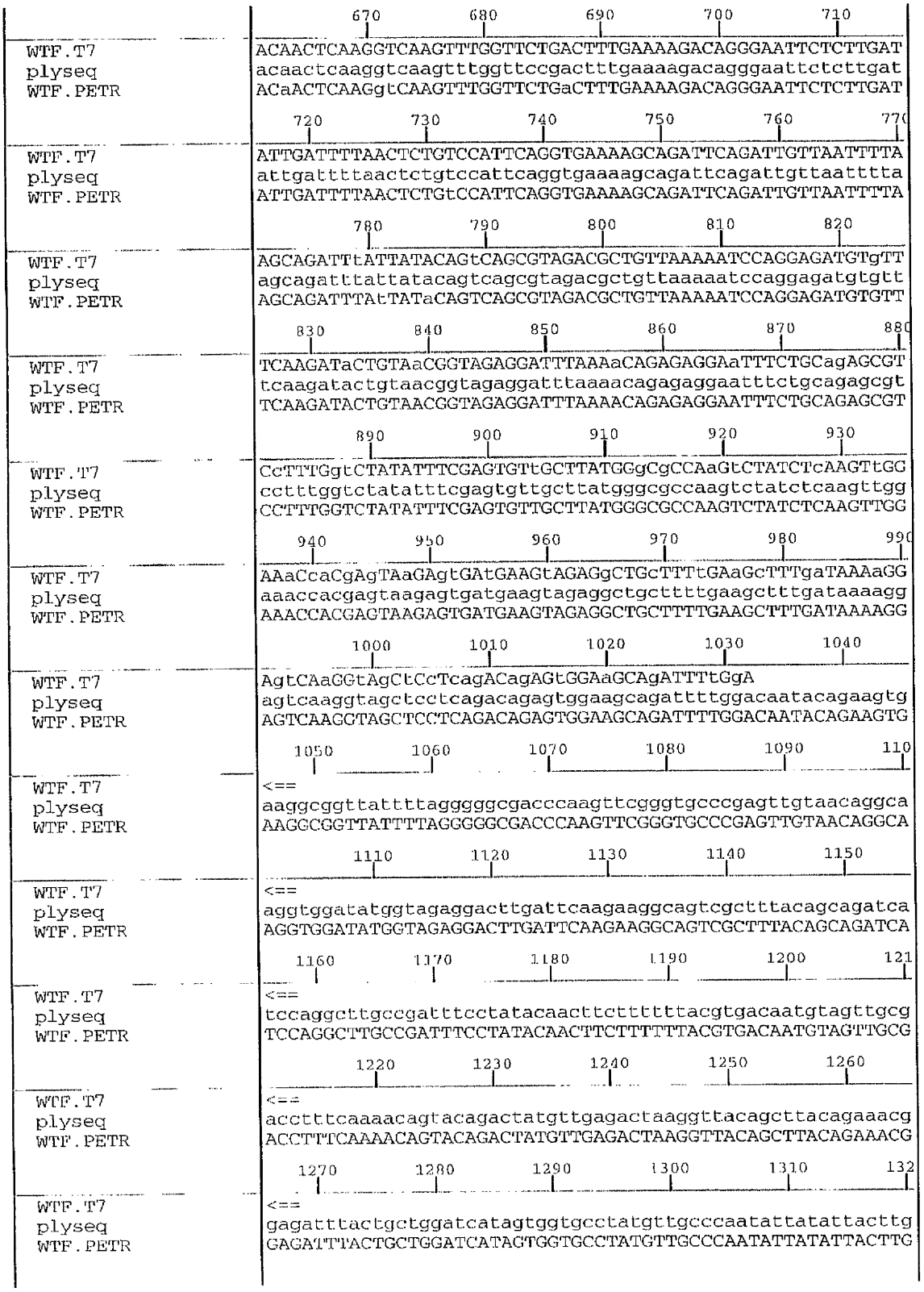


During severe pneumococcal infection, high concentrations of pneumolysin can lead to direct tissue damage and high levels of NO production from both macrophages and airway epithelial cells. This would lead to an overproduction of inflammatory mediators and could lead to sepsis and even death (top-left section of figure). Lower concentrations of pneumolysin may be beneficial to the host by stimulating appropriate mediator production leading to increased pneumococcal killing and clearance. Abbreviations: NO, nitric oxide; IL, interleukin; C3, complement component 3; TNF, tumour necrosis factor; IFNγ, interferon gamma.

APPENDIX

9.1 DNA sequence of wild type pneumolysin clone

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plyseq acaactcaaggtcaagtttggttccgactttgaaaagacagggaattctcttgat
WTF.PETR ACaACTCAAGgtCAAGTTTGGTTCTGaCTTTGAAAAGACAGGGAATTCTCTTGAT
720 730 740 750 760 770
WTF.T7 ATTGATTTTAACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTA
plyseq attgattttaactctgtccattcaggtgaaaagcagattcagattgttaatttta
WTF.PETR ATTGATTTTAACTCTGtCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTA
780 790 800 810 820
WTF.T7 AGCAGATTtATTATACAGtCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTgTT
plyseq agcagatttattatacagtcagcgtagacgctgttaaaaatccaggagatgtgtt
WTF.PETR AGCAGATTTAtTATaCAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTT
830 840 850 860 870 880
WTF.T7 TCAAGATaCTGTAaCGGTAGAGGATTTAAAaCAGAGAGGAaTTTCTGCagAGCGT
plyseq tcaagatactgtaacggtagaggatttaaaacagagaggaatttctgcagagcgt
WTF.PETR TCAAGATACTGTAACGGTAGAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGT
890 900 910 920 930
WTF.T7 CcTTTGGtCTATATTTCGAGTGTTtGCTTATGGgCgCCAaGtCTATCTcAAGTtGG
plyseq cctttggtctatatttcgagtgttgcttatgggcgccaagtctatctcaagttgg
WTF.PETR CCTTTGGTCTATATTTCGAGTGTTGCTTATGGGCGCCAAGTCTATCTCAAGTTGG
940 950 960 970 980 990
WTF.T7 AAaCcaCgAgTAaGAgtGAtGAAGtAGAGgCTGcTTTtGAaGcTTTgaTAAAaGG
plyseq aaaccacgagtaagagtgatgaagtagaggctgcttttgaagctttgataaaagg
WTF.PETR AAACCACGAGTAAGAGTGATGAAGTAGAGGCTGCTTTTGAAGCTTTGATAAAAGG
1000 1010 1020 1030 1040
WTF.T7 AgtCAaGGtAgCtCcTcagACagAGtGGAaGCAgATTTtGgA
plyseq agtcaaggtagctcctcagacagagtggaagcagattttggacaatacagaagtg
WTF.PETR AGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAGATTTTGGACAATACAGAAGTG
1050 1060 1070 1080 1090 1100
WTF.T7 <==
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1110 1120 1130 1140 1150
WTF.T7 <==
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WTF.T7 <==
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WTF.PETR ACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAACG
1270 1280 1290 1300 1310 1320
WTF.T7 <==
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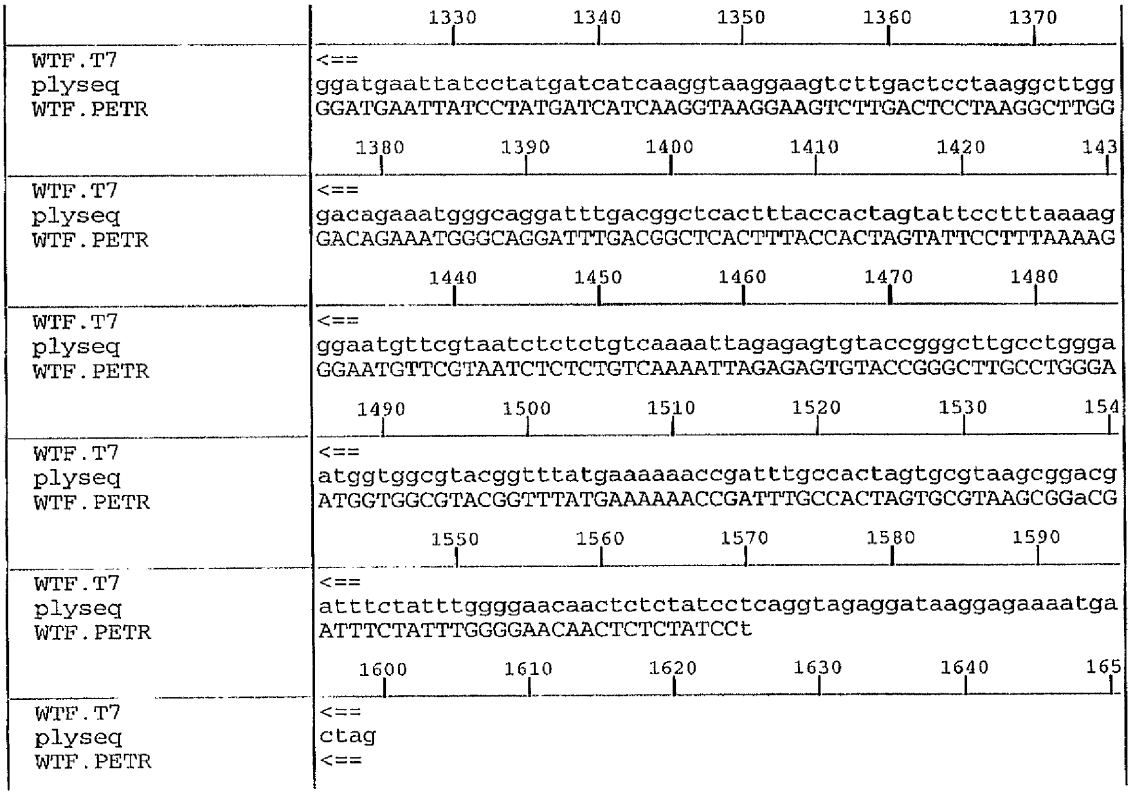

WTF.T7 = Pneumolysin sequence using forward primer (T7)
plyseq = wild type pneumolysin sequence
WTF.PETR = Pneumolysin sequence using reverse primer (PlypET Term)

9.2 Recipes

Nitrate Conversion Buffer

5 mg/ml NADPH	500 µl
41.5 mg/ml FAD	500 µl
34 mg/ml Nitrate reductase	50 µl freshly diluted in 450 µl dH_2O
0.5 M KH_2PO_4	500 µl

<u>BACTERIAL CELL CULTURE</u>

LB Broth/Agar

Bactotryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
dH_2O	950 ml

Adjust to pH 7.0 with NaOH. Adjust to 1 l.

Or add 25 g LB Broth (Oxoid) to 1 l dH_2O

Add 6 g Agar if required.

Kanamycin Stock (20mg/ml)

Kanamycin	200 mg
dH_2O	10 ml

Sterilise using 0.2 μm filters, aliquot and store at -20°C

Ampicillin Stock (100mg/ml)

Ampicillin	1 g
dH_2O	10 ml

Sterilise using 0.2 μm filters, aliquot and store at -20°C

PBS (1x)

0.14 M NaCl	8 g
2.68 mM KCl	0.2 g
1.76 mM KH_2PO_4	0.24 g
0.01 M Na_2HPO_4	1.44 g

IPTG (1M)

IPTG (Melford Laboratories)	2 g
dH_2O	10 ml

Sterilise using 0.2 μm filters, aliquot and store at -20°C

RECIPES USED FOR MOLECULAR BIOLOGY EXPERIMENTS WITH DNA

DNA Loading Buffer (6x)

Bromophenol blue	0.025 g
Sucrose	4 g
dH_2O	10 ml

1Kb Ladder (Gibco)

1 Kb ladder	100 μl
loading buffer	250 μl
dH_2O	940 μl

The ladder is at a concentration of 1 μg/ml. In this mix there is 100 μg ladder in 1290 μl, so in 10 μl there is 775 ng. The brightest band of the ladder on a gel contains 10% of the ladder, so if 10 μl is loaded it has 77.5 ng of DNA.

1% Agarose Gel

Agarose	2 g

TAE (1x)	200 ml

Microwave to dissolve agarose. Cool to hand-hot and add

1 mg/ml Ethidium Bromide	100 µl

Ligation Reaction

pPCR-Script Amp SK(+) cloning vector (10 ng/µl)	1 µl
PCR-Script 10 x reaction buffer	1 µl
10 mM rATP	0.5 µl
PCR product	2-4 µl
Srf I restriction enzyme (5 U/µl)	1 µl
T4 DNA ligase	1 µl

dH_2O to a final volume of 10 µl

Mix the ligation reaction gently and incubate for one hour at room temperature. Heat for ten minutes at 65°C. Store on ice until ready for transformation reaction.

TE Buffer

1 M Tris.Cl (pH 7.4)	1 ml
250 mM EDTA (pH 8.0)	400 µl

dH_2O to 100 ml

Restriction Digest

To 3 µl of miniprep DNA (0.5-3 µg) 7 µl of mastermix was added.

Mastermix: 1 µl of appropriate 10 x reaction buffer (supplied with the enzyme)
0.4 µl of each restriction enzyme
dH_2O to 7 µl

Restriction enzymes (purchased from Promega) were supplied at 10 units/µl. For each 10 µl reaction, 4 units of each enzyme were used with the appropriate reaction buffer.

Phenol:Chloroform Extraction of DNA

An equal volume of mixed phenol and chloroform was added to the DNA sample, which was then vortexed and centrifuged for one minute at 16000 x g. The top layer was removed and transferred to a new tube. 0.1 volumes of 3 M sodium acetate (pH 7.0) was added and mixed with 2 volumes of ethanol. This was incubated on ice for 15 minutes then centrifuged at 12000 x g for ten minutes at 4°C. The supernatant was discarded, 100 µl of

70% ethanol was added and the sample centrifuged again. The ethanol was removed and the DNA pellet was air-dried.

TRANSFORMATION PROTOCOLS

Novablue Singles (Novagen)

Thaw 50 µl of competent cells per reaction, on ice and mix gently to ensure cells are evenly suspended. Add 1 µl of DNA solution directly to the cells. Stir gently to mix. Incubate on ice for five minutes. Heat the tubes for exactly 30 seconds in a 42°C waterbath. Place on ice for two minutes. Add 250 µl of SOC media to each tube. Shake at 37°C for 30 minutes prior to plating on appropriate selective media and incubating overnight at 37°C.

Solopack Supercompetent Gold Cells (Stratagene)

Thaw one tube of competent cells per reaction, on ice and mix gently to ensure cells are evenly suspended. Add 1 µl β-mercaptoethanol, mix tubes gently and incubate on ice for ten minutes. Add 0.01-50 ng of DNA solution directly to the cells and stir gently to mix. Incubate on ice for 30 minutes. Heat the tubes for exactly 60 seconds in a 54°C waterbath. Place on ice for two minutes. Add 150 µl of SOC media to each tube. Shake at 37°C for one hour prior to plating on appropriate selective media and incubating overnight at 37°C.

RECIPES USED FOR MOLECULAR BIOLOGY EXPERIMENTS WITH RNA

Formaldehyde Agarose (FA) Gel Electrophoresis

1.2% Agarose Gel

1.2 g agarose

10 ml 10 x FA gel buffer

RNase-free water added to 100 ml.

The mixture was heated in a microwave until melted and left to cool to 65°C in a water bath. For each gel, 20 ml was transferred to a falcon tube, 360 µl of 37% (12.3 M) formaldehyde (Sigma) and 2 µl of 1 mg/ml Ethidium Bromide was added.

10x FA Gel Buffer

200 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

1x FA Gel Running Buffer

100 ml 10 x FA gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

5x RNA loading buffer

16 µl saturated aqueous bromophenol blue solution*

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde

2 ml 100% glycerol

3.084 ml formamide

4 ml 10 x FA gel buffer

RNase-free water to 10 ml.

*To make a saturated solution, add bromophenol blue to distilled water. Mix and continue to add more until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipette the saturated solution.

SDS-PAGE RECIPES

10% Separating gel

dH_2O	4.05 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
10% SDS	100 µl
30% Acrylamide/Bis Solution, 19:1 (5%) (Acrylamid:N,N'-Methylenbisacrylamid) (Biorad Laboratories)	3.3 ml
10% Ammonium persulfate	50 µl
Temed (N,N,N',N'-Tetramethylethyleneoliamine)	5 µl

Stacking gel:

dH_2O	6.1 ml
1.5 M Tris-HCl pH6.8	2.5 ml
10% SDS	100 µl
30% Acrylamide/bis	1.33 ml
10% Ammonium persulfate	50 µl
TEMED	10 µl

1.5M Tris-HCl (pH 8.8)

Tris base	18.15 g
dH_2O	50 ml

Add concentrated HCl to pH 8.8

Add dH_2O to 100 ml

1.5 M Tris-HCl (pH 6.8)

Tris base	18.15 g
dH_2O	50 ml

Add concentrated HCl to pH 6.8

Add dH_2O to 100 ml

Sample Buffer (5x)

1.5 M Tris-HCl (pH 6.8)	0.6 ml
50% glycerol	5 ml
10% SDS	2 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1 ml
H_2O	0.9 ml

Electrophoresis Buffer (5 x)

Tris base	9 g
Glycine	43.2 g
SDS	3 g
dH_2O	600 ml

Destain

Methanol	400 ml
Acetic Acid	100 ml
dH_2O	500 ml

Coomassie Stain

Coomassie Blue	1 g
Destain	1 l

BUFFERS USED FOR WESTERN BLOTS

Transfer Buffer

25 mM Tris Base	3.03 g
192 mM Glycine	14.4 g

20% Methanol	200 ml

Add H_2O to 1 l. pH should be 8.1-8.4 (do not adjust)

Tris-NaCl pH 7.4

Tris Base	1.2 g
NaCl	8.7 g
Concentrated HCl	800 µl

H_2O to 1 l. pH should be 7.4

TBS Buffer

10 mM Tris.Cl	1.57 g
150 mM NaCl	8.77 g
H_2O	1 l

TBS-Tween/Triton Buffer

20 mM Tris.Cl	3.14 g
500 mM NaCl	29.22 g
Tween 20	500 µl
Triton-X-100	2 ml
H_2O	to 1 l

Developer

4-chloro-1-naphthol	30 mg
Methanol	10 ml
Tris-NaCl	50 ml
H_2O_2 (30% w/v)	30 µl

The blot was incubated with the developer in the dark until protein bands were visible. The blot was then rinsed with H_2O and left to air dry.

BUFFERS USED FOR CHROMATOGRAPHY ON THE BIOCAD® 700E PERFUSION CHROMATOGRAPHY WORKSTATION

All buffers were made using milliQ water and 0.2µm filtered and degassed.

100 mM EDTA/ 1M NaCl

EDTA	18.6 g
NaCl	29.22 g
H_2O	500 ml

500 mM EDTA

EDTA	93.06 g
H_2O	500 ml

NB: EDTA will not dissolve until NaOH is added to pH 8.0.

2M HCl

HCl	84.76 ml
H_2O	415.24 ml

50mM Hepes

Hepes (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) (Sigma)	5.96 g
H_2O	500 ml

1M Imidazole

Imidazole extra pure (BDH)	34.04 g
H_2O	500 ml

3M NaCl

NaCl	87.66 g
H_2O	500 ml

1M NaOH / 1M NaCl

NaCl	29.22 g
NaOH	20 g
H_2O	500 ml

100mM $NiSO_4$

$NiSO_4$	13.142 g
H_2O	500 ml

Buffer A - PBS (without NaCl)

KCl	0.2 g
KH_2PO_4	0.24 g
Na_2HPO_4	1.44 g
H_2O	1 l

0.02% Sodium Azide

Sodium Azide	0.1 g
H_2O	500 ml

NB: Add sodium azide to water.

CELL CULTURE REAGENTS

To make up culture media for small airway epithelial cells, each singlequot (listed below) was added to 500 ml of small airway basal media (All BioWhittaker).

7.5 mg/ml BPE (Bovine Pituitary Extract) (CC-4002), 2ml

0.5 mg/ml Hydrocortisone (CC-4031), 0.5 ml

0.5 μg/ml hEGF (human recombinant epidermal Growth Factor) (CC-4230), 0.5 ml

0.5 mg/ml Epinephrine (CC-4221), 0.5 ml

10 mg/ml Transferrin (CC-4205), 0.5 ml

5 mg/ml Insulin (CC-4021), 0.5 ml

0.1 μg/ml Retinoic Acid (CC-4085), 0.5 ml

6.5 μg/ml Triiodothyronine (CC-4211), 0.5 ml

50 mg/ml Gentamicin, 50 μg/ml Amphotericin-B (CC-4081), 0.5 ml

50 mg/ml BSA-FAF (Bovine Serum Albumin-Fatty Acid Free) (CC-4162), 5 ml

PROPIDIUM IODIDE STAINING OF DNA

Permeabilisation buffer

2% FBS

2 mM EDTA pH8.0

0.1% w/v saponin

Blocking Buffer

10% goat serum

1% BSA

0.02% NaN_3

LIST OF SUPPLIERS

- Affymetrix UK Ltd. Voyager, Mercury Park, Wycombe Lane, Wooburn Green, High Wycombe HP10 0HH, UK.
- Aldevron, 3233 15th St S Fargo, ND 58104, USA.
- Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.
- Applied Biosystems, Kelvin Close, Birchwood Science Park North, Warrington, WA3 7PB, UK.
- BDH Laboratory Supplies, Poole, Dorset. BH15 1TD, UK.

- Bio-Rad Laboratories Ltd. Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, UK.
- BioWhittaker UK Ltd., BioWhittaker House, 1 Ashville Way, Wokingham, Berkshire. RG41 2PL, UK.
- Calbiochem-Novabiochem UK Ltd. Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR, UK.
- Constant Systems Ltd, Low March, Daventry, Northants. NN11 4SD, UK.
- Costar UK Ltd, 1 The Valley Centre, Gordon Road, High Wycombe, Buckinghamshire, HP13 6EQ, UK.
- DuPont Chemoswed, P.O. Box 839, SE-201 80 Malmö, Sweden.
- Dynatech Laboratories, Ltd., Billingshurt, UK.
- E & O Laboratories, Burnhouse, Bonnybridge, Stirlingshire, FK4 2HH, UK.
- Gibco Invitrogen (Life Technologies) Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF. UK.
- Greiner Bio-One Ltd. Brunel Way, Stroudwater Business Park, Stonehouse, Glos. GL10 3SX, UK.
- Hybaid, Action Ct. Ashford Rd. Ashford, Middlesex, TW15 1XB, UK.
- JASCO Ltd, 18 Oak Industrial Park, Chelmsford Road, Great Dunmow, CM6 1XN, UK.
- Kodak Ltd, Liverpool, UK.
- Melford Laboratories Ltd, Suffolk, UK.
- Menzel-Glaser, Germany.
- Millipore (U.K.) Limited, Units 3&5, The Courtyards, Hatters Lane, Watford, WD18 8YH, UK.
- New England Biolabs, 73 Knowl Piece, Wilbury Way, Hitchin, Herts, SG4 0TY, UK.
- Novagen Inc., 601 Science Drive, Madison, WI 53711, USA.
- Oxoid, Wade, Hants, UK.
- Promega, Southampton, UK.
- Qiagen UK Ltd, Crawley, West Sussex, UK.
- Shandon, Pittsburgh, PA, USA.
- Sigma-Aldrich, Dorset, UK.

- Stratagene, La Jolla, USA.
- Vector Laboratories Inc. Burlingame. CA 94010, USA.
- Zeiss, Woodfield Road, Welwyn Garden City, Herts. AL7 1LU, UK.

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