STUDIES ON BACTERIAL LUNG INFECTIONS IN CYSTIC FIBROSIS

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A ma maman, Aicha (merci pour tout) et
ma grand-mère Breuleux.

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ABBREVIATIONS

API	Analytical profile index
ARDS	Acute respiratory distress syndrome
BPI	Bacterial permeability-increasing protein
CAP	Cationic antimicrobial protein
CDM	Chemically defined medium
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
DAG	Diacylglycerol
DHA	Docosa-hexaenoic acid
DMSO	Dimethyl sulphoxide
DTA	Ethylenediaminetetraacetetate
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbant assay
ENaC	Epithelial sodium channel
GPI	Glycosyl phosphatidylinositol
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
ICAM	Intercellular adhesion molecule
ICE	Interleukin-1β converting enzyme
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGIF	Interferon-γ-inducing factor
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
Kb	Kilobase
kDa	Kilodalton
KDO	2-keto-3-deoxyoctulosonic acid
LBP	LPS binding protein
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MDP	Muramyl dipeptide
MH	Muller Hinton
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NC	Nitrocellulose
NF	Nuclear factor
NMR	Nuclear magnetic resonance
NO	Nitric oxyde
NOS	NO synthase
OM	Outer membrane
OMP	Outer membrane protein
opz	Opsonised zymosan
PCT	Procalcitonin
PG	Prostaglandin
PKC	Protein kinase C
PLA	Pro-inflammatory phospholipase A

PLC	Phospholipase C
PM	Plasma membrane
PMA	Phorbol myristate acetate
PMB	Polymyxin B
PMN	Polymorphonuclear
ppm	Parts per million
Rf	Relative front
RLU	Relative light unit
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
spp	Species
TBS	Tris buffered saline
TGF	Tumour growth factor
TK	Tyrosine kinase
TLC	Thin layer chromatography
TNF R1-R2	TNF-α receptor antagonist
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
TTBS	Tween-Tris buffered saline
v/v	Volume for volume
VCAM	Vascular cell adhesion molecule
w/v	Weight for volume

CHAPTER 1. INTRODUCTION

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is the commonest lethal inherited disease in Northern Europe. It affects 1 in 2500 live births in Britain. There is a frequency of 1 in 25 carriers of the CF recessive gene in the white population, the consequence being 350 children born with CF each year (Elborn *et al.*, 1994). 'Cystic fibrosis of the pancreas' was first described by Anderson in 1938 as pancreatic lesions. Abnormally high levels of sodium and chloride in the sweat of CF children were then observed by Di Sant'Agnese *et al.* (1953). The diagnostic test for CF based on the measurement of increased sweat electrolytes levels was developed in 1959 by Gibson and Cooke.

In CF patients, a malfunction of several organs including the lungs, the gastrointestinal tract, the pancreas, the liver, the vas deferens and the sweat glands, was found to be due to defective chloride transport across epithelia (Quinton, 1983). In the lungs the modified chloride transport causes a decrease of chloride secretion in the lumen and an increase of the resorption of sodium. Therefore the flux of water into the lumen is reduced. The mucus is dehydrated and gets thick, consequently the mucocilia are unable to clean the lungs effectively. Bacteria are then able to colonise the bronchopulmonary tract. In the gastrointestinal tract blockages named 'meconium ileus' can occur. Most CF patients have poor pancreatic function as a result of viscid secretions which block the pancreatic ducts preventing the release of enzymes (Kubesch *et al.*, 1993). Blockage in the vas deferens leads to male infertility and damage to the liver provokes cirrhosis (FitzSimmons, 1993).

1.1.1 Genetics of CF

The CF gene is found on the long arm of chromosome 7. It is 250 kb long, contains 27 exons and codes for the CF transmembrane conductance regulator protein, CFTR (Rommens *et al.*, 1989). The CFTR protein is considered to have a cell membrane transport function (Riordan *et al.*, 1989). It is thought to function as the main cellular chloride channel (Bear *et al.*, 1992). The CFTR protein has been detected in the following organs: respiratory tract, gastrointestinal tract, reproductive tract, kidney and sweat glands (Colins, 1992). More than 200 disease-causing mutations have been identified in the CFTR gene but one, ΔF508, occurs in over 70 % of cases. This mutation involves the deletion of three bases (CTT) from exon 10 of chromosome 7, resulting in the loss of a phenylalanine residue at position 508 of the 1480 amino acid CFTR protein.

1.1.2 Role of the CFTR Mutation in Lung Disease

There are two hypotheses on the role of the CFTR mutation in the high susceptibility of lung infection in CF. The first one, described by Matsui *et al.* in 1998, focuses on the dehydrated mucus in the CF airways. CFTR regulates other ion channels including the epithelial sodium channel, ENaC (Stutts *et al.*, 1997). CFTR decreases the number of times the ENaC is open. In CF, Na^+ absorption is increased because there is no inhibition of the ENaC by the defective CFTR. Chloride ions are drawn out of the airway surface fluid following the flow of sodium ions, then the water follows by osmosis, leaving dehydrated mucus. The second hypothesis is based on the theory that normal lungs have low concentrations of Na^+ and Cl^- and therefore allow salt-sensitive antimicrobial

agents to keep the airways free from bacteria. In the CF lungs, the defective CFTR channel stops chloride from following the sodium into the epithelium. High levels of salt in the airway surface fluid inactivates antibiotics (Smith *et al.*, 1996; Zabner *et al.*, 1998).

The chloride channels could influence fatty acid production. There is a two-fold increase in phospholipid-bound arachidonic acid and phospholipid-bound docosa-hexaenoic acid (DHA) in cells from CF mice. As a result, there is a higher level of arachidonic acid through activation of prostaglandin (PG). Prostaglandin can increase inflammation and mucus production (Fricker, 2000).

Normal tracheal epithelial cells express antimicrobial peptides which kill *P. aeruginosa,* this expression is impaired in CFTR defective cells (Ko *et al.*, 1997).

1.1.3 Lung Infections in CF

Lungs of CF babies are structurally normal at birth but following infections high levels of mucus are secreted (Girod *et al.*, 1992). Pulmonary infection in CF involves only a few species of bacteria, which colonise with a common chronology: *Staphylococcus aureus* in infancy, *Haemophilus influenzae* in the early years, *Pseudomonas aeruginosa* in early teens, *Burkholderia cepacia* and mycobacteria in the late teens (Govan and Nelson, 1992). The nontuberculous mycobacteria are often not cultured because they are overgrown by *P. aeruginosa* or killed in the bacterial decontamination process used to recover them (Bange *et al.*, 1999). Infections are localised in the major and minor airways rather than in the alveoli. Localised infections at non-pulmonary sites or systemic infections are rare. Viruses like RSV and fungi like *Aspergillus* spp. are also found in the lungs

of CF patients. In this study attention is concentrated upon *P. aeruginosa* and *B. cepacia* which are difficult to treat with antibiotics and are ultimately responsible for the death of CF patients.

1.1.3.1 *Staphylococcus aureus*

Before the use of antibiotics a *S. aureus* infection would be lethal for the CF child. Nowadays *S. aureus* infections are treated with oral flucloxacillin with or without fusidic acid, or in the case of severe infections with vancomycin. *S. aureus* causes tissue damage which primes the lungs for colonisation by *H. influenzae* and *P. aeruginosa* (Littlewood *et al.*, 2000a). *S. aureus* produces teichoic acid and exopolysaccharides which enable the bacteria to adhere to the respiratory epithelium in the CF lungs.

1.1.3.2 *Haemophilus influenzae*

H. influenzae is an intermittent and important pathogen in causing exacerbation. It may be treated with amoxycillin but 15 % of strains are resistant. Cephalosporins or tetracycline could alternatively be used. New macrolides like azithromycin are effective against *H. influenzae* and they achieve high tissue concentrations (Littlewood *et al.*, 2000a).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is non-pathogenic to the normal respiratory tract. *P. aeruginosa* has, over the last decades, become the most frequent pathogen in the lower respiratory tract of patients with CF. Chronic *P. aeruginosa* infection is now responsible for the large majority of excess morbidity and mortality in these patients since, once established, the organism cannot be eradicated from the lower respiratory tract (Koch and Høiby, 1993). Chronic *P. aeruginosa* infection is, however, preceded by a period of intermittent colonisation which averages 12 months (Johansen and Høiby, 1992). In CF lungs, colonisation by *P. aeruginosa* is a poor prognostic indicator. Initial colonisation could be due to the ability of *P. aeruginosa* to adhere to buccal cells, its presence in the gastrointestinal tract cannot be considered as a reservoir for lung colonisation. Colonisation of the airways is usually with non-mucoid strains which go through phenotypic adaptation once in the lung (Govan and Nelson, 1992). Primary colonisation with mucoid strains has been observed, also colonisation with more than one strain (Doggett, 1969). But there is evidence from genotyping that strains obtained from sputum or throat swabs might not to be representative of all strains actually in the lungs (Smith *et al.*, 1998) and problems in growing or recognising species occur in some clinics (Shreve *et al.*, 1998).

1.2.1 *Pseudomonas aeruginosa* Transmission and Cross-Infection

The appearance of *Pseudomonas* in the 1960s was related to the creation of centre-based care. In Denmark, it has been observed that 57 % of patients in centres were colonised whereas 27 % of patients were colonised in non-centre treated conditions (Høiby and Pedersen, 1989). A number of epidemiological

studies using different typing methods for *P. aeruginosa* suggest that a significant proportion of transmission of *P. aeruginosa* to CF patients is due to a direct patient-to-patient contact which is enhanced by the urban localisation (Farrell *et al.*, 1997). In addition, transmission of *P. aeruginosa* to CF patients may occur via contaminated environmental reservoirs by direct hand contact as well as by aerosols (Döring *et al.*, 1991).

1.2.2 Surface Antigens of *Pseudomonas aeruginosa* in CF

The outer membrane of *P. aeruginosa* is an important determinant of resistance to antibiotics and host defences. Studies on its structure will therefore help in the design of more effective therapy and might also be exploited in the diagnosis of colonisation.

The outer membrane (OM) protein profile of *P. aeruginosa* has been characterised by Hancock *et al.* (1990). It contains the following proteins:

- OprC and OprE, general porins (Yoshihara and Nakae, 1989)
- Esterase (Ohkawa, 1979)
- OprP, a phosphate porin induced in low phosphate medium (Hancock, 1982)
- OprB (or protein D1), a porin for glucose and xylose (Hancock and Carey, 1980; Trias, 1988)
- OprD (or protein D2), a substrate-selective porin containing a specific binding site for amino acids such as lysine also which is used by the antibiotic imipenem (Trias and Nikaido, 1990)
- OprF porin, a general porin, loss of which could be related to antibiotic resistance (Hancock, 1986)

- OprG, expression depends on the growth conditions, it could have a role in fluoroquinolone uptake (Chamberland *et al.*, 1989) or in low-affinity iron uptake (Yates *et al.*, 1989)
- OprH (or Protein H1), overexpressed in low Mg^{2+}, Ca^{2+}, Mn^{2+} and Sr^{2+} media or under all growth conditions in polymyxin-resistant strains
- OprL (or Protein H2), a peptidoglycan-associated lipoprotein which anchors the outer membrane to the peptidoglycan layer in the cell wall
- OprI, a lipoprotein, also involved in attaching the outer membrane to the peptidoglycan.

LPS (lipopolysaccharide), is an amphiphilic macromolecule located on the surface of the OM. Lipid A is the lipid component of LPS, it constitutes its toxic and immunostimulatory component leading to fever, vascular leakage, myocardial depression and shock (Rietschel *et al.*, 1984).

1.2.3 Mucoid Strains

Most *P. aeruginosa* stains are able to produce the exopolysaccharide, alginate. Expression of alginate is responsible for the mucoid phenotype which is frequently encountered in *P. aeruginosa* isolates from CF. In response to a change of environment the non-mucoid strain derepresses the *alg* genes which would normally allow the production of low levels of alginate (Flynn and Ohman, 1988). Alginate is a strongly anionic polysaccharide composed of linear 1-4 linked β-D-mannuronic acid and its C5 epimer, α-L-guluronic acid (Linker and Jones, 1966). This exopolysaccharide binds water and divalent cations to form a flexible gel. The CF lungs have high concentrations of calcium which induce alginate gel formation and enable the formation of microcolonies which

can measure up to 60 μm. The emergence of mucoid strains correlates with a poor patient outlook (Pedersen *et al.*, 1992). Alginate has an important role in the pathogenesis of the respiratory tract in CF. The biofilm prevents the phagocytosis of bacteria because it masks complement on the bacterial surface and *Pseudomonas* OM antigens from antibodies (Stiver *et al.*, 1988). Antibodies against alginate cannot mediate phagocytosis of *P. aeruginosa,* they lack opsonic killing activity (Pier, 1998). Biofilm stimulates the release of oxygen radicals from polymorphonuclear (PMN) leukocytes which results in greater lung damage (Hutchison and Govan, 1999). Alginate scavenges reactive oxygen intermediates and hypochlorite generated by phagocytic cells (Learn *et al.*, 1987; Simpson *et al.*, 1989).

1.2.4 Role of *Pseudomonas aeruginosa* in Inflammation

Because of the defective mucociliary clearance in CF patients, *P. aeruginosa* adheres to tracheal cells and mucin (the major component of mucus). A number of cell surface adhesins, lectins and exoenzyme-S are associated with the cell's lipopolysaccharide (LPS) (Irvin *et al.*, 1989). This range of adhesive mechanisms helps to explain how *P. aeruginosa* can persist in the lungs and induce an immune response (Elborn and Shale, 1990) with production of specific antibodies. In CF these antibodies seem to have a low avidity and a low opsonophagocytic capacity which could explain the progressive destruction of the lungs (Ciofu *et al.*, 1999). The normal lung cells internalise *P. aeruginosa* through CFTR which acts as a receptor for the ingestion of *P. aeruginosa* only. This is a critical event in the clearance of the airway from early *P. aeruginosa* infection (Pier, 1998). The immune-mediated inflammation of the

bronchopulmonary tract leads to tissue damage and loss of lung function (Høiby *et al.*, 1987).

1.2.5 Treatment of *Pseudomonas* Infection in CF Patients

The more effective treatment of *P. aeruginosa* infection has been central to the increased survival of CF patients. Nebulised Colomycin® (colistin E), used both to eradicate early *P. aeruginosa* infection, either alone (Littlewood *et al.*, 1985) or with oral ciprofloxacin (Valerius *et al.*, 1991; Frederiksen *et al.*, 1997) and to stabilise chronic infection (Jensen *et al.*, 1987), has been a central component of the modern treatment package in most European clinics. The improved condition and survival of people with CF has undoubtedly paralleled the increased use of antibiotics and improved nutrition achieved by aggressive nutritional support and the more effective acid resistant enzymes. It is important to stress that all these improvements have been introduced and evaluated at CF centres where the staff have the opportunity of treating large numbers of patients.

1.2.5.1 Role of Colomycin® in CF

Colomycin® is an interesting drug in the context of CF, having been resurrected in the 1980s as *P. aeruginosa* proved an increasing problem in children with CF. Many were in excellent condition and without chronic infection, *Staphylococcus aureus* having been successfully avoided or treated (Weaver *et al.*, 1994), only to deteriorate following the onset of *P. aeruginosa* infection. The drug's activity against *P. aeruginosa*, the very rare occurrence of resistance and the ability to deliver it by nebuliser resulted in its introduction and increasing use both to eradicate early *P. aeruginosa* and to stabilise those who had acquired chronic

infection. The problems of resistance to other anti-pseudomonal antibiotics for the treatment of exacerbations has led to an increasing use of Colomycin® by the intravenous route (Conway *et al.*, 1997; Ledson *et al.*, 1998).

A combination of ciprofloxacin, trimethoprim and either sulphodiazine or sulphamethoxazole was suggested for oral treatment of bacterial infection in CF patients. This treatment could stop the use of intravenous injections and reduce the appearance of resistance (Richards *et al.*, 1998).

1.3 *Burkholderia cepacia* Infection in CF

B. cepacia is a Gram-negative, motile, aerobic rod first described by Burkholder (1949) as a phytopathogen causing soft rot of onion bulb. It is an opportunistic pathogen which colonises the lungs of approximately 10 % of CF patients (Sajjan *et al.*, 1992). Widely different clinical responses to colonisation have been observed, ranging from no effect on clinical status to development of necrotising pneumonia with a progression to respiratory failure and rapid death (referred to as *B. cepacia* syndrome). Colonisation of the lungs by *B. cepacia* increases in the presence of *P. aeruginosa* whose exoproducts modify the cell surface (Saiman and Prince, 1993) there is also potential for interspecies signalling between *P. aeruginosa* and *B. cepacia* in the CF lung (McKenney *et al.*, 1995). *B. cepacia* binds to CF mucus glycoproteins via its pili (Sajjan *et al.*, 1995). There could be another adhesion receptor which is a lipid (Sylvester *et al.*, 1996). There are five different types of pili distinguished by their characteristic morphology (Goldstein *et al.*, 1995). This variety in pilus structure could explain how *B. cepacia* can colonise the unusual environment of the lungs and why a high adhesion to mucin is correlated with a poor prognosis for CF patients (Sajjan and Forstner, 1992). In contrast to *P. aeruginosa,* isolation of mucoid forms of *B. cepacia* from CF

patients is rare (Govan and Deretic, 1996). It has been shown that only genomic typing is a good way of finding out the origin of an epidemic of *B. cepacia* (Ouchi *et al.*, 1995). ET12 (or CF5610) is the most highly transmissible strain associated with colonisation and death in CF patients (Govan and Deretic, 1996; Govan *et al.*, 1993).

1.3.1 Transmission of *Burkholderia cepacia*

The patient-to-patient transmission in hospitals or through social contact has increased the number of colonised patients (Walters and Smith, 1993). There have also been reports of transmission between non-CF and CF patients (Holmes *et al.*, 1999). Segregation policies have been effective in reducing the rate of transmission between CF patients.

1.3.2 Surface Antigens of *Burkholderia cepacia*

Parr *et al.* (1987) and Gotoh *et al.* (1994) have described the following outer membrane proteins in *B. cepacia*:

- 81 kDa, a porin
- 40 kDa, a porin
- 36 kDa
- 24.5 kDa
- 17 kDa
- 14.5 kDa

B. cepacia strains isolated from the CF lungs generally produce rough LPS rather than smooth LPS associated with environmental isolates (Govan, *et al.*, 1993). Rough LPS is typically sensitive to the bactericidal activity of serum and is usually less virulent (Pier and Ames, 1984) than smooth LPS. An interesting problem arises: how does *B. cepacia* survive and cause bacteraemia in the CF lungs?

1.3.3 Role of *Burkholderia cepacia* in Inflammation

The 36 kDa and 24.5 kDa outer membrane proteins are heat-modifiable proteins (Anwar *et al.*, 1983) and are subunits from which the 81 kDa protein is constructed (Parr *et al.*, 1987). These OM proteins are antigenic and a specific immunological response can be observed. Different antibodies are directed against the 36 and 24.5 kDa proteins and the 81 kDa protein. When patient serum

was pre-absorbed with purified LPS, the immunological response did not decrease (Lacy *et al.*, 1997). Antibodies prevent progression of the disease but not colonisation (Burnie *et al.*, 1995). OM proteins could therefore be used in the detection of *B. cepacia* colonisation, but IgG and IgA antibodies to *B. cepacia* OM cross-react with those of *P. aeruginosa* (Aronoff *et al.*, 1991). LPS could also be used for the early detection for *B. cepacia* especially because there is no cross-reaction with *P. aeruginosa* LPS (Nelson *et al.*, 1993). Despite the rise in IgA and IgG antibodies against LPS in *B. cepacia* colonised patients, these antibodies do not protect the pulmonary milieu of CF children from infection.

1.3.3.1 Antimicrobial Resistance

The increased number of patients colonised with *B. cepacia* could be explained by multi-drug resistance of the bacterium. For example, penicillin G can be utilised as a substrate (Beckman and Lessie, 1979) and the *B. cepacia* outer membrane is 10 times less permeable than that of *E. coli* (Parr *et al.*, 1987). Multiple antibiotic resistance (trimethoprim, ciprofloxacin and chloramphenicol) could be due to an outer membrane lipoprotein which enables the efflux of antibiotics (Burns *et al.*, 1996) but further studies have shown that alteration in both porins and LPS structure is needed (Rajyaguru and Muszynski, 1997). Resistance to aminoglycosides and polypeptide antibiotics like colistin and polymyxin B could be due to the LPS structure. High levels of 4-amino-4-deoxyarabinose form ion pairs with negatively charged phosphate molecules present in adjacent LPS (Cox and Wilkinson, 1991; Vaara, 1992). The *B. cepacia* LPS has low levels of 2-keto-3-deoxyoctulosonic acid (KDO) and phosphate compared to other Gram-negative bacteria and this could explain polycationic antibiotic resistance. It has recently been reported that lactoferrin reduces the

MICs of doxycycline for *B. cepacia in vitro* (Alkawash *et al.*, 1999) but further clinical trials are needed to determine whether this observation has clinical significance.

1.3.3.2 Resistance in the CF Lung

It has been reported that, even if *B. cepacia* is susceptible to antibiotics *in vitro*, there is no reduction of bacterial load in sputum (Nelson *et al.*, 1993). These observations lead to the hypothesis that *B. cepacia* strains have adapted to the role of human intracellular pathogens, living in epithelial cells and phagocytes (Burns and Clark, 1992). The CF lung may have a different gas composition, with an increased carbon dioxide concentration and a lower oxygen concentration. Susceptibility of *B. cepacia* to β-lactam antibiotics is reduced when incubated in an atmosphere of 5 % carbon dioxide (Corkill *et al.*, 1994). Susceptibility of *B. cepacia* to ciprofloxacin and tobramycin increased in conditions of oxygen depletion (McKenney and Allison, 1997). Growth in nutrient depleted conditions has also been shown to increase resistance to antimicrobial agents (Cozens and Brown, 1983).

1.3.3.3 Virulence Determinants of *Burkholderia cepacia*

The pathogenicity of *B. cepacia* is relatively unclear but several putative virulence factors of *B. cepacia* have been identified. However their relevance to colonisation and damage to the CF lungs has not yet been fully described. Isolates from CF patients more frequently produce: catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginase and trypsin. They

also reduce nitrate to nitrite, hydrolyse urea and xanthine and lyse bovine red blood cells (Gessner and Mortensen, 1990).

1.3.3.3.1 Protease

B. cepacia secretes two proteases, a 32 kDa enzyme with antigenic similarity to *P. aeruginosa* elastase and a 40 kDa protease with much lower activity and only produced by some strains of *B. cepacia* (Kooi and Sokol, 1996). The 32 kDa protease cleaves gelatine, hide powder and collagen but not human immunoglobulins. In rat lungs purified proteinase causes bronchopneumonia, characterised by polymorphonuclear cell infiltration and proteinaceous exudation. Immunisation of rats with this proteinase was not protective against subsequent lung infection with *B. cepacia* (McKevitt *et al.*, 1989).

1.3.3.3.2 Lipase

Phospholipase C (PLC) has been associated with lung cytopathology because it cleaves phophotidylcholine, a major lung surfactant. PLC activity amongst strains of *B. cepacia* can vary from 40 % to 70 % (Vasil *et al.*, 1990; Nakazawa *et al.*, 1987). However, unlike *P. aeruginosa*, the level of PLC activity of *B. cepacia* does not correlate with haemolytic activity but is associated with haemolytic activity in some strains of *B. cepacia.*

1.3.3.3.3 Haemolysin

Recently a new haemolysin has been isolated from the highly transmissible ET12 strain of *B. cepacia* (Hutchison *et al.*, 1998). This lipopeptide toxin produced

pores in human red blood cells of less than 20 nm, and at low levels, apoptosis in neutrophils. High levels of haemolysin resulted in the release of both cathepsin G and elastase from human neutrophils. It is possible that this toxin contributes to the severe inflammatory response due to *B. cepacia.*

1.4 LPS

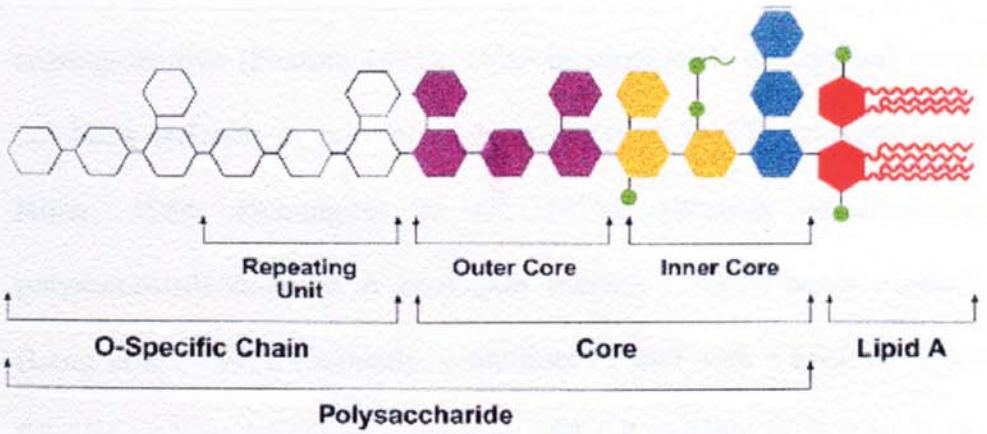
LPS was discovered at the end of the nineteenth century by Richard Pfeiffer (Westphal *et al.*, 1977). It was described as a heat stable and cell-associated amphiphilic macromolecule, located on the bacterial cell surface (Rietschel and Brade, 1992). LPS is released, free or complexed with protein, when bacteria multiply or die (Freudenberg *et al.*, 1991).

1.4.1 Structure of LPS

On the surface of Gram-negative bacteria is located a polysaccharide chain covalently bound to a lipid A. Lipid A is the endotoxic principle of LPS (Galanos and Freudenberg, 1993) it is the only lipid found on the outer leaflet of the OM and is responsible for the permeability of hydrophobic molecules (Labischinski *et al.*, 1985; Kropinski *et al.*, 1982). Lipid A can be cleaved at the KDO 1 residue of the inner core by mild acid. The polysaccharide portion constitutes of a repeated oligosaccharide chain, the O-specific chain linked to the core oligosaccharide (Fig 1.1).

A gene cluster, *rfb* is responsible for synthesis of the O-specific chain which is characteristic of a strain of bacteria. The O-specific chain enables the enterobacteria to survive *in vivo*, it makes the bacteria resistant to phagocytosis and killing by complement (Poxton, 1995). The outer core is less variable and the

inner core of all bacteria contains KDO. For example in *P. aeruginosa* the inner core has an L-glycero-D-manno-heptose which carries a carbamoyl residue in position 7 (Beckmann *et al.*, 1995).



Phosphate Monosaccharide Ethanolamine

Long chain (hydroxyl) fatty acid

Figure 1.1 Bacterial Lipopolysaccharide (LPS)

1.4.2 LPS Antigens as Markers of Colonisation

LPS being the virulent factor of bacteria, its presence in serum has been studied in patients with sepsis. The conclusion was that LPS levels in the blood could not be used as a diagnostic for sepsis (Silverman and Ostro, 1999). There are a great number of different monoclonal antibodies directed against the LPS molecule (Andersen *et al.*, 1996). High levels of antibodies to endotoxins are a good prognostic indicator in sepsis patients but not in CF because more inflammation is produced. Therefore the

antibodies produced in serum were IgG to the core region and the O antigen of LPS.

Immunisation against LPS could be of great advantage. The different studies have not been successful yet. Various problems occurred, for example the antibody produced had limited ability to bind to native LPS. Also it has been impossible to find an antibody which binds to all LPS molecules and which is cross-protective (Poxton, 1995). Also in some trials the applied vaccines have not been efficient in preventing lung infection in CF patients (Langford and Hiller, 1984; Pennington *et al.*, 1975), although administration of a polysaccharide-exotoxin A conjugate vaccine revealed some clinical efficacy (Lang *et al.*, 1995). Currently, a multicentre trial with a bivalent *P. aeruginosa* flagella vaccine including more than 500 CF patients in Europe is in progress (Döring and Dorner, 1997).

1.5 Inflammation

LPS of Gram-negative bacteria is the primary stimulus for the host response. In CF lungs chronically colonised with *P. aeruginosa,* there is a continuous stimulation of the immune system by LPS. This eventually leads to the patient's death.

1.5.1 Role of LPS from *Burkholderia cepacia* in Inflammation

LPS from *B. cepacia* is not responsible for transmission or colonisation of the CF lungs (Evans *et al.*, 1999) but, like LPS from *P. aeruginosa,* it plays a major role in inflammation. LPS from *B. gladioli,* a closely related phytopathogen, shows an endotoxic response and cytokine stimulation greater than that from *E. coli* and

P. aeruginosa (Shaw *et al.*, 1995). Moreover Zughaier *et al.* (1999) showed that, on a weight for weight basis, *B. cepacia* LPS had the same ability to stimulate TNF-α from a human monocyte cell line via a CD14 pathway as *E. coli* LPS, but the activity was four to eightfold greater than LPS from *P. aeruginosa* and *Stenotrophomonas maltophilia.* The cytokine response contributes to the processes that cause lung damage associated with *P. aeruginosa* (Greally *et al.*, 1993), this could explain the rapid deterioration in health of CF patients when colonised with *B. cepacia.* Increased expression of complement receptor 3, important for neutrophil transmigration into alveolar spaces, and priming of neutrophil respiratory burst activity, was observed for both clinical and environmental isolates of *B. cepacia* (Hughes *et al.*, 1997). LPS from the strain ET12 has been shown to upregulate the production of inducible nitric oxide synthase and inflammatory cytokine mRNA in CF and non-CF leukocytes (Hutchison *et al.*, 1998). Other *B. cepacia* exoproducts have also been shown to induce IL-8 release from epithelial cells and human monocytes (Palfreyman *et al.*, 1997).

1.5.2 Humoral Binding Proteins

HDL (high density lipoprotein) (Flegel *et al.*, 1989) and BPI (bactericidal permeability-increasing protein), which is found at a concentration of 1 ng/ml in the serum of normal individuals, attenuates the effects of LPS (Weiss *et al.*, 1992). Cationic proteins like CAP 18 (Sawa *et al.*, 1998), CAP 37, P15A/ P15B, (Hirata *et al.*, 1994), albumin, transferrin, lactoferrin, haemoglobin, lysozyme and a 28 kDa mannose-binding protein associate with LPS but do not change the endotoxic activity (Morrison *et al.*, 1998).

LBP (LPS binding protein) increases the endotoxin activity, even femto- or picogrammes of LPS become active when bound to LBP (Tobias *et al.*, 1988). Despite showing 45 % homology of amino acid sequence, BPI and LBP have opposite effects on LPS activity. LBP catalyses the transfer of LPS aggregates to other LPS binding proteins like CD14. The complex represents the main pathway by which cells are stimulated to respond to Gram-negative bacteria. In contrast, BPI contributes to the extracellular and intracellular antibacterial activity of polymorphonuclear leukocytes by altering bacterial membrane integrity (Tobias *et al.*, 1997). Upon the binding of BPI to lipid A, the bacteria stop dividing but the inhibitory effects are reversible. After longer incubation, irreversible damage to the cytoplasmic membrane occurs leading to cell death (Elsbach, 1994). BPI has a much higher affinity for LPS than LBP and blocks the interaction of LBP to LPS by global alterations of the LPS aggregates (Tobias *et al.*, 1997).

1.5.3 Effect of LPS on Cellular Targets

PMN phagocytose bacteria and bacterial membranes. They have enzymes which transform (de-O-acylate and dephosphorylate) LPS and lipid A into nontoxic material (Munford and Hall, 1989). Their cytoplasm also contains BPI, CAP 18 and CAP 37 which neutralise LPS. PMN can bind to endothelial cells and penetrate through vessel walls into tissue and cause inflammation. They also secrete cytokines which participate in the self-regulation and regulation of lymphocytes, monocytes and platelets (Labro, 1998). LPS causes polyclonal stimulation of proliferation, differentiation and secretion of antibodies by B-lymphocytes (Anderson *et al.*, 1973). In T-lymphocytes (Th1 type) LPS activates proliferation and secretion of lymphokines (Labro, 1998).

Monocytes and tissue macrophages are long-lived. They originate from blood monocytes that settle in tissue and differentiate into resident macrophages. Macrophages have the role of presenting antigens to the B and T lymphocytes. They also remove damaged cells form the body (Labro, 1998) and produce IL-1, IL-6, IL-8, macrophage migration-inhibitory factor (Bernhagen *et al.*, 1995) and TNF-α. These cytokines help to eliminate bacteria by causing fever, leukocytosis, attracting defence cells to the site of infection and initiating the acute-phase response (Warren and Chedid, 1987). Followingbphagocytosis of bacteria there is also production of reduced oxygen species (like superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide) and active metabolites of arachidonic acid (like prostaglandin, thromboxane and leukotrienes) and linoleic acid. Monocytes from normal and CF patients have the same superoxide anion production after LPS stimulation (Thomassen *et al.*, 1990).

Vascular cells (endothelial or smooth muscle cells) secrete IL-1, IL-6 and IL-8 (Schönbeck *et al.*, 1995) prostaglandin, nitric oxide (NO), platelet-activating factor, interferon and adhesion molecules. These latter are activated by LPS or IL-1 and TNF-α (Doherty *et al.*, 1989).

1.5.4 Induction of the Host Response

LPB is a 60 kDa acute-phase glycoprotein synthesised in the liver. It is present in the plasma and binds to LPS. At low concentrations of LPS it disaggregates LPS micelles and binds at a 1:1 ratio. It facilitates the transfer of LPS to CD14 but is not necessary for the binding of LPS to CD14 (Hailman *et al.*, 1994). The aggregate conformation plays a role in the activity of the lipid A, and the presence of negative charges on the lipid A is a prerequisite for biological activity

(Schromm *et al.*, 1998). CD14 is located on the membrane of monocytes and polymorphonuclear cells. It is a 55 kDa glycosyl phosphatidylinositol (GPI) anchored protein with no intracellular domain. There also is a soluble sCD14 (2-6 μg/ml in serum) which enables endothelial cells that do not express CD14 to respond to low levels of LPS. sCD14 has an LPS-neutralising action and modulates endotoxin activity in the plasma (Hiki *et al.*, 1999). In the case of high endotoxin concentrations, 0.1 to 10 μg/ml, it has been observed that cellular stimulation can be CD14-independent. LBP can transport other negatively charged lipids (Schromm *et al.*, 1996) and can insert them into the lipid matrix of the cell, and cause activation. The internalisation of LPS is not necessary for the activation of monocytes but is necessary for the clearance of LPS from the circulation. There is no correlation between internalisation and activation, internalisation can take up to 6 hours, but there is a correlation between the kinetics of internalisation and the LPS structure. The more hydrophobic the LPS, the faster the kinetics (Lentschat *et al.*, 1999). The LPS-CD14 complex enhances activation of tyrosine kinases like *ras* and *raf* and other mitogen-activated protein (MAP)-kinases which rapidly phosphorylate the tyrosine residues of some proteins. These phosphorylated proteins activate the cell and regulate the intracellular signalling pathways (Murphy *et al.*, 1998)(Fig 1.2). The second messenger inositol-1,4,5-triphosphate mobilises calcium from internal stores and diacylglycerol (DAG) stimulates protein kinase C (PKC). Both increases of free calcium levels and PKC activation play a role in cellular activation. For example, stimulation of PKC alone is sufficient to trigger PGE_2 production but not IL-1 production (Abu-Lawi and Sultzer, 1994). Intracellular calcium elevation alone does not induce cytokine gene expression and calcium levels do not seem to change greatly to LPS response (Ohmori and Hamilton, 1992). But later Watanabe showed that LPS enhances the transient increase of intracellular

calcium which plays a critical role in TNF-α mRNA expression (Watanabe *et al.*, 1996). After stimulation, levels of calcium must fall otherwise cell apoptosis would occur. PKC stimulates an active efflux of calcium through the plasma membrane and levels of intracellular calcium decrease. Calcium is released from the stores to maintain homeostasis (Kong *et al.*, 1993). Monocytes also have a calcium-binding receptor, an increase in extracellular calcium stimulates the release of IL-6 and could be involved in bone remodelling (Yamaguchi *et al.*, 1998).

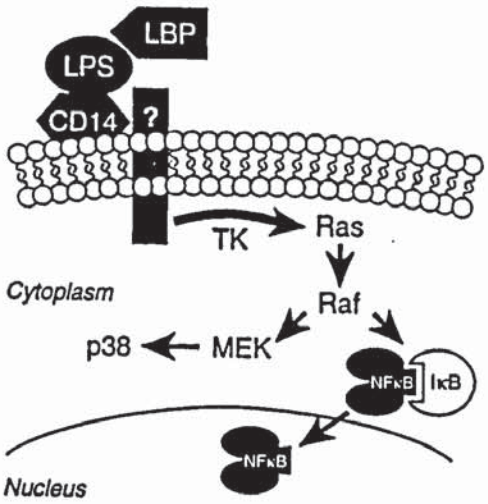


Figure 1.2 Activation of macrophages by LPS (Murphy *et al.*, 1998). Endotoxin (LPS) binds to the plasma protein lipopolysaccharide-binding protein (LBP), which then transfers LPS to the CD14 molecule on the cell surface. Intracellular signal transduction may occur via an unknown protein, initially through tyrosine kinase(s) (TK). MAP kinase/extracellular signal-regulated kinase (MEK); NFκB (nuclear factor κB); I κB (inhibitory κB)

1.5.5 Host Response

1.5.5.1 Oxidant Formation

NADPH oxidase is present in neutrophils, macrophages and monocytes. The degradation of oxygen leads to the production of superoxide anion radical $O_2^{\cdot-}$ There is also production of HOCl which is a powerful oxidant but is non-toxic. It is bactericidal and activates the precursors for metalloproteinases such as collagenase and gelatinase. The product of H_2O_2 and $O_2^{\cdot-}$, $HO^{\cdot}$ causes damage to the lung and induces the expression of the IL-1 and TNF-α genes.

NO is formed in epithelial cells, macrophages, neutrophils, mast cells, endothelial cells, autonomic neurons, smooth muscles cells and fibroblasts using

a constitutive enzyme, NOS (NO synthase) via a L-Arg-dependent pathway. NO induces airway and vascular smooth muscle cell relaxation. The latter results in enhanced blood flow and therefore delivery of leukocytes. NO has an important role in the killing of bacteria in the macrophages and even Gram-positive exotoxins stimulate the NO production and the killing of intracellular parasites (Cunha *et al.*, 1993). In the case of sepsis, there is a systemic vasodilatation that leads to shock.

1.5.5.2 Proteinases

Matrix metalloproteinases are produced by macrophages and neutrophils. Elastase can destroy the integrity of the alveolar wall through cleavage of elastin. Collagenase has a similar effect through breakdown of collagen type 1. Selectins and other adhesion molecule (ICAM-1 and VCAM-1) help the leukocytes to localise at the site of inflammation (Lukacs and Ward, 1996).

1.5.5.3 Cytokines

Cytokines allow cell-to-cell communication. TNF-α and IL-1β are the cytokines responsible in the early immune response. In the CF lungs chronically colonised by *P. aeruginosa*, the macrophages continuously produce pro-inflammatory cytokines like IL-1, IL-6, IL-8 and TNF-α. These cytokines gradually cause cachexia, hyperglobulinaemia and infiltration of the airways by neutrophils (Bonfield *et al.*, 1995). This leads to the destruction of the airways. IL-1β and TNF-α have been shown to stimulate the production of IL-1 and IL-8 by the epithelial cells (Sar *et al.*, 1999). Mast cells are also known to produce a wide range of cytokines (Möller *et al.*, 1998).

During the inflammatory response there also is a production of anti-inflammatory cytokines. They are soluble cytokine receptors: IL-1-receptor antagonist (IL-1RA) and TNF-α-receptor antagonists, TNF-R1 and TNF-R2. The IL-1RA could be a good marker of the chronic infection of CF lungs and high levels were detected in patients with poor pulmonary function (Kronborg *et al.*, 1993).

IL-8 is a chemotactic and activating factor for neutrophils, playing a role in the generation of dense neutrophil accumulation in the acute pneumonia and ARDS (acute respiratory distress syndrome).

β-lactam antibiotics could enhance the release of nitrite reductase from *P. aeruginosa*. *Pseudomonas* nitrite reductase is responsible for IL-8 production by bronchial epithelial cells (Sar *et al.*, 1999) which increase the inflammation.

IL-10 is a cytokine synthesis inhibitory factor, it decreases LPS-induced TNF-α and IL-1 synthesis and suppresses inflammation (Wolter *et al.*, 1999). It induces fever, production of endogenous adrenaline, noradrenaline and glucocorticoids. In CF patients the level of IL-10 decreases.

TNF-α was first described as inhibiting and killing tumour cells. Its gene is located on the short arm of chromosome 6. It is the first cytokine produced, it mediates the upregulation of vascular adhesion molecule and activates the inflammatory cytokine cascade. TNF-α stimulates the intracellular production of IL-1 but not its secretion (Bahl and Foreman, 1994).

TNF-α also induces a signal transduction pathway that leads to cellular apoptosis. This process eliminates infected and damaged cells without causing inflammation.

IL-1 is the second cytokine produced. There are two forms of IL-1, IL-1α and IL-1β, both agonists to IL-1R. The maximum secretion for IL-1β appears 2 hours

after LPS stimulation and the secretion of IL-1α 13 hours later (Bahl and Foreman, 1994).

IL-1β is produced in significant quantities, it is synthesised as a 31 kDa cytoplasmic precursor pIL-1β which must be cleaved at the Asp^{116}-Ala^{117} to give the active form, the 17 kDa mIL-1β. Cleavage is produced by an IL-1β-converting-enzyme (ICE) or caspase 1. ICE is found in its inactive p45 form, in the cytoplasm of stimulated and nonstimulated monocytes (Yamin *et al.*, 1996). The active form, made of an equimolar ratio of 10 kDa and 20 kDa proteins p10 and p20, (Ayala *et al.*, 1994) is located on the cell membrane. pIL-1β is processed through the ICE channel and the mature IL-1β and a propiece are secreted (Singer *et al.*, 1995)(Fig.1.3). LPS has been shown to enhance both the rate of transcription of IL-1β and the accumulation of IL-1β transcript but does not affect the level of caspase 1. Calcium ionophores enhance the rate of IL-1 release and also stimulate the processing of its precursors to their mature form (Suttles *et al.*, 1989). ICE also leads to cleavage and release of interferon-γ-inducing factor (IGIF, IL-18) (Schumann *et al.*, 1998).

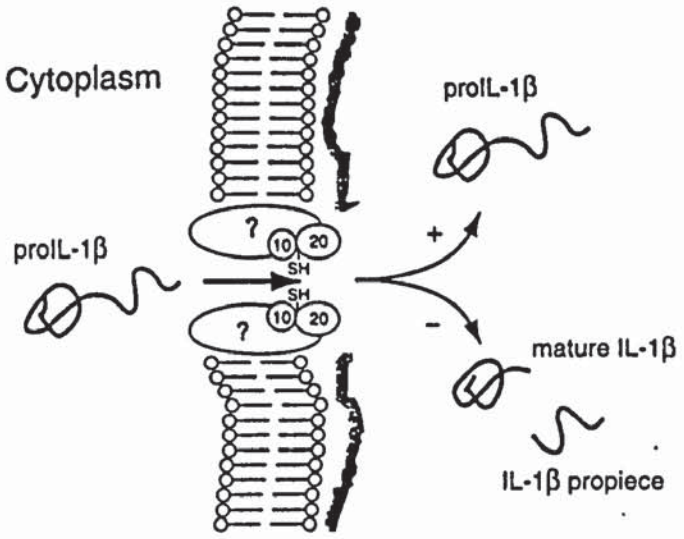


Figure 1.3 Secretion of IL-1β (Singer *et al.*, 1995)

At the site of inflammation, matrix metalloproteinases, stromelysin-1 (MMP-3), gelatinase A (MMP-2) and B (MMP-9) process pIL-1β into its active form, this could explain why chronically ill patients have only 0.1 to 10 ng/ml of IL-1β in their serum but up to 1 μg/ml of IL-1β at the site of inflammation (Schönbeck *et al.*, 1998). However MMP1, 2, 3 and 9 also play a negative control role by degrading IL-1β (Ito *et al.*, 1996).

1.5.5.4 Other Virulence Factors

P. aeruginosa produces other extracellular molecules which are more toxic than LPS, the exo-enzymes: exotoxin A, elastase and alkaline protease.

Exotoxin A suppresses the production of LPS-induced TNF-α by alveolar macrophages. TNF-α is known to increase host resistance to bacterial infection, therefore exotoxin A could aggravate the infection (Hirakata *et al.*, 1999).

1.5.5.5 Procalcitonin

Procalcitonin (PCT) is a 116 amino acid peptide produced in thyroid C cells. It is transformed into calcitonin by proteolysis. Calcitonin is a 32 amino-acid hormone involved in skeletal homeostasis. By reducing osteoclast activity it helps maintaining calcium stores in the body. Calcitonin also has effects in the kidney, the central nervous system, the respiratory and gastrointestinal tracts and the reproductive system.

It has recently been reported that PCT is a marker of severe infection (Al-Nawas and Shah, 1998; Monneret *et al.*, 1998). Healthy patients have a serum PCT concentration of less than 0.15 ng/ml, patients with a positive bacterial culture of any source have a serum concentration of 3.6 ± 1.3 ng/ml (Whang *et al.*, 1998). In Ugarte's study (1999) the cut off value for bacteraemia was 0.6 ng/ml. There is no augmentation of the serum concentration of calcitonin in infected patients (10.5 ± 3.8 pg/ml). During severe infection PCT is not only produced by the thyroid but also by mononuclear cells (Oberhoffer *et al.*, 1999) and by other tissues not yet identified (Assicot *et al.*, 1993). These other tissues might lack the enzymes that transform PCT into calcitonin, this could explain why the level of calcitonin does not change during infection. LPS is a potent inducer of PCT, as well as TNF-α, IL-1β, IL-2 and IL-6 (Oberhoffer *et al.*, 1999). LPS has been shown to trigger initial PCT synthesis and has greater effects on PCT release than the cytokines. The anti-inflammatory cytokine IL-10 has no effect on PCT levels. It has also been reported that a high serum level of PCT can help differentiate between acute bacterial meningitis and viral meningitis (Viallon *et al.*, 1999). PCT seems to be a good marker of bacterial infection (Gendrel and Bohuon, 1997) and of the severity of the infection (Ugarte *et al.*, 1999). It might be useful in the detection of bacterial infection of the lungs in CF patients.

The weight-reducing hormone leptin has also been investigated because of the anorexia usually present during sepsis. Levels of leptin did not increase during acute-phase response to LPS (Bornstein *et al.*, 1998).

1.6 Polymyxin

The polymyxins are a group of lipopeptide antibiotics isolated from the spore-forming soil bacterium, *Bacillus polymyxa* (Koyama *et al.*, 1950). They are bactericidal at low concentrations to many Gram-negative bacteria, including *P. aeruginosa* (Barnett *et al.*, 1964). Five major, chemically distinct members of the group were recognised and designated as polymyxin A, B, C, D and E. Colistin (Colomycin®) was isolated in 1950 from a related strain, *Bacillus colistinus*. It is identical to polymyxin E, and has the characteristic polymyxin structure comprising a cyclic heptapeptide joined to a tripeptide chain with a single fatty acid substituent. For parenteral and aerosol therapy colistin is used in the form of the sodium salt of the negatively–charged derivative, colistin sulphomethate, which is hydrolysed to the active, positively charged colistin base after administration, releasing formaldehyde and bisulphite (Barnett *et al.*, 1964).

1.6.1 Structure of Polymyxin

Polymyxin B (PMB) is a decapeptide characterised by a heptapeptide ring containing four 2,4-diaminobutyric acids. An additional peptide chain covalently bound to the γ-amino group carries an aliphatic chain attached to the peptide through an amide bond. The molecule carries five positively charged residues of diaminobutyric acid (Storm *et al.*, 1977)(Fig 1.4).

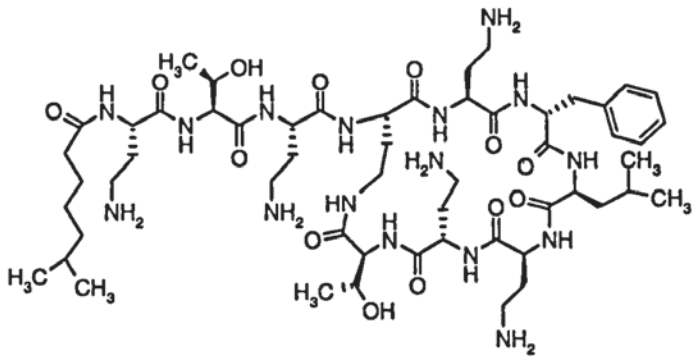


Figure 1.4 Polymyxin B structure

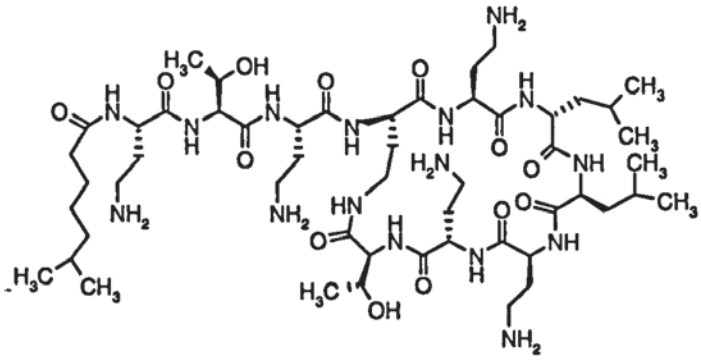


Figure 1.5 Colistin E structure

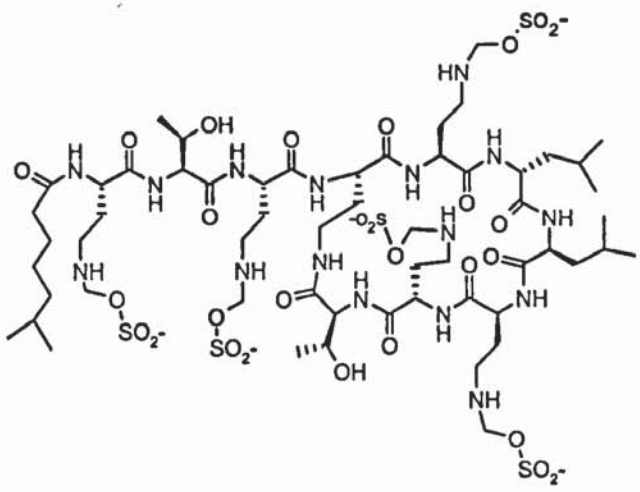


Figure 1.6 Colistin sulphomethate (Colomycin®) structure

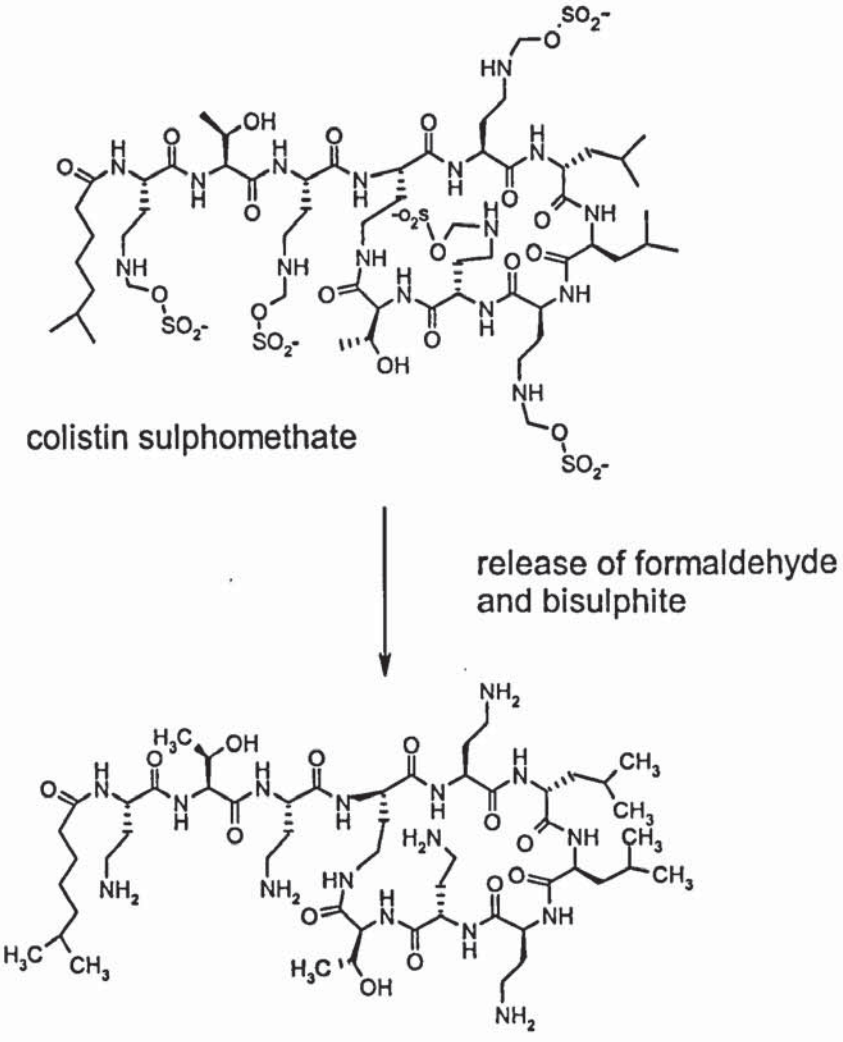


Figure 1.7 Proposed conversion of colistin sulphomethate to colistin

1.6.2 Mode of Action of Polymyxins

Polymyxin competitively displaces magnesium and calcium ions in the OM. The peptide amino groups of PMB are thought to bind to the anionic phosphate groups present in the core and lipid A of adjacent LPS molecules on the outer face of the OM (Nummila *et al.*, 1995). The fatty acid chain of PMB associates with the fatty acids in lipid A. These hydrophobic interactions are the main forces for the association of PMB and LPS (Srimal *et al.*, 1996) and are independent of pH or salt concentration (Thomas and Surolia, 1999). PMB breaks up the LPS molecular structure by insertion of peptides in the fatty acid chain and detoxifies the LPS (Pristovsek and Kidric, 1999). The permeability barrier posed by the OM is disrupted, allowing penetration of polymyxin and other molecules to the cytoplasmic membrane. PMB also induces its own uptake. Aminoglycosides and cationic antimicrobial peptides such as insect cecropins and mammalian neutrophil defensins might also access their targets using this pathway (Hancock and Chapple, 1999; Hancock *et al.*, 1995). Mechanisms of bactericidal and cytotoxic effects are still not clear (Oh *et al.*, 1998; Wiese *et al.*, 1998). The permeabilisation of the OM alone cannot be the cause of the antibacterial effect of PMB since the deacylated PMB nonapeptide form of PMB shows similar effect without affecting bacteria (Vaara 1992; Morris *et al.*, 1995). Lesions induced by PMB in the OM enable PMB to gain access to the plasma membrane (PM) (Dixon and Chopra, 1986; Wiese *et al.*, 1998). The lethal action of polymyxin results from its interaction with anionic phospholipids in the cytoplasmic membrane, causing irreversible damage to the permeability barrier by affecting active transport and respiration (Storm *et al.*, 1977). By comparison LaPorte (1977) demonstrated that the interaction of immobilised PMB with the OM alone is sufficient to block the respiration and growth of *E. coli*. According

to Oh *et al.* (1998) PMB disturbs the phospholipid composition of the OM and the PM.

1.6.3 Resistance to Polymyxin

Despite the widespread use of polymyxin in treatment of *P. aeruginosa* lung infections in CF for over 30 years, resistance in clinical isolates is very uncommon. The possibility of inducing genetically stable resistance against PMB by mutagenesis *in vivo* is low (Oh *et al.*, 1998). However, it is possible to generate resistance *in vitro* (McLeod and Spector, 1996) and studies have identified the different mechanisms involved:

1.6.3.1 Mutational Resistance

When grown in a low Mg^{2+} medium *P. aeruginosa* over-expresses the outer membrane protein OprH (H1) which binds to sites in the LPS normally occupied by divalent metal cations (Nicas and Hancock, 1980) and only a small number of sites are susceptible to PMB and gentamycin (Nicas and Hancock, 1983). Cells grown in these conditions are resistant to the distortion and the permeabilising action of polymyxin upon the outer membrane (Shand *et al.*, 1988). The antibacterial action of EDTA against *P. aeruginosa* was reduced when Mg^{2+} was added. Mg^{2+} is thought to replace missing cations from the outer membrane and therefore restore the damages caused by permeabilising agents (Ayres *et al.*, 1998).

1.6.3.2 OM Alteration

Not all resistant strains of *P. aeruginosa* produce OprH (Gilleland and Beckham, 1982), stepwise adaptation in increasing amounts of PMB results in OM protein alteration and LPS alteration (Conrad and Galanos, 1989). These two mechanisms involving changes in LPS and OprH are distinct (Moore *et al.*, 1984). The esterification of the phosphate groups present in the core lipid A of LPS in *E. coli* and *Salmonella typhimurium* with ethanolamine or aminoarabinose residues reduces the net anionic charge of the LPS and prevents polymyxin binding (Someya *et al.*, 1979; Helander *et al.*, 1996). Studies on *Salmonella minnesota*, *Yersinia enterocolitica* and *Proteus mirabilis* showed that the presence of 4-amino-4-deoxy-L-arabinose bound to the inner core of the LPS could be responsible for the bacterial resistance towards polymyxin (Boll *et al.*, 1994). However recent studies have shown that the presence of 4-amino-4-deoxy-L-arabinose in *P. aeruginosa* does not correlate with PMB resistance (Conrad *et al.*, 1999).

A reduced amount of LPS in the OM of *P. aeruginosa* could also result in resistance to polymyxin (Bell *et al.*, 1991).

1.6.4 Genetically Engineered Antibiotics

The cationic antibacterial peptides, MBI-27 and MBI-28 bind to purified and whole-cell LPS from *P. aeruginosa*. They prevent TNF-α secretion by macrophages stimulated with LPS and block endotoxemia (Gough *et al.*, 1996). They have an advantage over β-lactams and quinolones, which are known to promote endotoxin release. Some β-lactam antibiotics like ceftazidime, ofloxacin and levofloxacin produce sudden release of endotoxin from Gram-negative

bacteria which has a harmful effect on the host by activating macrophages (Kirikae *et al.*, 1998).

1.7 Tetracyclines

Tetracyclines belong to a family of antibiotics that have a broad spectrum, they are active against Gram-positive, Gram-negative bacteria and parasites (Chlamydiae, mycoplasmas, rickettsiales and protozoa). Chlortetracycline was the first tetracycline to be discovered in 1948 and the first one, with tetracycline, to be used. The discovery of oxytetracycline and demethylchlortetracycline enabled chemists to develop semisynthetic tetracyclines like doxycycline (1967), minocycline (1972) and methacycline. Minocycline is active against bacteria resistant to the first antibiotics discovered (Perdue and Standiford, 1999).

Tetracyclines are bacteriostatic, they block protein synthesis by binding to the 30S subunit of the ribosome. They prevent the aminoacyl-tRNA from binding to the mRNA-ribosome complex (Goldman *et al.*, 1983). Other tetracyclines like chelocardin, 6-thiatetracycline and anhydrotetracycline are bactericidal, they attack the cytoplasmic membrane. They are cytotoxic and are not used for treatment.

Tetracyclines penetrate the cells through hydrophilic pores in the outer membrane by passive diffusion. An active transport enables further penetration through the inner membrane. At high concentrations tetracyclines also inhibit protein synthesis in mammalian cells. Recently minocycline has been shown to be active, *in vitro*, against *B. cepacia* and *Stenotrophomonas maltophilia* isolated from CF patients (Kurlandsky and Fader, 2000).

1.7.1 Structure

Tetracyclines have a common 4-ring carbocyclic structure with different substitutions at carbons 5, 6 or 7 (Figure 1.8)

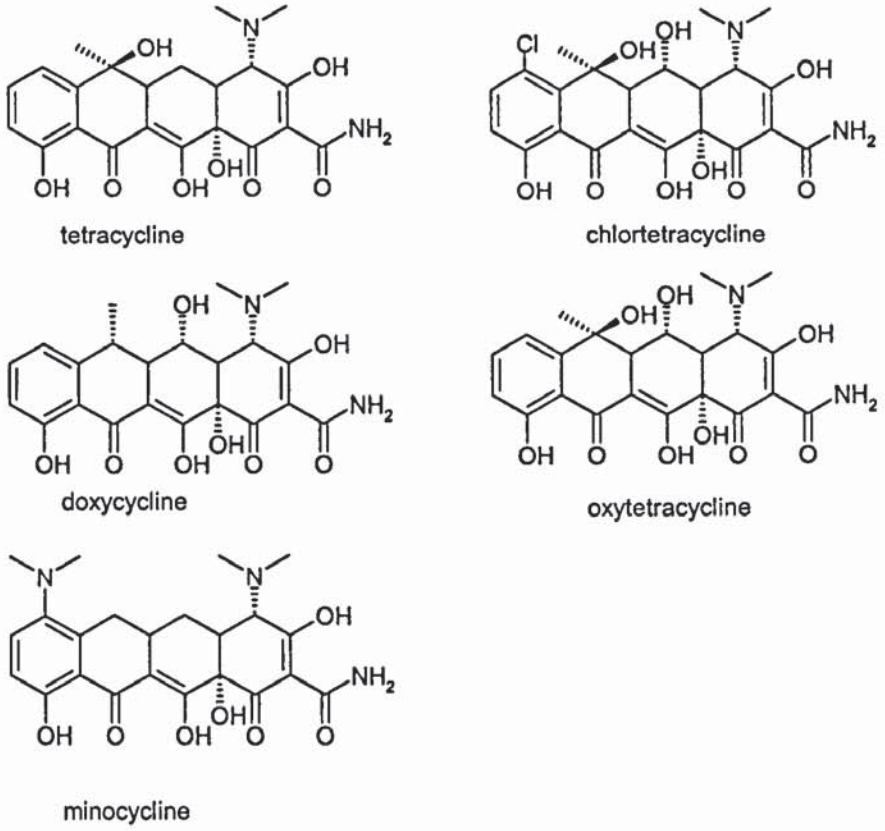


Figure 1.8 Structure of clinically useful tetracyclines

These substitutions result in a variation in the pharmacokinetics of the different tetracyclines. For example, in gastrointestinal absorption where the affinity for multivalent cations is important, protein binding and in antimicrobial activity. Doxycycline and minocycline are the most active of tetracyclines. Penetration in many tissues depends on the lipid solubility. Doxycycline is five times more lipophilic than minocycline which is 10 times more lipophilic than tetracycline (Barza *et al.*, 1975).

The MIC of doxycycline for most bacteria is 4 μg/ml, for *P. aeruginosa* it is 25 μg/ml (Perdue and Standiford, 1999).

1.7.2 Serum Concentration

There is a peak of tetracycline concentration four hours after oral administration and 30 min after intravenous injection. A single oral dose of 500 mg of tetracycline gives a serum concentration of 3 to 4 μg/ml and a 200 mg dose of doxycycline or minocycline gives a serum concentration of 1.5 to 2.5 μg/ml. A single intravenous dose of doxycycline or minocycline gives a serum concentration of 4 to 10 μg/ml. The serum concentration increases slightly with continued administration (Perdue and Standiford, 1999).

1.7.3 Anti-inflammatory Effects of Tetracyclines

It has been well reported that tetracyclines have anti-inflammatory as well as antibiotic effects (Labro, 1998). Tetracyclines act at different levels. They can inhibit MMPs, like collagenase and gelatinase (Israel *et al.*, 1998; Sorsa *et al.*, 1998) which have an important role in tissue destruction in arthritic joints, as well as osteoclast collagenase and parathyroid-hormone which cause bone resorption (Bax *et al.*, 1993). At low concentrations (5-10 μg/ml) doxycycline inhibits type 1 collagen synthesis by hypertrophic chondrocytes (cartilage). At higher doses (40-80 μg/ml) doxycycline affects the synthesis of other proteins like proteoglycan (Davies *et al.*, 1996). Doxycycline reduces superoxide production by PMN at concentrations of 20 and 40 μg/ml and suppresses phorbol myristate

acetate (PMA)-induced mononuclear cell binding (Gabler *et al.*, 1992). Also doxycycline decreases NO levels in mouse peritoneal cells but does not inhibit the anti-inflammatory cytokine IL-10 (D'Agostino *et al.*, 1998). Myers (1995) showed that oxytetracycline had no effect on humoral immunity but that at 500 μg/ml it reduced the production of intracellular oxygen radicals but had no effect on TNF-α secretion. On the contrary, Shapira *et al.* (1996) showed that cytokine secretion by human monocytes stimulated with 1 μg/ml of LPS was inhibited by tetracycline (50 to 500 μg/ml) but this concentration did not affect cytokine mRNA accumulation. Tetracycline seems to inhibit the TNF-convertase, a TNF-specific MMP and therefore blocks secretion (Shapira *et al.*, 1997).

The mechanism of action of the tetracyclines is yet not clear and they seem to act via different pathways (Sorsa *et al.*, 1998). For example, the inhibition of TNF-α is not due to the inhibition of LPS-induction of NO secretion and vice-versa (Shapira *et al.*, 1998). Tetracycline inhibits NO synthase activity but not its synthesis. Different concentrations of minocycline and doxycycline inhibit either NO or PGE_2, so there is an independent effect on their production (Attu *et al.*, 1999). Chemically modified non-antimicrobial tetracyclines inhibit pro-inflammatory phospholipase A (PLA_2) and MMP and would be ideal to treat diseases like pancreatitis and inflammatory bowel disease (Pruzanski *et al.*, 1998).

1.7.4 Effect of Tetracyclines on Calcium

The chelating effect of tetracyclines on calcium and magnesium is suspected to reduce bioavailability of these cations (Lambs *et al.*, 1984) but this does not seem to be the cause of the inhibitory effects shown previously. Minocycline could be a partial agonist of osteoclast Ca^{2+} receptor. Minocycline changes the response to an external calcium concentration elevation but not to calcitonin (Donahue *et al.*, 1992). Minocycline at concentrations of 0.1 to 100 μg/ml increases cytosolic calcium in osteoclasts which has an anti-bone resorptive effect. This mechanism is also used by calcitonin, elevated external calcium concentration, perchlorate ion, veropamil, ionomycin (Bax *et al.*, 1993). Parathyroid hormone (1,25-dihydroxyvitamin D) and prostaglandin stimulate the increase of intracellular calcium, the result being the activation of osteoblasts (Donahue *et al.*, 1992). It is therefore possible that tetracyclines stimulate intracellular calcium increase in osteoblasts.

In rheumatoid arthritis minocycline increases intracellular calcium in T cells promoting a tolerogenic state, T cells being critical components of the pathogenesis of rheumatoid arthritis (Sewell *et al.*, 1995).

1.7.5 Other Antibiotics with Anti-inflammatory Effects

Other antibiotics are known to have anti-inflammatory effects. For example, Sassa *et al.* (1999) have reported that clarithromycin and roxithromycin inhibit MMP-9, TGF-β and TNF-α in rat tumours with a transient increase in IL-6. There was no such effect with cefotiam and gentamicin.

Polymyxin was shown to give a dose-dependent increase in monocyte complement C3 production on IL-1 and IL-6 release (Høgåsen and Abrahamsen, 1995). PMB inhibited LPS-induced IL-6 and TNF-α release by cells expressing membrane-bound CD14 (Iwagaki *et al.*, 2000). Anti-inflammatory effects of PMB could also be due to the fact that PMB is an inhibitor of PKC; PKC being a major step in the activation of macrophages (Casnellie, 1991).

Azithromycin is an azalide member of the macrolide group of antibiotics. Despite its broad spectrum of activity, *P. aeruginosa* is generally resistant to azithromycin. Azithromycin penetrates through macrophage membranes by active transport and is active against intracellular pathogens like *Mycobacterium avium.* Azithromycin uptake is increased by stimulation of the macrophage by TNF-α or IFN-γ (Bermudez *et al.*, 1991). Paradoxically azithromycin decreases levels of TNF-α and IL-1 produced by human macrophages (Khan *et al.*, 1999). Sub-MICs of azithromycin (2 μg/ml) inhibit exotoxin A, protease, elastase and PLC production by *P. aeruginosa* without affecting growth. Azithromycin has therefore virulence-suppressing effects (Mizukane *et al.*, 1994).

Fosfomycin is not related to any of the major classes of antibiotics but has a good spectrum of bactericidal effect. It also has anti-inflammatory effects. For example it reduces the levels of TNF-α, IL-1β and IL-6 in sera of mice with gut-derived sepsis (Labro, 1998). Also levels of TNF-α and IL-1β were decreased in mice treated with fosfomycin 10 min before LPS injection (Matsumoto *et al.*, 1999).

1.8 Aims of the Project

The majority of CF patients will become colonised with *P. aeruginosa* during their early life, however, in recent years *B. cepacia* has proved to be a great threat due to its antibiotic resistance and the dramatic inflammatory response it induces. *P. aeruginosa* and *B. cepacia* infection is usually fatal in CF patients. A test to detect the early colonisation with either of the bacteria would be important to enable prompt therapy aimed at slowing progression from colonisation to full infection. Part of the study was therefore directed towards the development of ELISA tests to detect antibody levels in serum and/or saliva.

The polymyxin antibiotic colistin E (available as Colomycin® (Pharmax)) forms an important part of the therapy of lung infections in CF. There have been very few reports of resistance occurring in clinical isolates, despite over 30 years of use of colistin. Recently reports were made of the isolation of low-level resistance in *P. aeruginosa* samples from the CF clinic at Booth Hall Hospital, Manchester. Investigation of these strains was undertaken to determine the basis of the resistance and to evaluate the threat posed by the spread of such strains. Particular attention was given to the molecular basis of the resistance and the involvement of alterations in outer membrane proteins such as H1 and the nature of the LPS produced by the resistant strains.

The initial aims of the project were therefore:

1. To establish ELISA tests based on *P. aeruginosa* and *B. cepacia* antigens. These would be used to investigate the antibody response in CF patients in serum and saliva. The longer-term objective was to distinguish *B. cepacia* antibody

responses from *P. aeruginosa*, since all patients susceptible to colonisation by *B. cepacia* would already have *P. aeruginosa* infection. The development of saliva-based tests would be less invasive and more acceptable for screening individuals on a regular basis.

2. To investigate patterns of polymyxin resistance in isolates of *P. aeruginosa* from CF clinics. The objective was to establish the most frequent mechanism of resistance (i.e. over-expression of OprH or modification/reduction in LPS content). This might also be important in devising methods for neutralising LPS released in the lungs from *P. aeruginosa*. There is evidence that LPS from *B. cepacia* is an important virulence factor. Since it does not bind to polymyxin, an understanding of the interaction might aid the future design of *B. cepacia* LPS-binding agents. A reverse phase HPLC separation system was developed to aid future investigation of interactions between LPS and polymyxin.

3. Finally, the anti-inflammatory effects of antibiotics were investigated. Mainly, the effect of doxycycline because an improvement in lung function has been reported in clinical observation at Birmingham Children's Hospital in CF patients treated with the drug, even though doxycycline has no antimicrobial activity against *P. aeruginosa*. Compounds that would reduce or inhibit inflammation would help greatly in increasing the survival of CF patients colonised with *P. aeruginosa*.

CHAPTER 2: EXPERIMENTAL METHODS

2.1 Bacterial Strains and Growth Conditions

2.1.1 *Pseudomonas aeruginosa*

PAO1 (ATCC 15692) was used a reference strain. The sequence of the genome of this strain has been determined in the Pseudomonas Genome Project by the Cystic Fibrosis Foundation, University of Washington Genome Centre and PathoGenesis Corporation (http://www.pseudomonas.com).

P. aeruginosa antigen cocktail "PSA3" for ELISA studies was prepared according to the method of Pressler *et al.* (1994) using 17 strains provided by Prof. N. Høiby, Department of Clinical Microbiology, Rigshospitalet, Copenhagen. The 17 clinical isolates (designated 7LI to 7LVI, 7MI to 7MVI, and 7NI to 7NVI in the Microbiology Research Group culture collection, Aston University) were chosen to represent one example of each of LPS O serotypes, 1-17. The PSA3 antigen was prepared by growing each strain in nutrient broth (500 ml, 18 hours, 37 °C on orbital shaker). The cells were harvested by centrifugation (10,000 *g*, 10 min), washed once in water, resuspended in 20 ml of water and cells were then disrupted by sonication on ice (three cycles of 30 sec sonication with 30 sec intervening cooling) using a Soniprep disintegrator (MSE) operating at maximum power with a 1 cm diameter probe tip. The broken cell suspensions were centrifuged at 10,000 *g* for 10 min to deposit unbroken cells and wall fragments. The supernatants from each strain containing a mixture of cellular antigens were combined and diluted with water to a protein concentration of 10 μg/ml.

A series of clinical isolates of *P. aeruginosa* designated 6MI to 6MVI, 6NI to 6NVI, 6OI to 6OVI and 6PI (Microbiology Research Group culture collection, Aston University) were obtained from Booth Hall Children's Hospital, Manchester. The strains were isolated from cough and sputum samples from Cystic Fibrosis patients. They were identified by API 10S and API 20NE (BioMérieux, Marcy l'Etoile, France) and positive oxidase reaction (Cowan, 1974). Although they were initially reported as polymyxin resistant *P. aeruginosa* strains, some were subsequently shown to be *Proteus* or *Providencia* species and were not studied further.

2.1.2 *Burkholderia cepacia*

Isolates 5JIV, 5TIV, 5SI, 5PIV, J1948, 5YIV (Microbiology Research Group culture collection) were all obtained from CF patients (Baxter *et al.,* 1997).

2.1.2.1 Growth Conditions

Strains were grown on Nutrient Agar or Broth (Oxoid Ltd, Basingstoke), Mueller Hinton (MH) Agar or Broth (Oxoid), iron or magnesium-supplemented chemically-defined medium agar (designated CDM+Fe or CDM+Mg), low-iron or low-magnesium chemically-defined medium agar (designated CDM-Fe or CDM-Mg). The compositions of the chemically defined media are listed in Table 2.1.

Reagent	CDM +Fe Agar	CDM+Mg Agar
Glucose	48 mM	48mM
KCl	7.4 mM	7.4 mM
NaCl	6 mM	6 mM
$(NH_4)_2SO_4$	48 mM	48 mM
$MgSO_4.7\ H_2O$	0.5 mM	0.5 mM
MOPS	60 mM	60 mM
$K_2HPO_4.3H_2O$	21 mM	21 mM
Casamino acids	0.1 %	0.1 %
Agar	15 g/l	15 g/l
$FeSO_4.7H_2O$	0.12 mM	-

Table 2.1 Composition of defined media

Broths were made using the same substances without adding the agar. The CDM-Fe was prepared as for CDM+Fe without $FeSO_4.7H_2O$. The CDM-Mg was prepared as for CDM+Mg without $MgSO_4.7H_2O$ (AnalaR reagent, BDH).

2.2 Preparation of Outer Membranes - Sarkosyl Method

Outer membranes (OMs) of clinical strains of *P. aeruginosa* and *B. cepacia* were prepared by a method based on that of Lambert and Booth (1982). *B. cepacia* strains were grown in 2 litres of MH broth for 48 hours at 37 °C, harvested by centrifugation (10,000 *g* for 10 min), washed in saline and resuspended in water. Cells were then disrupted by sonication as described in section 2.1 for the PSA3 antigen, using three cycles of sonication for *P. aeruginosa* and ten cycles for *B. cepacia*. Sarkosyl detergent (N-lauroyl sarcosine, sodium salt, Sigma) was added to a final concentration of 2 % (w/v). Centrifugation at 10,000 *g* for 10 min deposited a pellet containing residual unbroken cells. The supernatant containing

intact outer membranes, sarkosyl-solubilised cytoplasmic membrane and cytoplasm was centrifuged at 25,000 *g* for 50 min. The pellet of outer membranes was resuspended in double-distilled water and stored at 20 °C for analysis by SDS-PAGE.

2.3 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE method of Lugtenberg *et al.*, (1975) was used to separate the different outer membrane proteins. *B. cepacia* OMs prepared as described above were denatured in sample denaturing buffer for 10 or 30 min at 100 °C. Aliquots of 10 µl denatured sample were loaded into separate lanes. A sample of a molecular weight protein marker mixture was also included. Samples were electrophoresed at 200 V for 50 min using 12 % (w/v) acrylamide gels (BioRad miniProtean II system). Gels were stained by immersing in 0.1% Coomassie Brillant Blue R-250 (Sigma) in 20 % (v/v) methanol-10 % (v/v) glacial acetic acid solution for 1 hour and destained in a 20 % (v/v) methanol-10 % (v/v) glacial acetic acid solution overnight. Gels were then rinsed in distilled water, stored in distilled water or dried on chromatography paper for 3 hours using a vacuum gel drier (BioRad).

P. aeruginosa OMs were denatured in sample denaturing buffer for 10 min at 100 °C. Samples were run on 15 % (w/v) acrylamide separating gels at 200 V for 50 min and stained as above.

2.4 Isolation of OM Proteins by Preparative SDS-PAGE and Electroelution

The BioRad Protean II xi cell system with gels containing single sample loading slots spanning each 12 % (w/v) gel was used for quantitative isolation of individual OM proteins. Aliquots of 200 µl denatured outer membrane preparation of the *B. cepacia* strains 5TIV and 5JIV were loaded into each well and electrophoresed at 200 V for 3 hours.

The separated protein bands were rapidly visualised by staining with copper chloride solution as follows (Lee *et al.*, 1987). After electrophoresis gels were washed for several seconds in distilled water, then immersed in 100 ml of a 0.3 M copper chloride solution ($CuCl_2$) and gently rocked for 5 min at room temperature. The gels were then washed for 3 min in distilled water to remove excess copper chloride and left in distilled water. Protein bands appeared white against the blue background of the gel.

The bands corresponding to the required outer membrane proteins were excised from the gels and the proteins recovered from the gel by electro-elution (BioRad Model 422 Electro-Eluter). The segments of gel containing protein bands of interest were placed in separate glass electro-elution tubes were filled with elution buffer (Tris base 3.0 g, glycine 14.4 g, SDS 1.0 g in 1 litre of water). Elution was carried out at a constant current of 8-10 mA/glass tube for 3 to 5 hours. About 400 µl of the solution containing the eluted protein was recovered from each tube using a plastic pipette. The eluted protein solutions were stored at –20 °C.

2.5 Preparation of Purified Lipopolysaccharide

LPS was prepared from whole cells by the hot phenol extraction method of Westphal and Jann (1965). Cells were harvested by centrifugation at 10,000 *g* for 10 min, washed in saline and centrifuged at 10,000 *g* for 10 min. The pellet was resuspended in distilled water, sonicated for 3 min on ice (30 sec intervals with 30 sec cooling) and incubated with DNase (0.125 mg/ml, Sigma, EC number 3.1.21.1) at 37 °C for 1 hour. An equal volume of phenol (80 % (w/v)) was added to the mixture and heated to 80 °C with stirring. After separation of the two phases by centrifugation at 10,000 *g* for 10 min, the upper aqueous phase was recovered and retained. The lower phenol layer was re-extracted with an equal volume of water. The combined upper aqueous phases were dialysed overnight against several changes of water to remove phenol. Magnesium sulphate was then added to the dialysate to a concentration of 10 mM to aid aggregation of LPS micelles. The LPS was then deposited by centrifugation at 35,000 rpm using a Beckman J8 ultracentrifuge with a Ti 80 rotor for 3 hours (average of 50,000 *g*). The LPS pellet was freeze-dried and stored at –20 °C.

2.6 Preparation of Purified Rough LPS

Whole cells of *P. aeruginosa* (strain 5MV) were grown in MH broth on a rotary shaker (18 hours, 37 °C), harvested by centrifugation (10,000 *g*, 10 min) and freeze dried. Approximately 5 g of freeze-dried cells were suspended in 20 ml of extraction mixture composed of 90 % (w/v) phenol, chloroform and petroleum spirit in the proportions 2:5:8 by volume. Bacteria and extraction mixture were stirred together for 2 min below 20 °C. The mixture was centrifuged (10,000 *g*, 15

min) and the supernatant containing extracted rough LPS was filtered through Whatman filter paper into a round-bottomed flask. The pellet was extracted again and the supernatant filtered into the flask containing the first filtrate. Chloroform and petroleum were removed by rotary evaporation. When solid material formed in the flask it was redissolved by the addition of a small volume of water. After complete removal of the organic solvents water was added dropwise until the LPS precipitated (about 0.4 ml). The LPS was then pelleted by centrifugation (5,000 *g*, 10 min). The centrifuge tubes were drained and the pellet was washed three times with 80 % (w/v) phenol and recovered by centrifugation each time. Finally the pellet was washed twice in ether and dried under vacuum. LPS was stored as a powder at 4 °C.

2.7 Gel Electrophoresis of LPS

The purified LPS prepared by phenol extraction was separated by electrophoresis on 12 % (w/v) acrylamide gels containing 6 M urea as described in section 2.3 and visualised by silver staining (2.7). Alternatively, bacterial OMs or whole cells were digested with proteinase K using the method of Hitchcock and Brown (1983). 100 µl of cells (equivalent to 1 mg/ml protein) were denatured at 100 °C with 80 µl of sample buffer. On cooling, 20 µl of sample buffer containing proteinase K (2.5 mg/ml, 15 units/mg, Sigma) was added and the mixture incubated for a further 1 hour at 60 °C. Samples (10 µl) were then loaded on the 12 % (w/v) acrylamide gel containing 6 M urea and electrophoresis was carried out as described in section 2.3. Gels were then used for immunoblotting (2.9) or silver stained (2.8).

2.8 Silver Stain of LPS

LPS on the gels was visualised using the method of Tsai and Frasch (1982). Gels were immersed overnight in fixing solution containing 40 % (v/v) ethanol and 5 % (v/v) acetic acid. The fixing solution was replaced by an oxidising solution of 40 % ethanol/5 % acetic acid/1 % periodic acid for 1 hour. After 3 × 15 min washes with double distilled water the staining reagent (consisting of 2 ml of concentrated ammonium hydroxide added to 28 ml of 0.1 N NaOH and 5 ml of 20 % (w/v) silver nitrate and diluted to 150 ml with double distilled water). Gels were washed for 3 × 15 min in double distilled water. The formaldehyde developer was added (10 mg citric acid, 0.1 ml of 37 % (w/v) formaldehyde, water to 200 ml). Brown stained bands appeared within 5-30 min. When the LPS was stained to the desired intensity the developer was replaced with water to stop the reaction. Gels were photographed immediately.

2.9 Immunoblotting

Proteins or LPS separated by SDS-PAGE using single slots spanning the width of the gel were transferred onto nitrocellulose membranes (NC) with a Mini Transblot cell (BioRad) using the method of Towbin *et al.* (1979). Transfer was carried out in an ice-cooled transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3) at 100 V for 1 hour. A vertical strip from the NC was stained with 0.8 % (w/v) amido black (naphthol blue black, Sigma) in 0.7 % (w/v) glacial acetic acid in order to determine if the transfer had worked. The rest of the blots were blocked at 37 °C for 30 min with TTBS (Tween-Tris buffered saline, 0.3 % (w/v) Tween 20 (Sigma), 0.9 % (w/v) NaCl, 10 mM Tris adjusted to pH 7.4

with HCl). Blots were then rinsed three times in TBS (Tris-buffered saline, 0.9 % (w/v) NaCl, 10 mM Tris adjusted to pH 7.4 with HCl) and incubated overnight at 4 °C with patient's serum diluted 1:100 in TTBS. The NC membranes were then rinsed three times in TBS and incubated for 2 hours at 4 °C in anti-human IgG peroxidase conjugate (Sigma) in TTBS diluted according to the manufacturer's instructions. The serum-incubated blots were visualised after rinsing three times in TBS by adding the substrate buffer (see section 2.9) at 37 °C for 30 min. The reaction was stopped by flooding with distilled water.

Blots using *B. cepacia* whole cells, outer membrane proteins or eluted proteins were left overnight at 4 °C in serum previously incubated with PSA3 (50 µl serum, 50 µl PSA3 in 5 ml TTBS) or *B. cepacia* LPS (10 µg/ml), centrifuged at 13,500 rpm (Eppendorf micro centrifuge), and the pellet of antibody-antigen discarded.

2.10 ELISA Tests for Antibody Responses to *Pseudomonas aeruginosa* and *Burkholderia cepacia* Antigens

2.10.1 *Pseudomonas aeruginosa* PSA3 Antigen

The PSA3 antigen was prepared from 17 strains of *P. aeruginosa* using the method of Pressler *et al.* (section 2.1). The antigen, comprising the cell free extract from sonicated cells was stored at –20 °C until required. Microtitre plates (96-well Immulon 2 HB, Dynex Technologies, USA) were coated overnight at 4 °C with 100 µl of PSA3 (10.88 mg protein/ml) diluted 1000-fold in

carbonate/bicarbonate buffer (50 mM sodium carbonate adjusted to pH 9.6 with a 50 mM sodium bicarbonate solution) in each well. Plates were washed three times in TTBS and stored at –20 °C.

2.10.1.1 Serum IgG

Plates were incubated at 4 °C overnight with 100 µl of patient serum, diluted 1:100 in TTBS in the first well and at doubling dilutions across the plate. Plates were washed three times in TTBS and 100 µl of anti-human IgG (gamma chain) specific peroxidase conjugate (Sigma) diluted 1 in 10,000 in TTBS were added to each well, incubated at 4 °C for 2 hours. Plates were washed three times in TTBS and developed with 100 µl of substrate buffer (10 mg 3,3',5,5'-tetramethylbenzidine in 1 ml DMSO (Sigma), 100 ml acetate/citrate buffer pH 6, 40 µl of 6 % H_2O_2) for 5 min. The reaction was stopped by adding 100 µl of 2 M sulphuric acid. The absorbance at 450 nm was measured using an Anthos 2001 reader (Labtec).

2.10.1.2 Salivary IgA

Saliva samples were collected on three separate sialopaper strips (2 cm × 0.5 cm, OraFlow Inc., USA) inserted in the mouths of patients who had previously fasted for 3 hours. The strips were stored at –70 °C prior to elution of antibody by immersion in TTBS. Single saliva-coated strips were weighed and the volume of saliva was calculated assuming a strip dry weight is 8 mg (previously determined by weighing 20 separate unused strips). TTBS was added to the strips to give a

dilution of a 100-fold (assuming 10 mg saliva = 10 µl) and incubated for 2 hours at room temperature to elute the IgA from the strips. Samples were then applied to the plates as described for serum IgG. Detection of IgA antibodies was made by using anti-human IgA (alpha chain) HRP conjugate diluted according to the manufacturer's instructions.

2.10.2 *Pseudomonas aeruginosa* Rough LPS Antigen

LPS was prepared using the method described in section 2.6. Immulon 2 HB plates were coated for two hours at room temperature with 100 µl/well of LPS in carbonate buffer at concentrations ranging from 1 pg/ml to 10 µg/ml. Plates were washed and blocked with TTBS for two hours at room temperature. Plates were stored at –20 °C. The minimum concentration of LPS needed to give a significant response to a positive serum control was 1 µg/ml.

2.10.3 *Burkholderia cepacia* Outer Membrane Proteins Prepared by Electroelution from SDS-PAGE Gels

Plates were coated with one of each eluted protein using 0.1 ml of eluted protein in 50 ml of coating buffer (carbonate/bicarbonate buffer) as described above for PSA3. Serum from a CF patient infected with *B. cepacia* was used. In some experiments serum was first incubated for 3 hours with PSA3 at 4 °C (50 µl serum, 50 µl PSA3 in 5 ml TTBS) to absorb any cross-reacting antibody to *P. aeruginosa* and centrifuged for 2 min (13,500 rpm in an Eppendorf

microcentrifuge) to remove any immune complexes formed. The ELISA test was run again with the purified serum.

2.11 Anti-*Pseudomonas aeruginosa* IgG EIA

The anti-*P. aeruginosa* IgG EIA (Mediagnost) is a sandwich enzyme immunoassay. Serum samples were diluted and added, for 2 hours, to the wells of a microtiter plate, which had been previously coated with the *P. aeruginosa* antigens alkaline protease, elastase or exotoxin A. After washing, the conjugate (anti-human IgG peroxidase-labelled immuno-globulin) was added and incubated for 2 hours at 37 °C. The wells were washed and the substrate was added and incubated for 30 min at room temperature. The stop solution was then added and the optical density was measured at 450 nm. A patient was regarded sero-positive when the serum was positive for one or more of the antigens.

2.12 Immunoluminometric Assay for the Detection of Procalcitonin (PCT)

Concentration of PCT in CF patients' serum was quantitated using the LUMItest PCT (B.R.A.H.M.S Diagnostica Gmbh). Briefly, two antigen-specific monoclonal antibodies bind PCT at two different sites (the calcitonin and katacalcin segments). One of the antibodies is luminescence labelled (the tracer) and the other one is coated on the tubes. Serum and tracer are incubated together in the tubes. The bound antibody captures PCT which is labelled by the tracer antibody. After washing the amount of tracer on the test tube wall is quantified by measuring the luminiscence signal (RLU) using a luminometer (Berthold LB952

T/16 AutoClinilumat) which is directly proportional to the PCT concentration in the serum sample. The assay sensitivity is 0.1 ng/ml.

2.13 Minimum Inhibitory Concentrations (MIC) of Colistin E (Colomycin®) and Polymyxin B (Aerosporin®) for Isolates of *Pseudomonas aeruginosa*

MICs were determined in nutrient broth by the microdilution method using an inoculum of 10^5 cells per ml with polymyxin B sulphate (Aerosporin®, Calmic Medical Division, London) and Colomycin® (Colistin Sulphomethate Sodium, Pharmax Ltd).

2.14 Thin Layer Chromatography (TLC)

Volumes of 2 µl of LPS, PMB, Colomycin® and Colymycin® (alternative form of colistin sulphomethate produced by Parke-Davies) at a concentration of 5 mg/ml in water, were applied to a TLC silica gel plate on polyester (Sigma-Aldrich) and dried. The TLC plate was then placed in a glass developing tank (Sigma) containing a solution of acetone, water, acetic acid and 2 N ammonia in a ratio of 15:5:1:2. After the migration of the compounds the plate was air dried and sprayed with ninhydrin (1 % (w/v) in acetone), heated at 100 °C for 5 min. Appearance of purple or brown spots revealed amino compounds. Alternatively the plates were sprayed with 50 % (v/v) sulphuric acid to reveal sugar residues on LPS.

2.15 High Voltage Paper Electrophoresis

Aliquots of 2 µl of PMB, colomycin and colymycin at a concentration of 5 mg/ml in water were applied to a strip of Whatman 3MM paper. The paper was soaked with 0.05 M phosphate buffer pH 7.0. Samples were run on a LBK Bromma 2117 Multiphor II horizontal Electrophoresis Unit at 200 V for 3 hours with cooling through the flat bed to 5 °C provided by circulating water from a LKB 2209 Multitemp device. The paper was then dried and amino groups revealed by ninhydrin spray at 100 °C for 5 min.

2.16 Separation and Analysis of Colomycin by HPLC

The HPLC system of Elverdam *et al.* (1981) was used. This comprised a C18 reverse phase column with an isocratic mobile phase containing acetonitrile at 22 or 23 % (v/v) and 77 % (v/v) buffer (0.023 M H_3PO_4, 0.01 M CH_3COOH and 0.05 M Na_2SO_4, adjusted to pH 2.5 with triethylamine). A Waters 600 Multisolvent delivery system was employed with a Nucleosil 250 × 0.5 mm OD5 column (5 µm support), a Pye Unicam LC3 UV detector operating at 220 nm and Omniscribe chart recorder. The flow-rate was 1 ml/min and the injection volume was 10 µl.

2.17 ^{31}P NMR

Samples of LPS were analysed using a 250 MHz Bruker instrument operating at 20 °C. The ^{31}P spectrum was obtained using 20 mg of LPS dissolved in 1 ml of

D_2O. The phosphorus chemical shift was measured as parts per million (ppm) relative to an internal reference of 80 % phosphoric acid.

2.18 Cell Culture

2.18.1 Primary Mouse Peritoneal Macrophages

To prepare mouse peritoneal macrophages adult male, MF1 outbred mice were killed by cervical dislocation under diethyl ether anaesthesia. The animal was swabbed with 70 % alcohol, the fur was removed with sterile scissors to reveal the abdominal wall. The peritoneal macrophages were extracted by injecting 10ml of warmed, sterile RPMI 1640 medium (Sigma) supplemented with 10 % foetal calf serum (Difco), 100 μg/ml penicillin G, 100 IU/ml streptomycin (Sigma) and 1 % glutamine (Sigma) into the peritoneum and gently massaging the area. The medium was then removed using a syringe (Plastipak) and centrifuged at 1600 RPMI for 10 min. The pellet was resuspended to a concentration of 10^6 cells/ml and 1 ml was poured into each well of a 24-well plastic plate (Tissue culture Cluster[24], Costar 3424 Mark II) and incubated in 5 % CO_2 and 95 % air at 37 °C overnight to allow the macrophages to adhere. Cell counts were made using haemocytometer. Next day the supernatant was removed and replaced by fresh warm medium. Various concentrations of LPS from rough strains of *P. aeruginosa* and *B. cepacia* and different antibiotics were added to the wells and the supernatant collected after various times for measurement of cytokine release.

2.18.2 Measurement of Oxidative Burst by Luminometry

Phagocytosis by murine peritoneal macrophages was assessed by measuring the oxidative burst, via enhanced chemiluminescence, using a Luminometer (Labtech International, Jade). Superoxide anions were converted by superoxide dismutase to hydrogen peroxide (H_2O_2) which reacted with lucigenin to generate light.

General equation:

$$\text{Reduced lucigenin} + H_2O_2 \rightarrow \text{Oxidised lucigenin} + \text{light}$$

A photomultiplier converted the light to an electrical signal, which is expressed as relative light units (RLU).

Murine peritoneal macrophages were harvested and resuspended at 1×10^6 cells per ml and 100 µl was added to a 75 × 11 mm plastic tube (Sarstedt) containing 100 µl lucigenin (5×10^{-4} M) (Sigma) and either 100 µl of opsonised zymozan (opz) or 100 µl of LPS from *P. aeruginosa*. Tubes were shaken gently and placed in the luminometer (BioOrbit 1253) and read immediately. Tubes were incubated at 37 °C in the dark and further chemiluminescence readings were taken at 1-5 min intervals over a 30 min period.

2.18.3 Cell Line

The cell line used to investigate the cytokine release was human lymphoblast U937 (Source: ECCAC: 85011440). U937 cells were routinely cultured in 75 cm^2 flasks (Falcon/Costar) in 1640 RPMI medium supplemented with the following

(final concentrations): 10 % FCS, 100 IU penicillin/streptomycin, 1 % glutamine. The process of passaging involved seeding in fresh pre-warmed medium at a ratio of 1:20. Passaging was carried out every 4-5 days. In order to differentiate the U937 monocytes into macrophages, the cells were centrifuged at 1600 rpm for 10 min (IEC Central-3C Centrifuge, International Equipment Company). The pellet was resuspended in fresh pre-warmed media at a concentration of 10^6 cells/ml. Cells were counted using a haemocytometer. Phorbol 12-myristate 13-acetate (PMA, Sigma) at a final concentration of 10^{-7} M was added to the media. One ml of cell suspension was then placed in each well of a 24-well plate and incubated for 48 hours in 5 % CO_2 and 95 % air at 37 °C. The supernatant was discarded and replaced with fresh pre-warmed medium and incubated for a further 24 hours.

2.18.4 Analysis of Supernatants

To assess whether cytokines were released by macrophages after activation with LPS, supernatants were collected and their contents analysed using commercial ELISA cytokine assay kits.

2.18.4.1 Collecting Supernatants

Supernatants were collected after the cells have been incubated for 5 hours with the stimulant and the antibiotics. In preliminary studies 5 hours was found to be the incubation time needed to give the highest cytokine concentration. The supernatants were then either used immediately or stored at –70 °C for no longer than 2 weeks.

2.18.4.2 Cytokines

The cytokines IL-1β and TNF-α were measured in the cell supernatants using either murine or human Quantikine ELISA kits (R & D Systems). The kits were sandwich ELISAs using affinity purified polyclonal antibodies specific for the cytokine pre-coated onto microtitre plate well strips. Standards, controls and samples were pipetted into the wells and any cytokine present was bound by the immobilised antibody. After removal of any unbound substances by washing, an enzyme-linked polyclonal antibody was added to the wells. The wells were washed again to remove any unbound enzyme-linked antibody. The substrate solution was added to each well, and incubated for the prescribed time (manufacturer's instructions). Stop solution was added and the optical density was measured at 450 nm with a correction wavelength of 570 nm using an Anthos 2001 plate reader (Labtec). The cytokine concentrations were then determined from the respective standard curves (Fig 2.1 and 2.2).

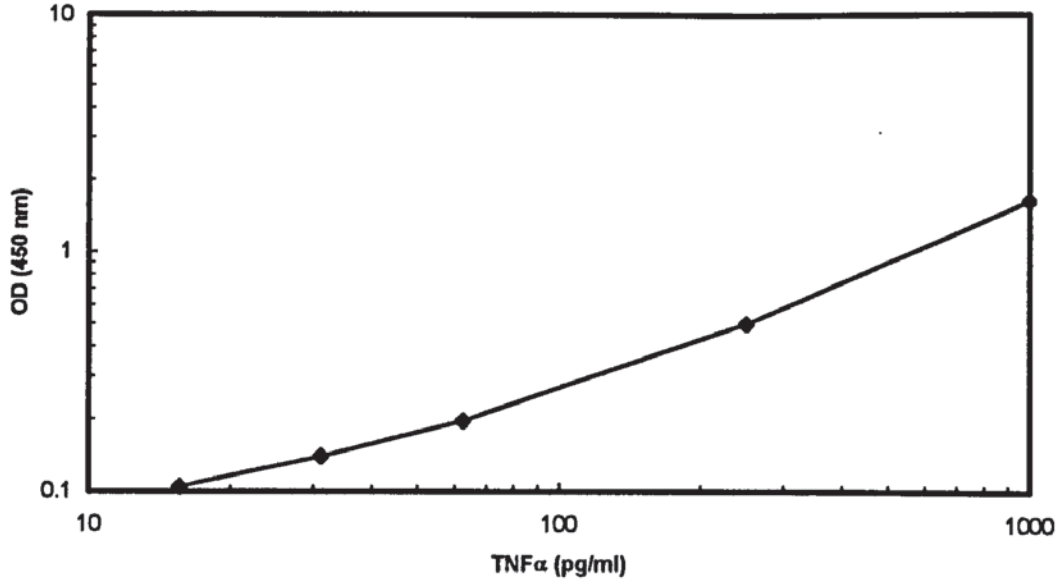


Figure 2.1 Standard curve for TNF-α

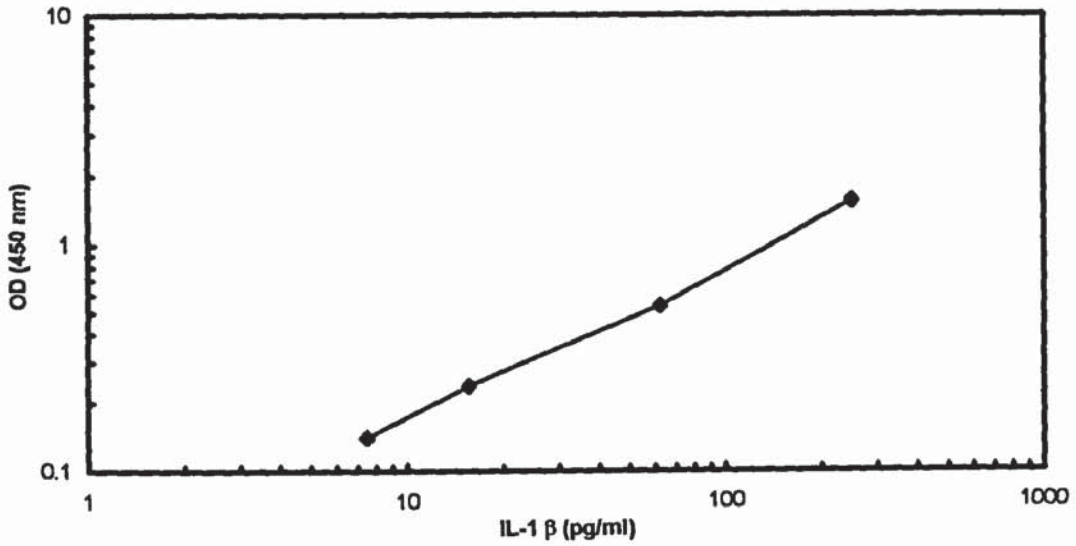


Figure 2.2 Standard curve for IL-1β

3.1 ELISA Test with *Pseudomonas aeruginosa* Antigen

Tests were carried out on serum and saliva samples taken from 105 patients attending day clinics at the Birmingham Children's Hospital. Four groups were studied: non-CF (n = 20), non-colonised CF (n = 32), intermittent CF i.e. previoulsly colonised but cough swab negative following antibiotic treatment (n = 22), and chronically colonised CF (n = 30). Levels of serum IgG and saliva IgA were measured by ELISA using the *P. aeruginosa* PSA3 antigen, the results are shown as plots of IgG against IgA for each patient in Figures 3.1-3.4 and summarised in Table 3.1.

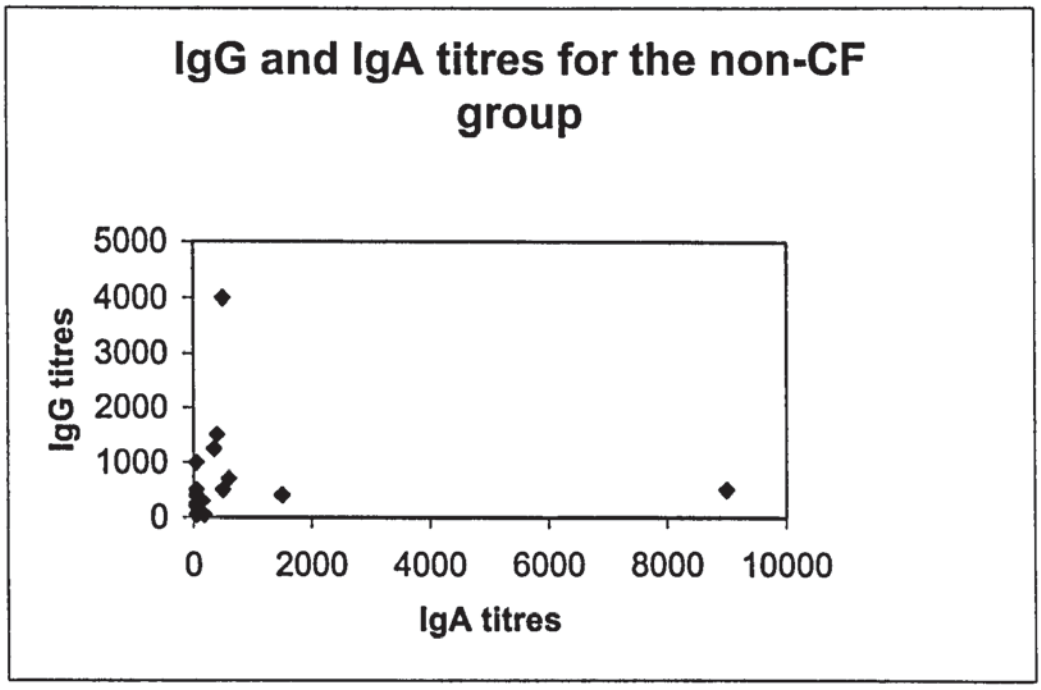


Figure 3.1 Serum IgG and saliva IgA titres towards PSA3 for individual patients in the non-CF group

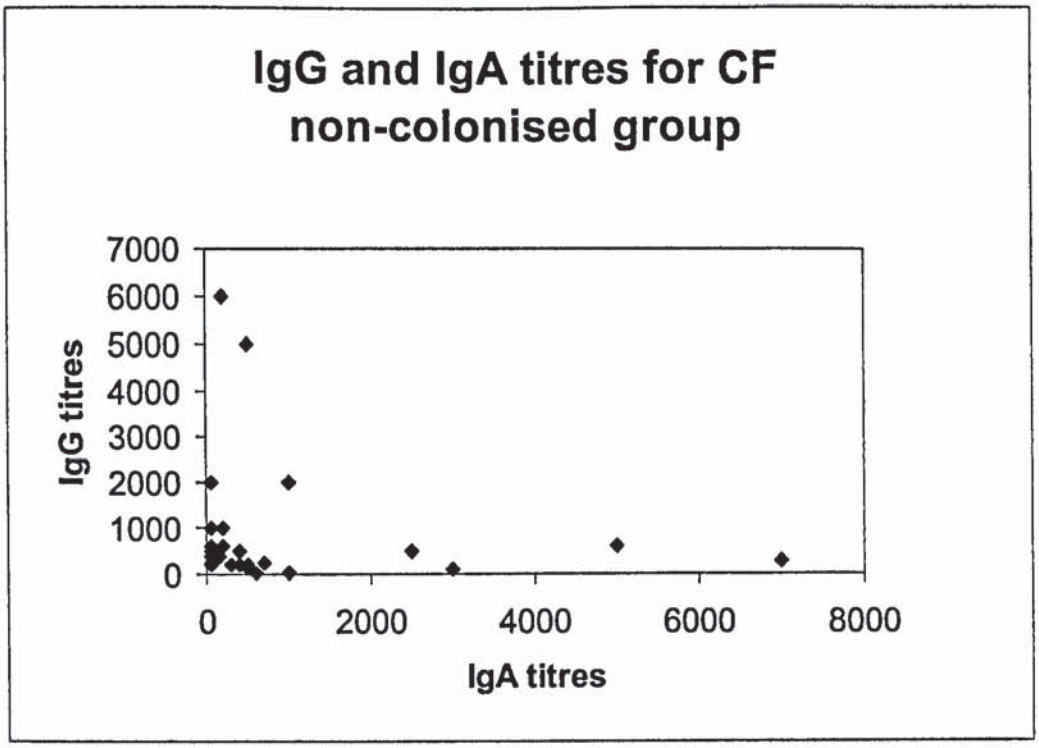


Figure 3.2 Serum IgG and saliva IgA titres towards PSA3 for individual patients in the CF non-colonised group

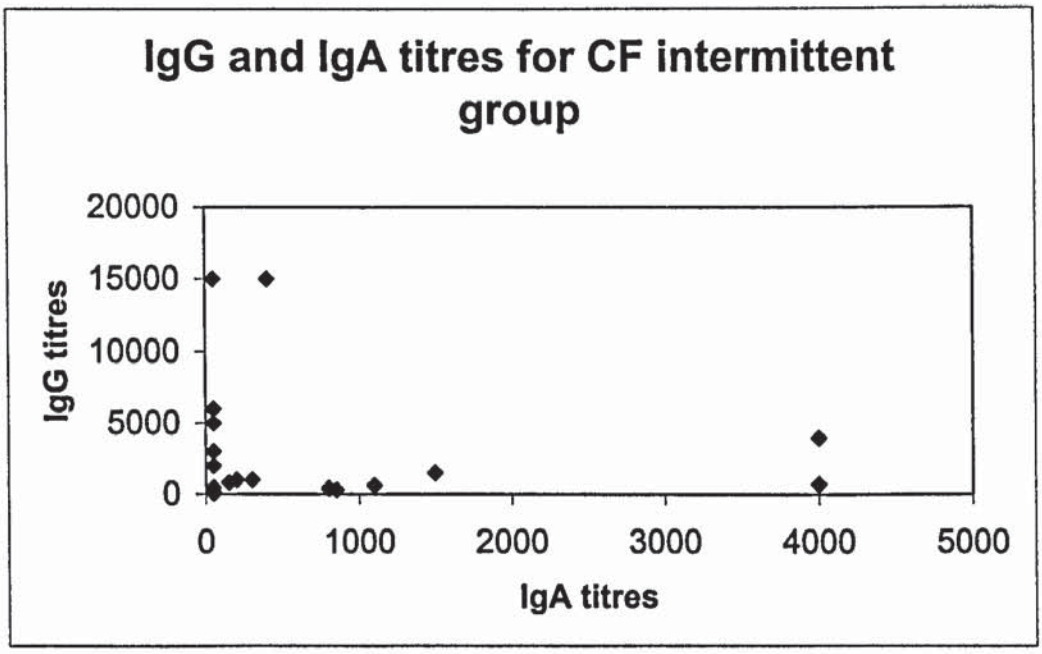


Figure 3.3 Serum IgG and saliva IgA titres towards PSA3 for individual patients in the CF intermittent group

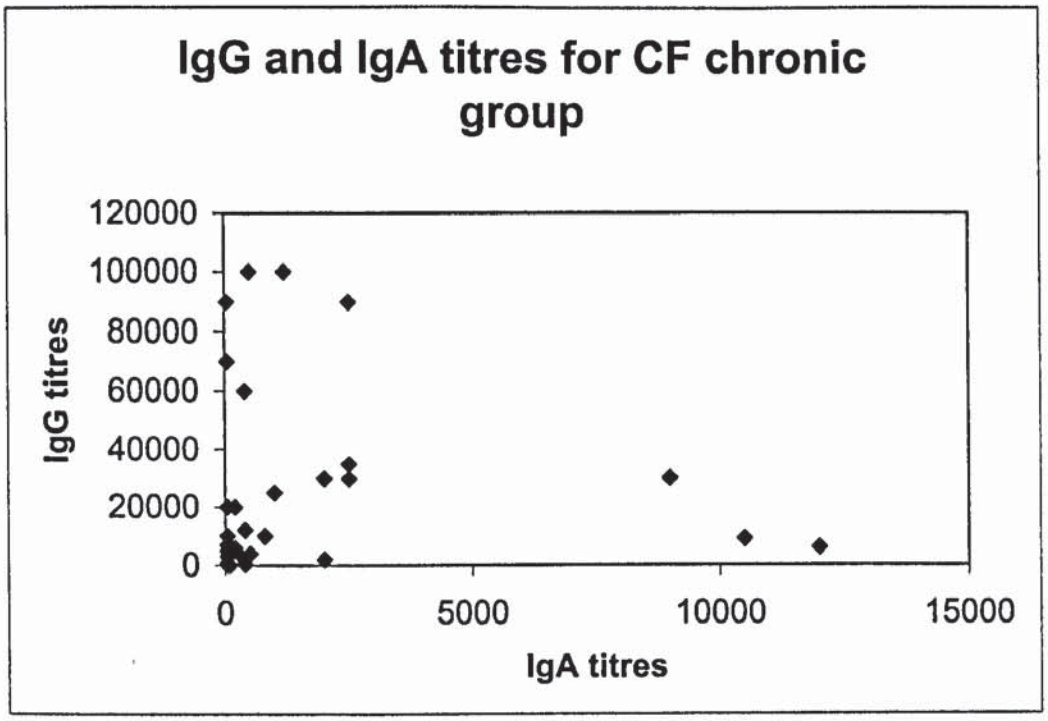


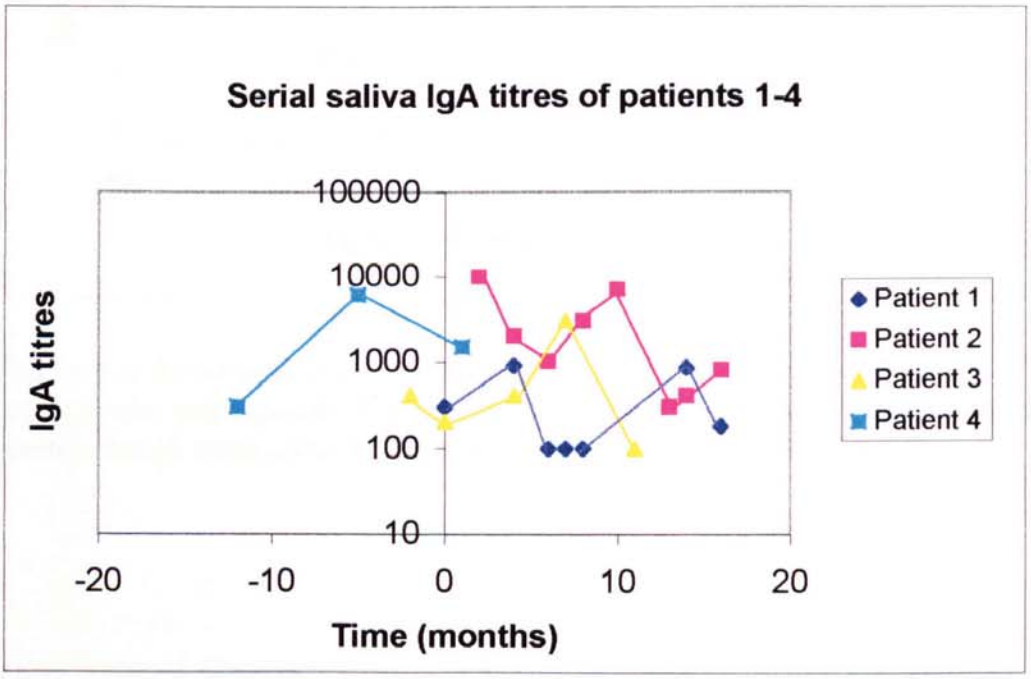
Figure 3.4 Serum IgG and saliva IgA titres towards PSA3 for individual patients in the CF chronic group

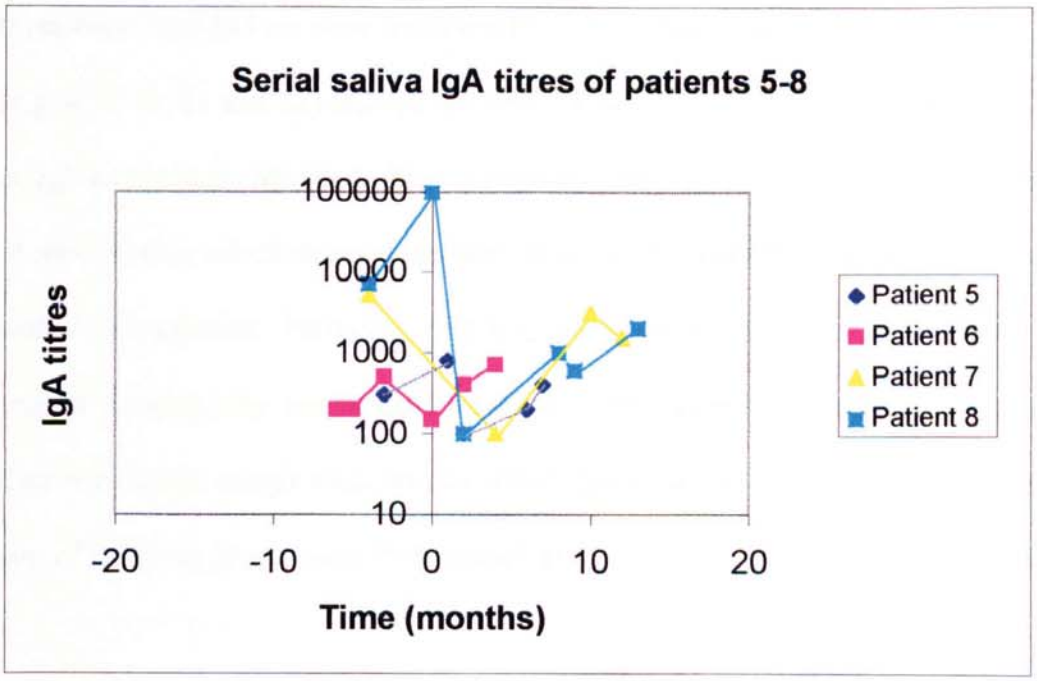
Patient group	Parameter	Saliva IgA	Serum IgG
Group 1 non-CF patients	**n**	21	21
	Mean	1037.5	632.5
	Median	50	400
	Range	50-9000	50-4000
Group 2 Non-colonised CF	**n**	30	31
	Mean	1151.7	1009.7
	Median	300	400
	Range	50-8000	50-6000
Group 3 Intermittently-colonised CF	**n**	18	19
	Mean	725	3008.2
	Median	125	1000
	Range	50-4000	50-15000
Group 4 Chronically-colonised CF	**n**	31	32
	Mean	1256.5	25562.9
	Median	400	10000
	Range	50-10500	100-100000

Table 3.1 Summary of saliva IgA and serum IgG titres towards PSA3 for patient groups shown in Figures 3.1-3.4

The chronically colonised CF group showed a clearly elevated serum IgG response to the PSA3 antigen in comparison with the CF and non-CF non-colonised groups. However, this difference was not seen for the equivalent salivary IgA levels. Furthermore, there was no correlation between serum IgG level and saliva IgA level in individual patients from any group. It therefore appears that salivary IgA levels would not be useful in distinguishing between colonised and non-colonised patients whereas the serum IgG levels show a clear correlation with infection. Measurement of saliva IgA levels might, however, be of value in monitoring individual non-colonised CF patients. During the course

of the study, 13 of the non-colonised CF patients became colonised with *P. aeruginosa* as determined by positive cough swabs. The results are shown in Figure 3.5 as serial salivary IgA titres on a time course where time zero is the date of obtaining the first positive cough swab.





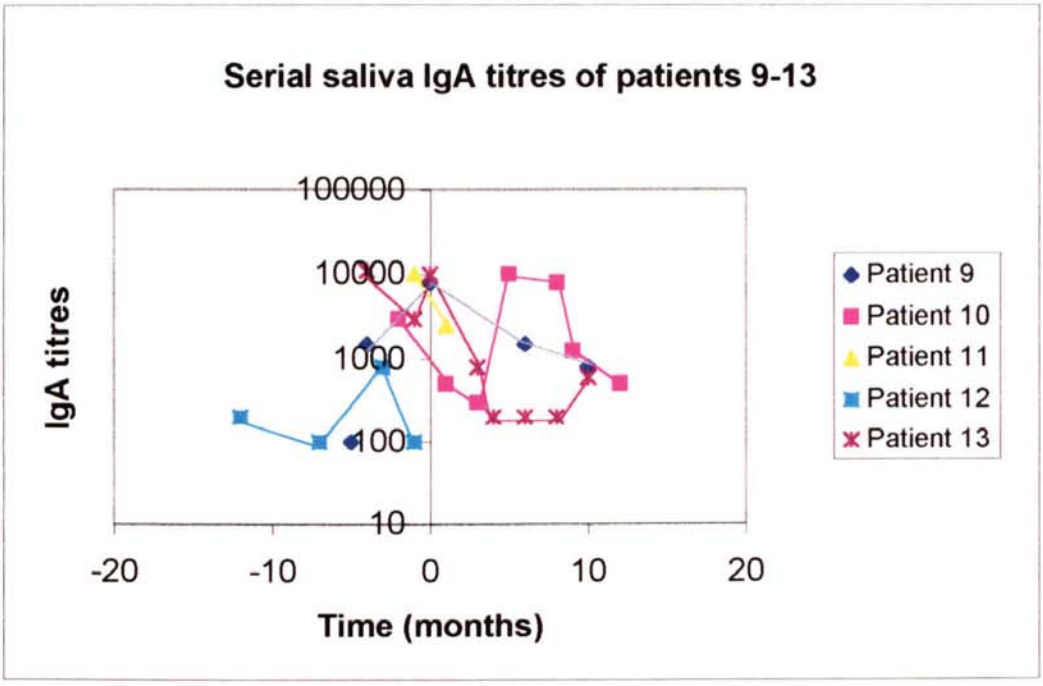


Figure 3.5 Serial salivary IgA titres towards PSA3 in 13 non-colonised CF patients who subsequently became colonised by *P. aeruginosa* (determined by positive cough swab culture). Time zero indicates the time of colonisation

There were considerable variations among the patients in the pattern of saliva IgA titres over time and no clear trend could be identified. However, some of the patients (e.g. 4, 6, 10, 11 and 12) showed an elevated salivary IgA response before the positive cough swabs were obtained. This experiment should be continued for a longer period of time, during which more of the non-colonised CF patients would become colonised with *P. aeruginosa*. However, this was not possible in the time available for this project. Clearly, any test method which provides an earlier diagnosis of colonisation than the current cough swab would allow appropriate therapy to be initiated with the aim of delaying progression from colonisation to active infection of the lungs.

3.2 Evaluation of Mediagnost Kit for Detection of *Pseudomonas aeruginosa* Colonisation

One commercial kit has been developed by Mediagnost (Tübingen, Germany) to identify colonisation through detection of elevated serum antibody to three major exocellular proteins of *P. aeruginosa,* exotoxin A, elastase and alkaline protease. The performance of this kit was tested on a selection of serum samples from each of the four groups. The results are shown in Table 3.2.

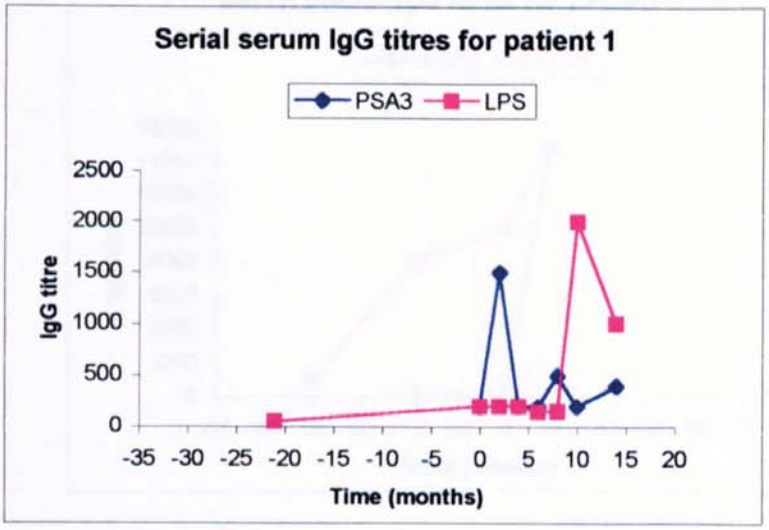
Mediagnost result	Group1 non-CF (n=8)	Group 2 CF non-Ps (n=28)	Group 3 CF intermittent (n=13)	Group 4 CF chronic (n=16)
Negative	7	20	10	0
Borderline	1	3	1	1
Positive	0	5	2	6
Chronic	0	0	0	9

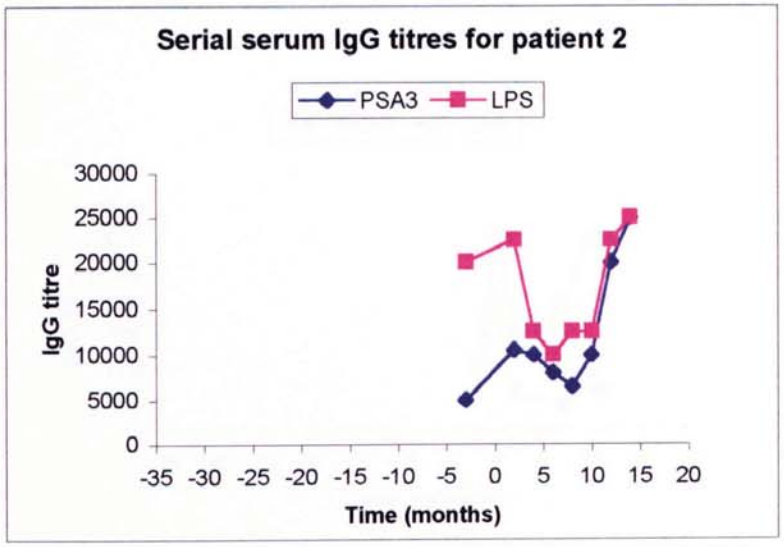
Table 3.2 Evaluation of Mediagnost test kit to detect *P. aeruginosa* colonisation in 4 groups of patients
Results show the number of patients in each group identified as negative, borderline, positive and chronic according to the manufacturer's instructions.

Out of the 65 Patients tested 47 gave results which were consistent with their clinical status. Seven of the eight patients in group 1 and 20 of the 28 patients in group 2 were confirmed as negative. Only two of the group 3 patients were positive by the test with one identified as borderline. Of the 16 group 4 patients, nine were chronic, six positive and one borderline. No false negatives were found in this group suggesting 100 % sensitivity. The three borderline and five positive detected in the group 2 patients indicate that they may have been colonised at this stage but remained undetected by clinical and microbiological criteria.

3.3 Comparison between Serum IgG Antibody Response to PSA3 and Rough LPS in CF Patients during Colonisation by *Pseudomonas aeruginosa*

The assay used to evaluate the levels of IgG antibodies in serum from CF patients employs PSA3, a mixture of cellular antigens from 17 different O-serotype strains of *P. aeruginosa*. This antigen mixture, containing a wide range of cellular antigens has been used previously to monitor serum antibody levels in CF patients (Pressler *et al.*, 1994). It has been assumed that the principal common antigenic component of *P. aeruginosa* is LPS (Pier, 2000). To investigate this further antibody levels against PSA3 were compared with those against LPS prepared from a single rough strain of *P. aeruginosa*, containing the core LPS common to all O serotypes and present in non-typable rough strains. Sera used for this study were obtained from 13 CF patients shown in Figure 3.5 who acquired *P. aeruginosa* during the time of the study. Results are shown in Figure 3.6. Time zero on the chart scale represents the date when *P. aeruginosa* was first grown from cough swabs.

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PSA3
LPS
IgG titre
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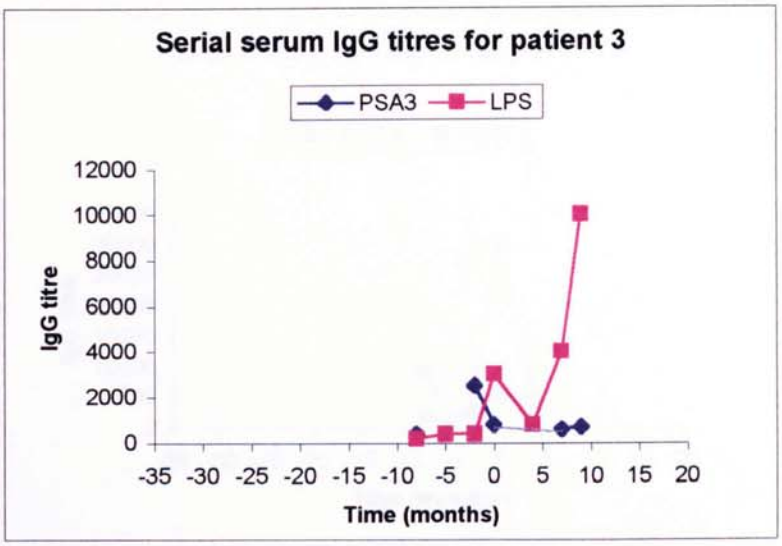
25000
20000
15000
10000
5000
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20
Time (months)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PSA3
LPS
IgG titre
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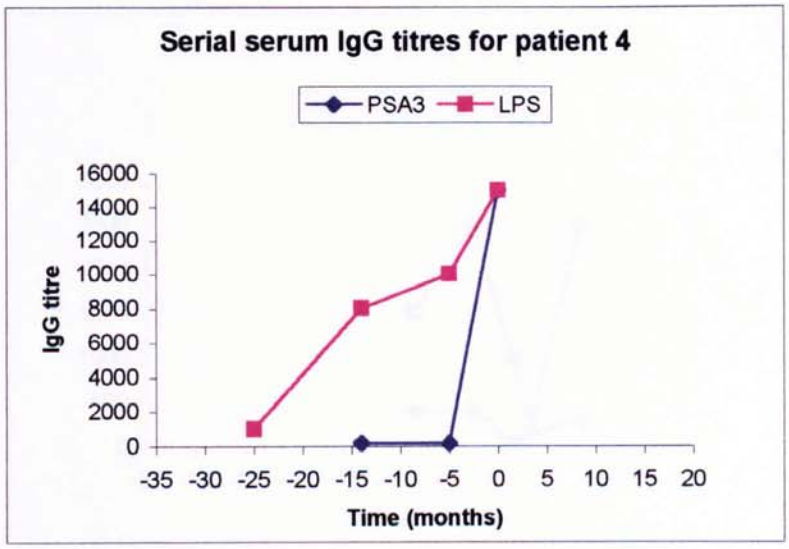
10000
8000
6000
4000
2000
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20
Time (months)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PSA3
LPS
IgG titre
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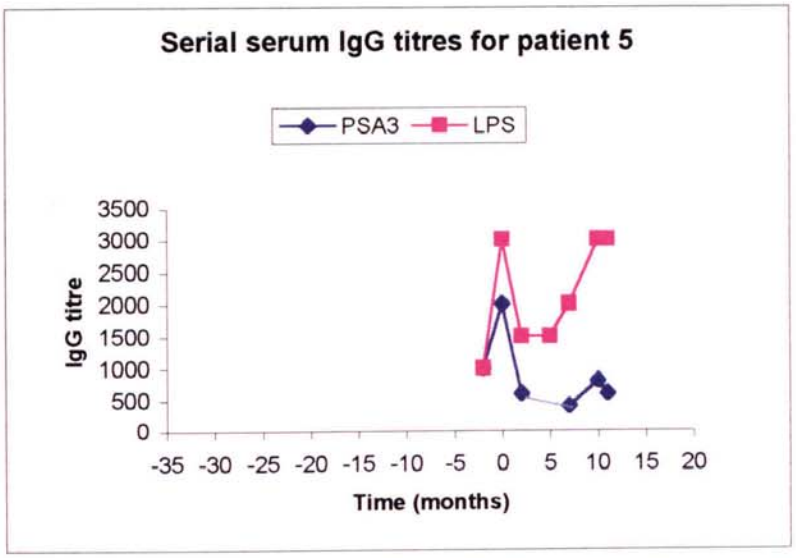
14000
12000
10000
8000
6000
4000
2000
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20
Time (months)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PSA3
LPS
IgG titre
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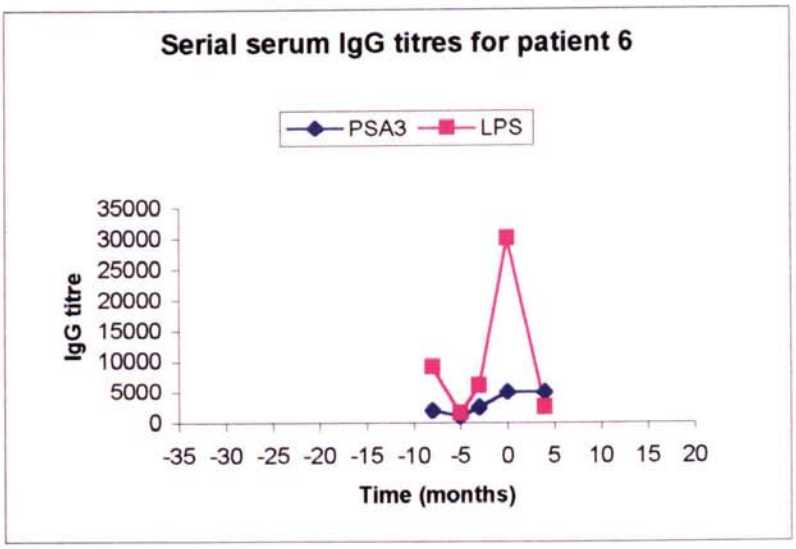
3000
2500
2000
1500
1000
500
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20
Time (months)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PSA3
LPS
IgG titre
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30000
25000
20000
15000
10000
5000
0
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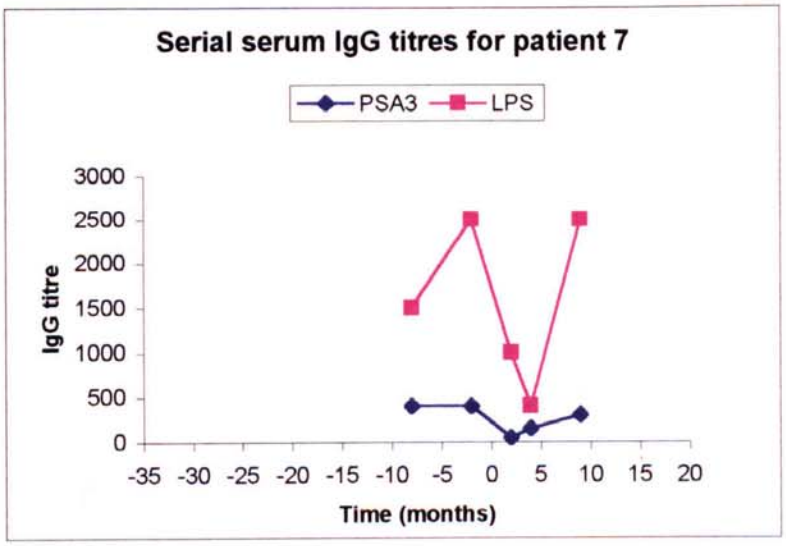
Time (months)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LPS
IgG titre
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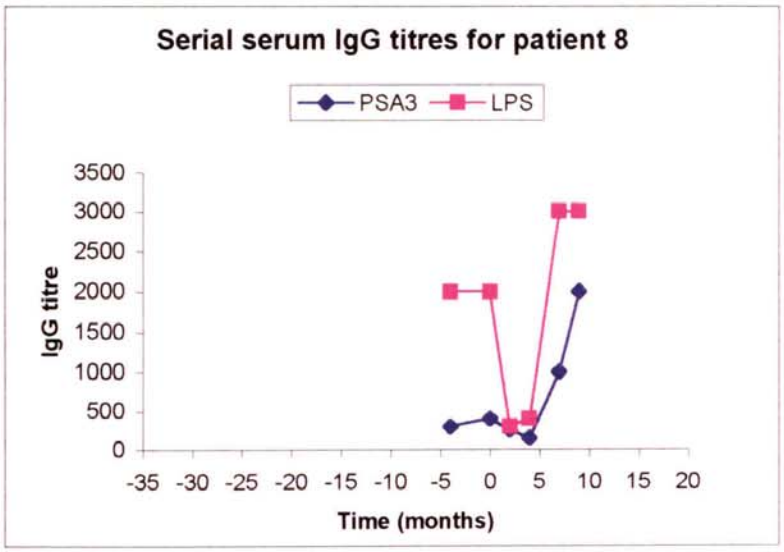
2500
2000
1500
1000
500
0
-35 -30 -25 -20 -15 -10 -5 0 5 10 15 20
Time (months)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PSA3
LPS
IgG titre
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3000
2500
2000
1500
1000
500
0
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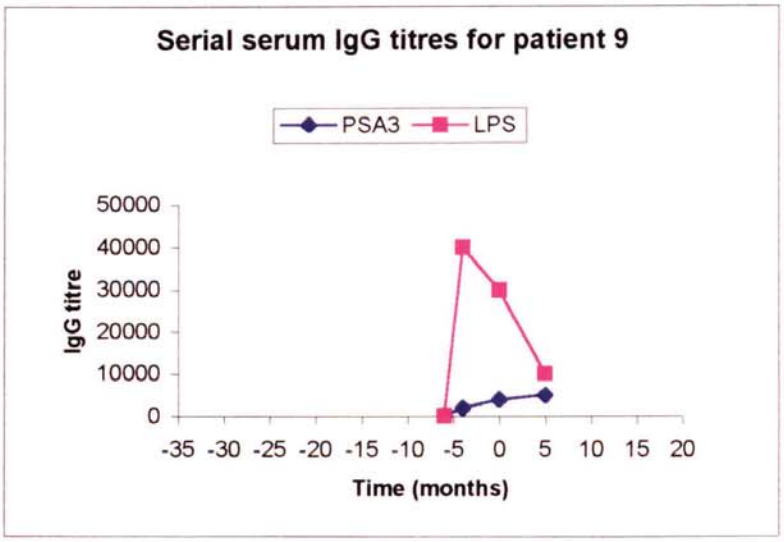
Time (months)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PSA3
LPS
IgG titre
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40000
30000
20000
10000
0
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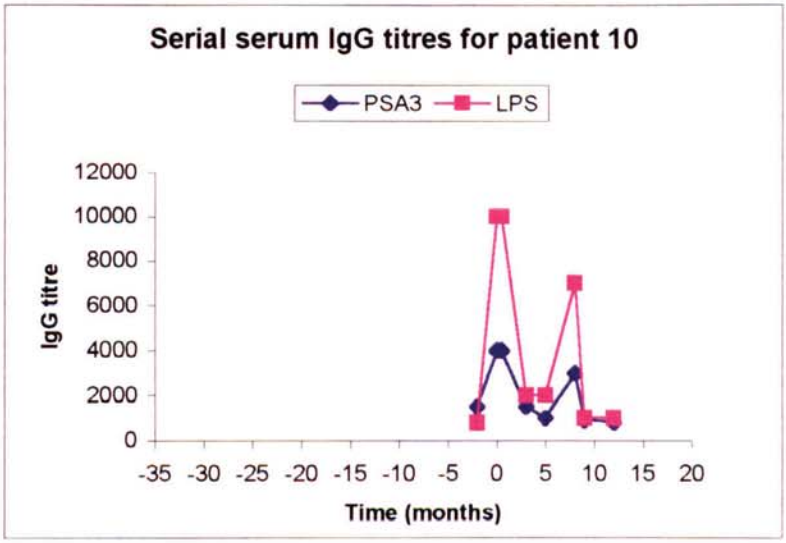
Time (months)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LPS
IgG titre
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10000
8000
6000
4000
2000
0
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Time (months)

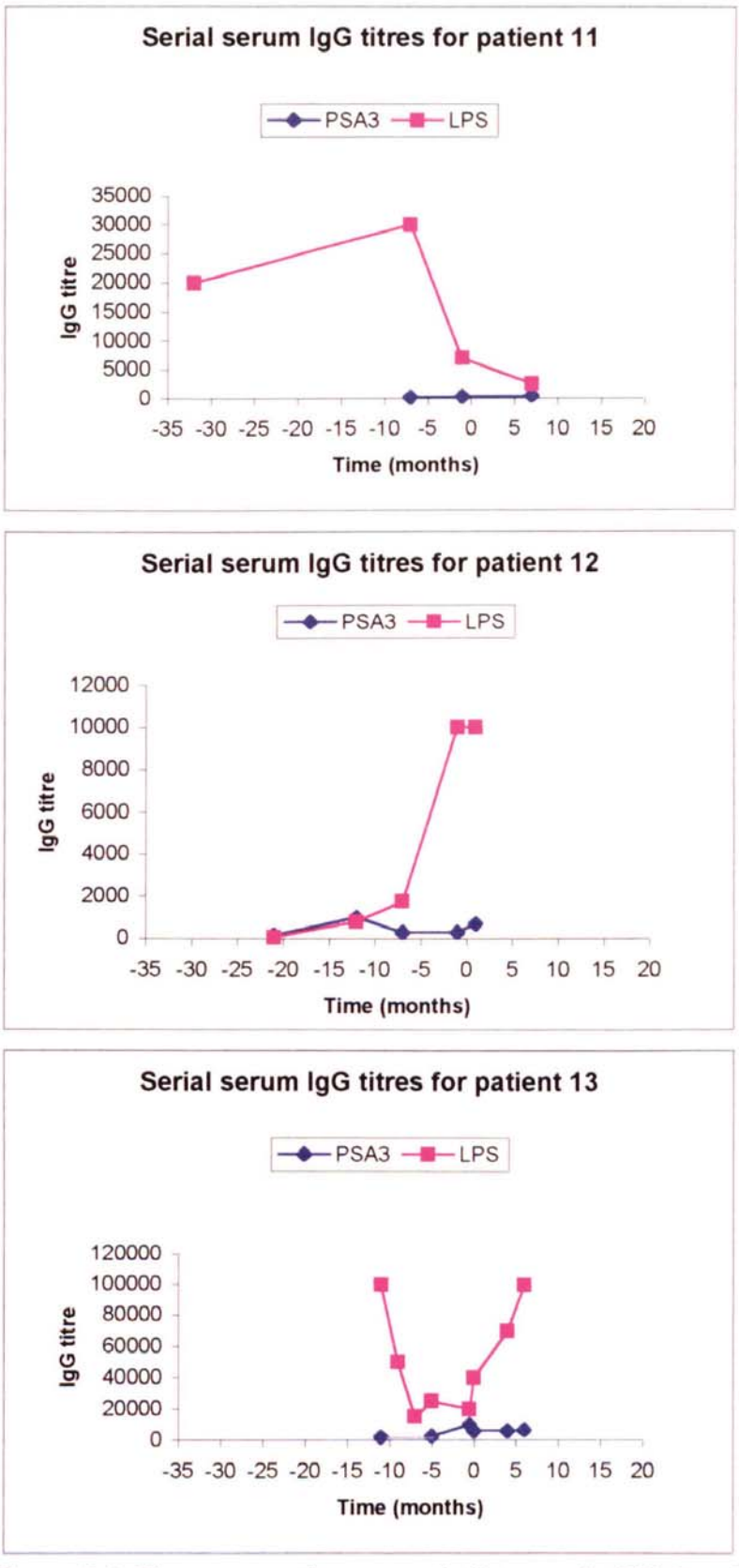


Figure 3.6 Time course for serum IgG towards PSA3 and rough LPS in 13 patients, time zero indicates the date of the first positive culture, cough swab or sputum

As found for the saliva IgA titres to PSA3, the serum IgG titres to PSA3 and rough LPS varied markedly between the different patients.

For patient 1 there was no increase of antibodies to either antigen at the time of colonisation but there was a peak level of PSA3 antibodies 2 months after time zero and a small increase after 7 months. In comparison a peak level of antibody to the rough LPS was reached 10 months after the first positive cough swab.

For patient 2 both markers give a peak level 2 months after time zero, but the levels of anti-rough LPS antibodies were twice as high than those of PSA3. Both antibodies reached similar peak levels at 14 months after the first colonisation.

Patient 3 showed a small increase of anti-rough LPS antibodies at time zero and a greater peak level at 10 months after time zero. By comparison, low titres of antibodies to PSA3 were obtained throughout the time period for this patient.

For patient 4 there was a gradual increase of antibodies to the rough LPS which started 15 months before the first diagnosed colonisation and reach the highest level at time zero. Antibodies to PSA3 give a peak level at time zero.

Patient 5 showed peak levels at time zero for both markers, the antibody level against rough LPS being higher than those against PSA3. Antibodies to rough LPS reached a peak level 10 months after colonisation, those to PAS3 rose a little at the same time.

Patient 6 only gave a peak level for antibodies to rough LPS whereas levels of PSA3 antibodies increased only slightly at time zero.

For patient 7 there were two peak levels for anti-rough LPS antibodies, the first three months before and the second 7 months after time zero. During the 20 months of the study period for this patient, levels of PSA3 antibodies stayed below a titre of 500.

For patient 8 levels of anti-rough LPS antibodies were already high at the time the study started and dropped after time zero. A fivefold increase appeared 5 months after the first colonisation. PSA3 antibodies only gave a peak level 8 months after time zero.

Patient 9 showed a peak level for anti-rough LPS antibodies 5 months before the first colonisation was diagnosed. Levels of PSA3 antibodies rose slowly during the 10 months study period.

For patient 10 there were similar peak levels for both markers at the time of colonisation and at 7 months after colonisation. Both times peak levels of LPS antibodies were twice as high as those of PSA3.

Patient 11 had the highest level of anti-rough LPS antibodies 8 months before the first colonisation was detected, levels dropped from there on. During the course of the study, levels of PSA3 antibodies remained low.

For patient 12 there was a peak level of anti-rough LPS antibodies at time zero whilst the level of PSA3 antibodies stayed very low through out the study.

Patient 13 gave two peak levels of anti-rough LPS antibodies 12 months before and 5 months after time zero. Levels of PSA3 antibodies did not increase during the 18 months study.

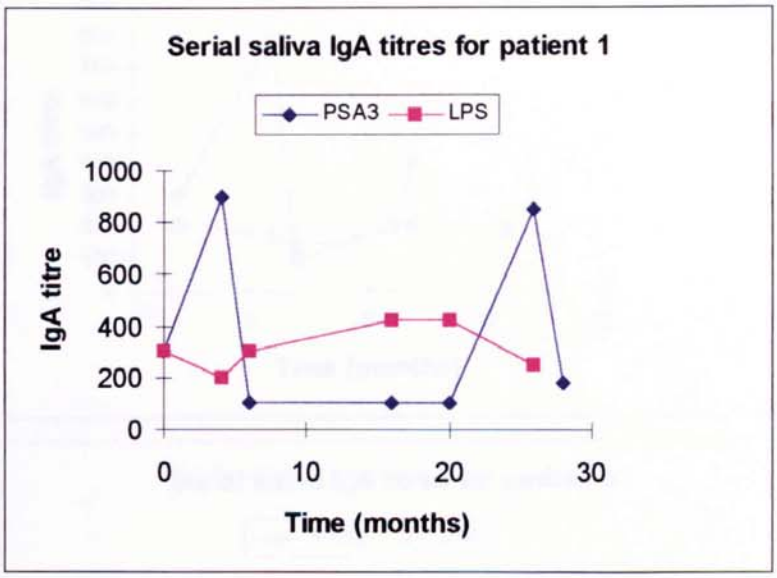
Overall seven out of the 13 patients had very low levels of PSA3 antibodies which did not give peaks during the time of the study. Ten patients had levels of anti-rough LPS antibodies rising up to at least 2000, 5 months before the time of colonisation, only three patients had peak levels for PSA3 antibodies during the same period. For only one patient (patient 1) PSA3 antibodies gave a peak level before rough LPS antibodies, but 2 months after the first colonisation.

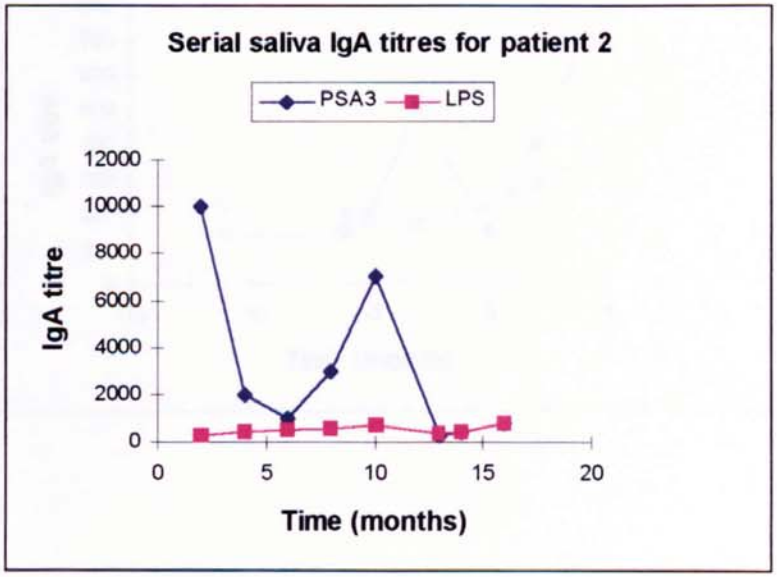
For the eight patients for which the study was carried out over 5 months after time zero, five showed peaks levels of anti-rough LPS antibodies between 5 to 15 months after colonisation but not for PSA3 antibodies. Sera from three patients showed peak levels for both markers at the same time during this period.

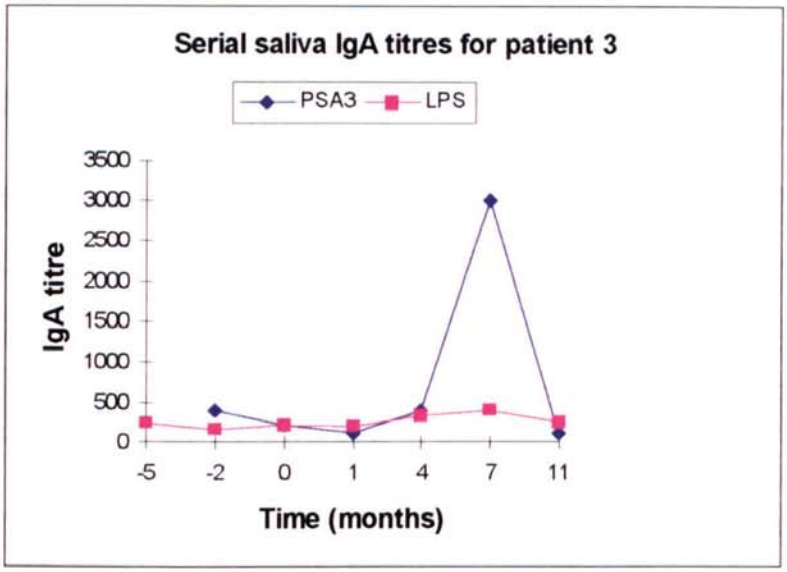
It can be concluded that this study that measurement of serum antibodies against rough (core) LPS gives a far more sensitive test for *P. aeruginosa* colonisation than does PSA3.

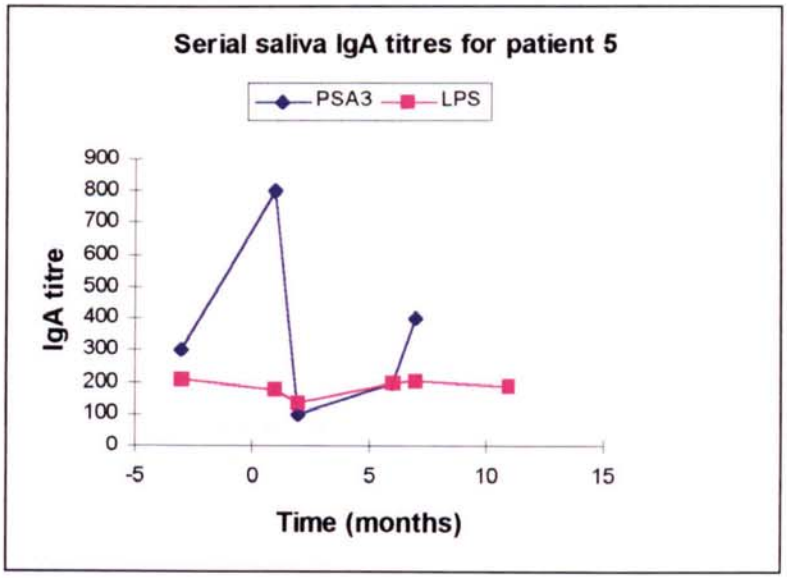
3.4 Comparison between Saliva IgA Antibodies to PSA3 and a Rough LPS from *Pseudomonas aeruginosa*

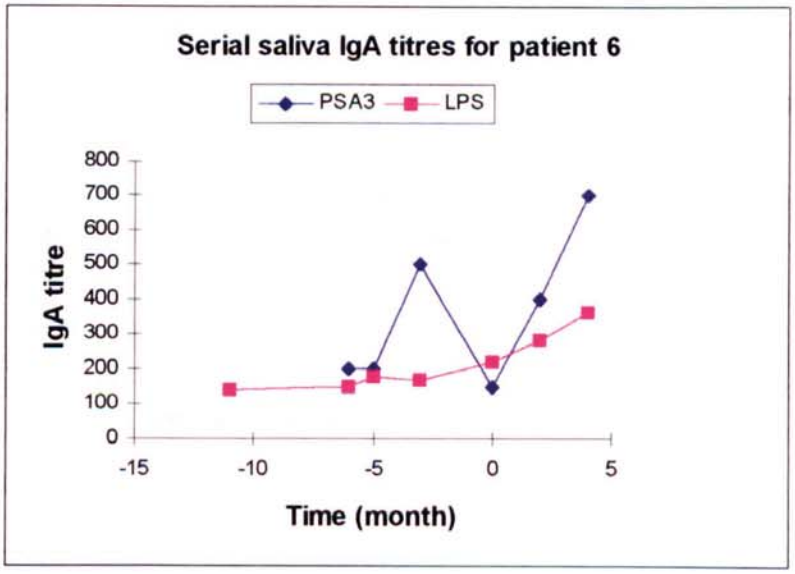
Because of the encouraging results obtained for the rough LPS in detecting elevated serum IgG titres, experiments were also conducted on saliva samples to measure IgA levels. The diagnosis of *P. aeruginosa* infection through detection of IgA in saliva would be less invasive than diagnosis via serum. Saliva samples from eleven CF patients were tested using ELISA plates coated either with PSA3 or LPS from a rough strain of *P. aeruginosa.* Results are shown in Figure 3.7.

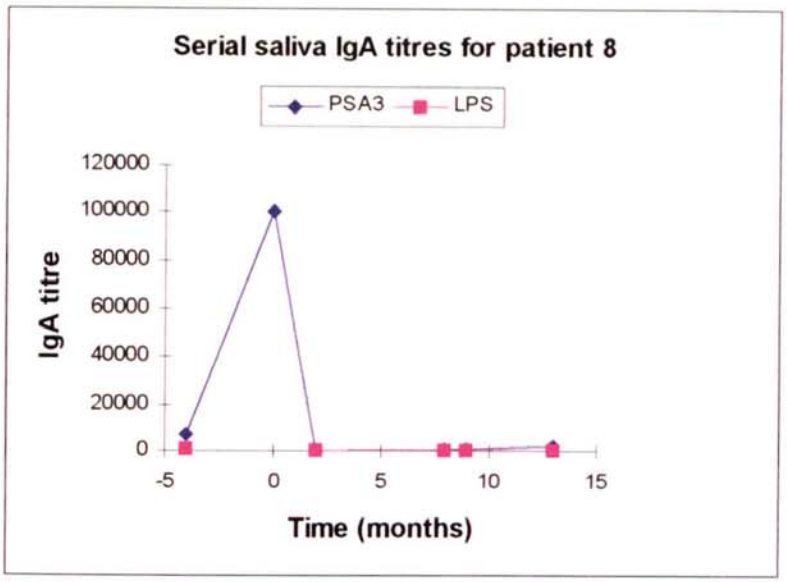


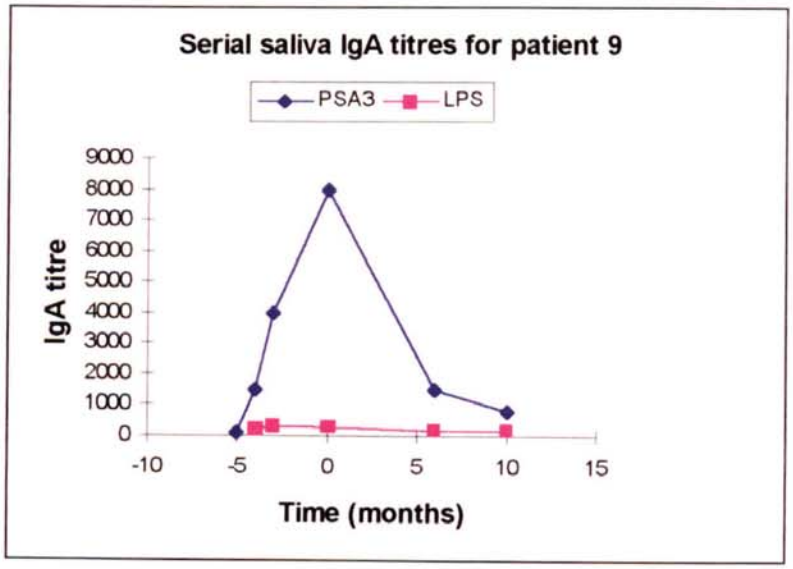


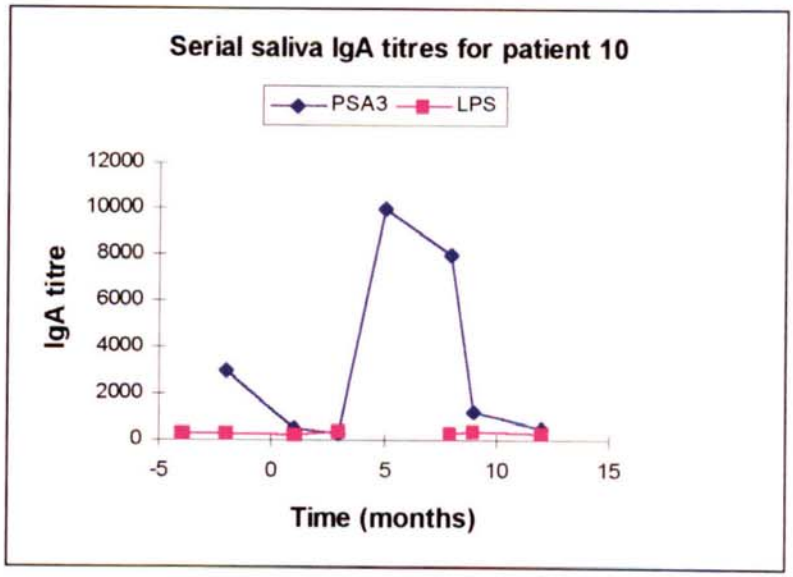

Serial saliva IgA titres for patient 3
PSA3
LPS
IgA titre
3500
3000
2500
2000
1500
1000
500
0
-5
-2
0
1
4
7
11
Time (months)

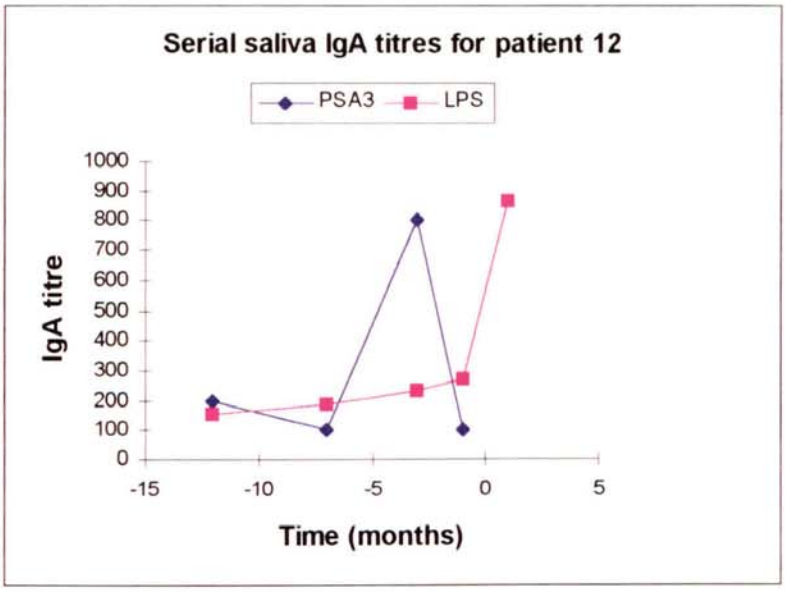

Serial saliva IgA titres for patient 5
PSA3
LPS
IgA titre
900
800
700
600
500
400
300
200
100
0
-5
0
5
10
15
Time (months)


Serial saliva IgA titres for patient 6
PSA3
LPS
IgA titre
800
700
600
500
400
300
200
100
0
-15
-10
-5
0
5
Time (month)


Serial saliva IgA titres for patient 8
PSA3
LPS
120000
100000
80000
60000
40000
20000
0
IgA titre
-5
0
5
10
15
Time (months)


Serial saliva IgA titres for patient 9
PSA3
LPS
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
IgA titre
-10
-5
0
5
10
15
Time (months)


Serial saliva IgA titres for patient 10
PSA3
LPS
12000
10000
8000
6000
4000
2000
0
IgA titre
-5
0
5
10
15
Time (months)



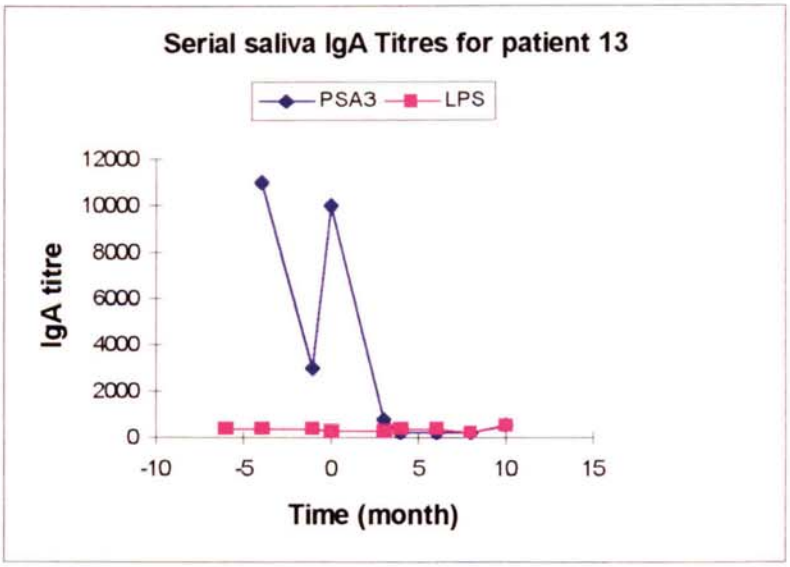


Figure 3.7 Time course for saliva IgA towards PSA3 and LPS in ten patients, time zero indicates the date of the first positive culture, cough swab or sputum

Out of the ten patients studied, only one showed a peak level of saliva IgA to LPS at the time of colonisation. For the other nine patients levels of saliva IgA antibodies to LPS stayed the same during the time of the study. Five patients gave peak levels of PSA3 antibodies at time zero, five had peak levels of PSA3 IgA during the 5 month period after the first positive cough swab. For eight patients, saliva samples were taken at least 5 months before colonisation, three of them gave peak levels of PSA3 IgA before time zero and other peaks at time zero or 2 months later.

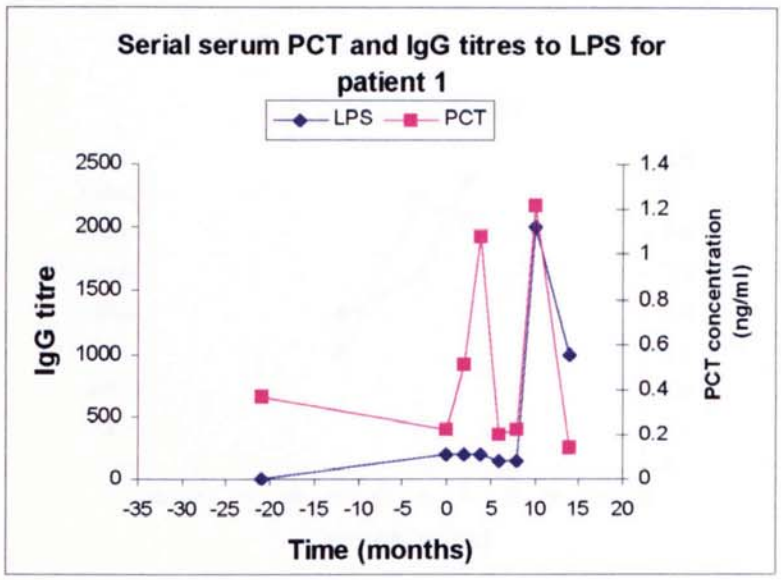
Overall, in contrast to the serum IgG titres which were higher to rough LPS than to PSA3, the PSA3 antigen generally produced higher saliva IgA titres. This suggests that some of the antigens present in the PSA3 are more readily detected by IgA in saliva than by IgG in serum. It is not possible to identify these antigens at present but they are presumably not present in the rough LPS antigen.

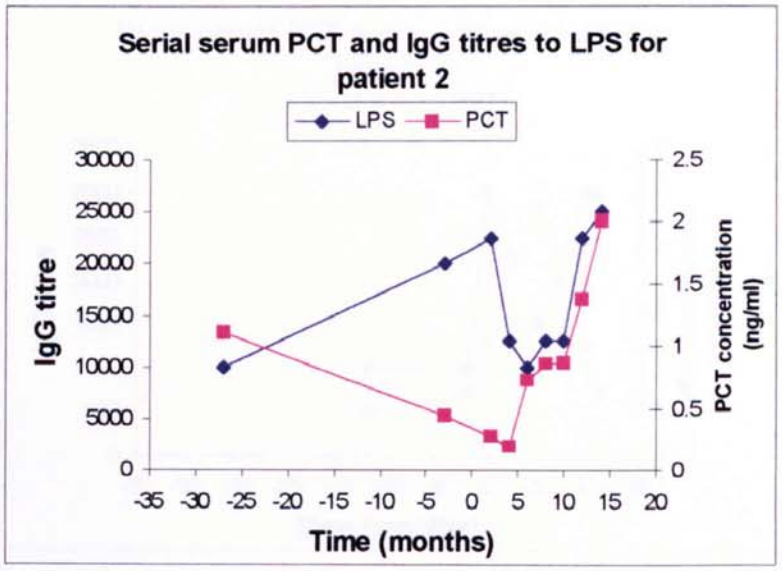
It appears from these results that the quantification of saliva IgA to LPS from *P. aeruginosa* cannot be used as a method of detection for *P. aeruginosa* colonisation in CF patients.

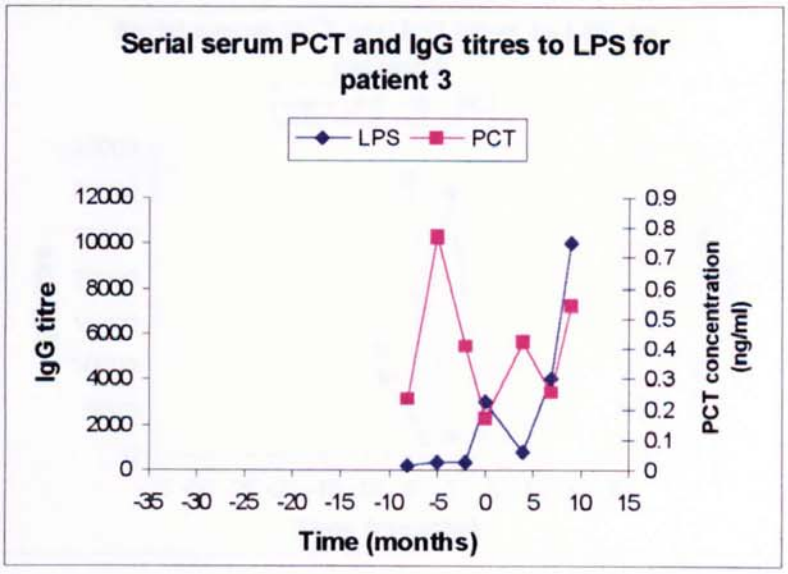
3.5 Evaluation of Procalcitonin (PCT) as a Marker of *Pseudomonas aeruginosa* Infection

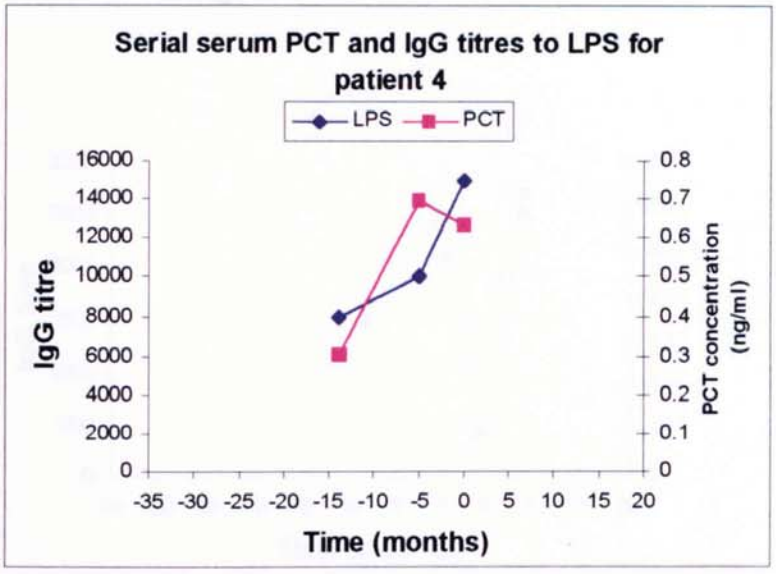
PCT has been considered as a potential marker of septicaemia. To investigate its potential as a marker of *P. aeruginosa* infection in CF, serum levels of PCT in CF patients who had become colonised with *P. aeruginosa* were measured and compared with serum levels of IgG antibodies to rough LPS. Results are shown in Figure 3.8.

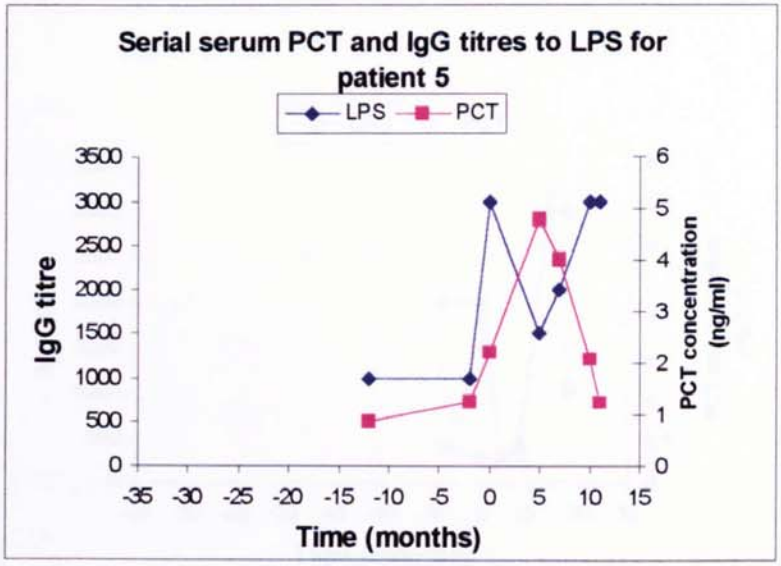
The zero on the time axis of the graphs represents the first time that *P. aeruginosa* was cultured from a cough swab.

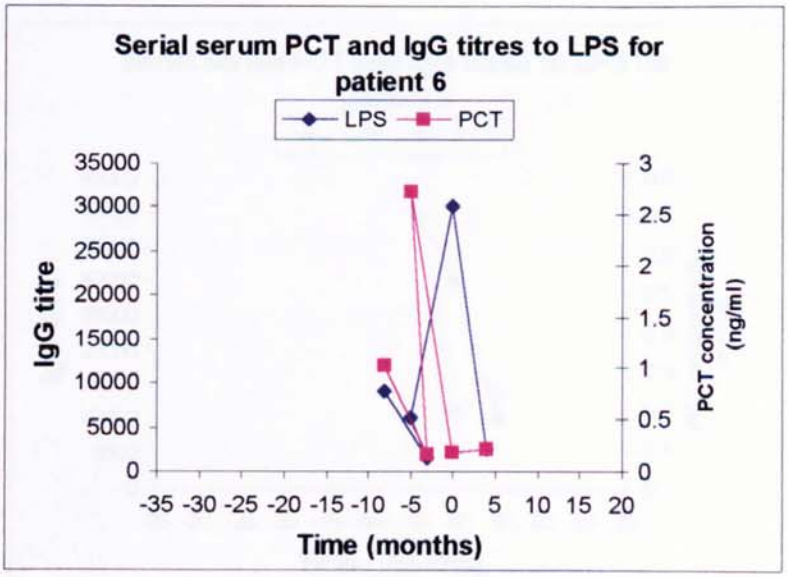

Serial serum PCT and IgG titres to LPS for patient 1
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

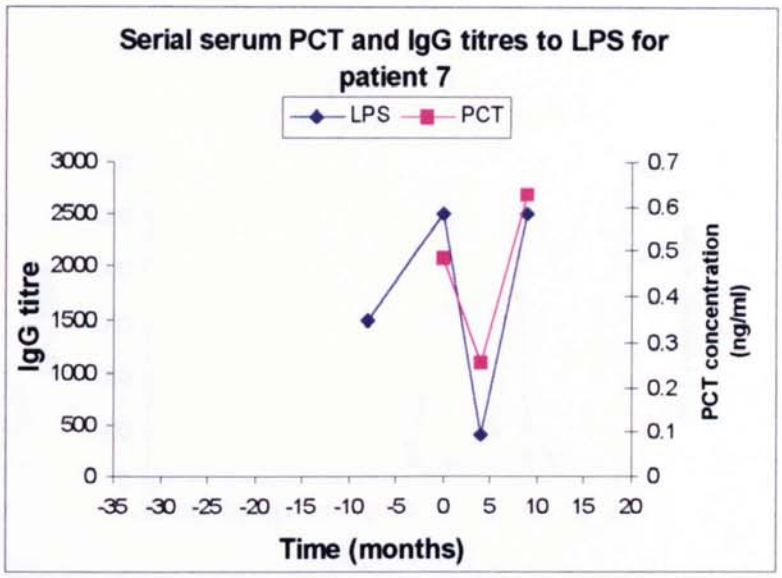

Serial serum PCT and IgG titres to LPS for patient 2
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

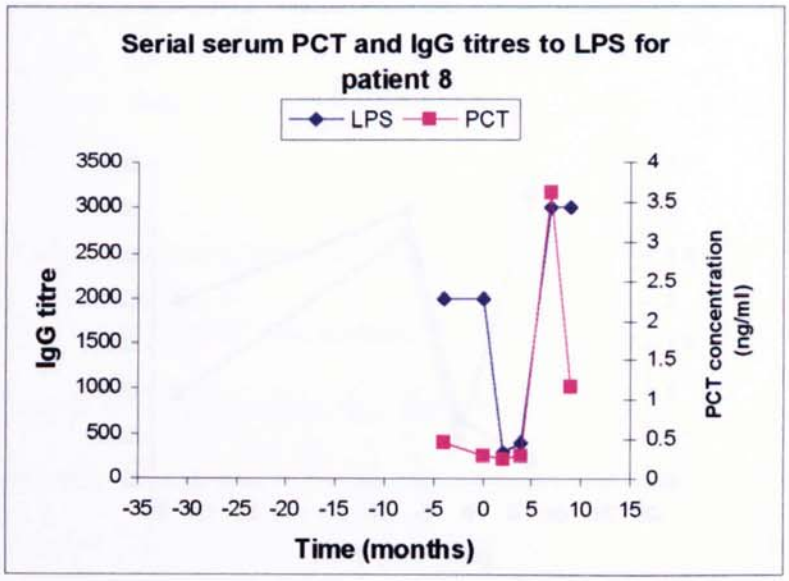

Serial serum PCT and IgG titres to LPS for patient 3
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

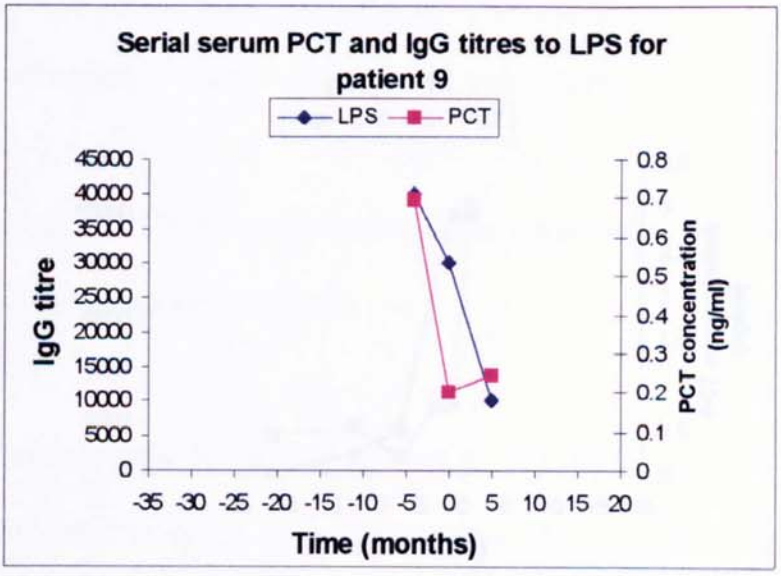

Serial serum PCT and IgG titres to LPS for patient 4
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

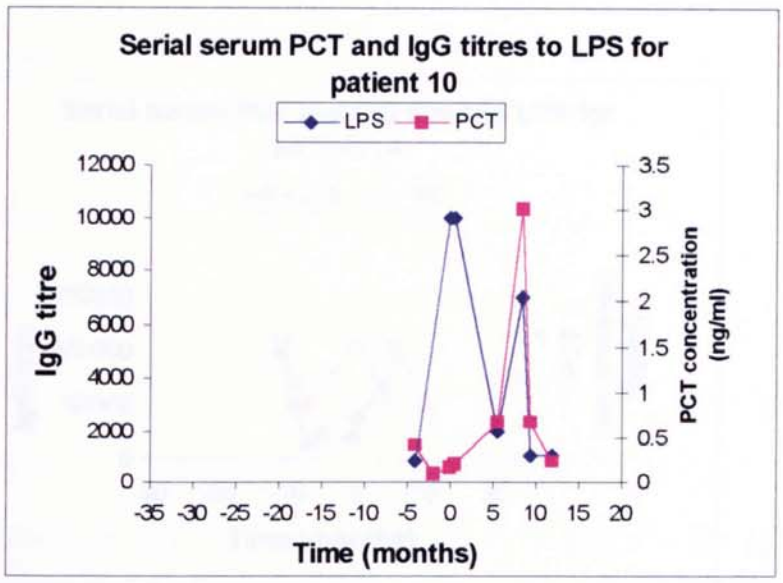

Serial serum PCT and IgG titres to LPS for patient 5
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

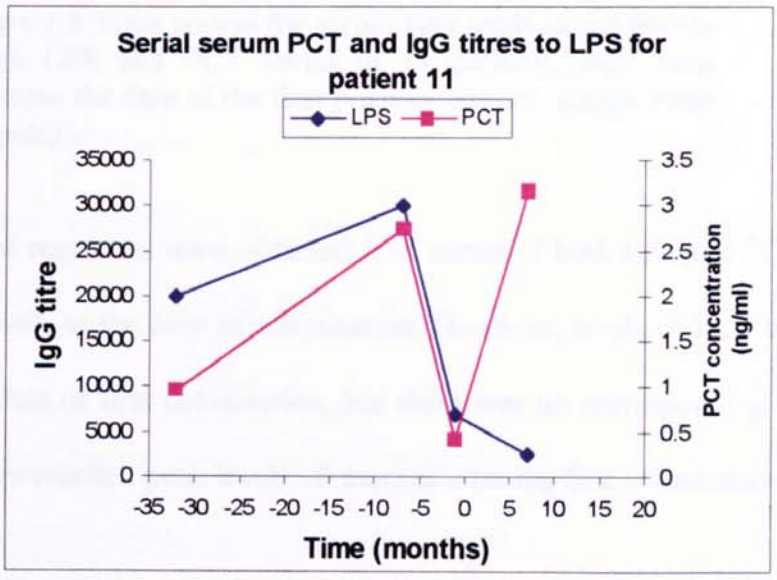

Serial serum PCT and IgG titres to LPS for patient 6
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

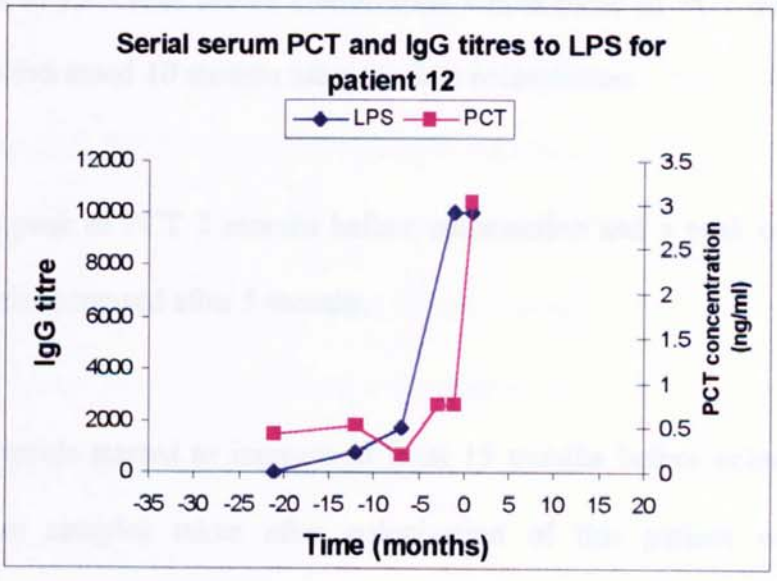

Serial serum PCT and IgG titres to LPS for patient 7
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)


Serial serum PCT and IgG titres to LPS for patient 8
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)


Serial serum PCT and IgG titres to LPS for patient 9
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)


Serial serum PCT and IgG titres to LPS for patient 10
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)


Serial serum PCT and IgG titres to LPS for patient 11
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)


Serial serum PCT and IgG titres to LPS for patient 12
LPS
PCT
IgG titre
PCT concentration (ng/ml)
Time (months)

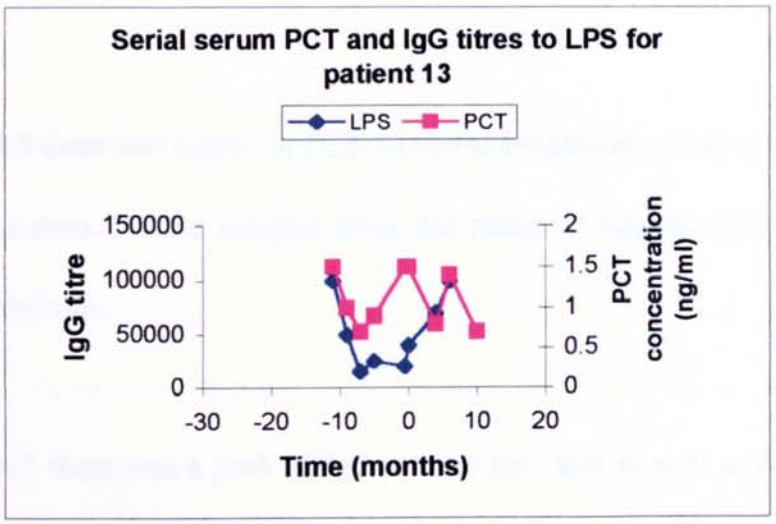


Figure 3.8 Time course for serum IgG antibodies towards rough LPS and PCT levels in 13 patients, time zero indicates the date of the first positive culture, cough swab or sputum

A range of different responses were obtained. For patient 1 both IgG and PCT failed to show elevated levels at the time of colonisation. However, levels of PCT started to rise following the date of first colonisation, but there was no corresponding increase in IgG. Both markers reached peak levels 10 months after the first colonisation.

For patient 2 levels of LPS rose before colonisation whilst those of PCT decreased, both markers levels increased 10 months after the first colonisation.

Patient 3 showed a peak of PCT 5 months before colonisation and a peak of IgG at time zero. Both levels increased after 5 months.

For patient 4 both levels started to increase at least 15 months before colonisation. Unfortunately serum samples taken after colonisation of this patient were not available at the time of the study.

Patient 5 gave a peak of IgG at the time of colonisation and a peak of PCT 5 months after colonisation. Levels of IgG increased again after months.

For patient 6 there was a peak of PCT 2 months before colonisation and a peak of IgG at time zero. Further samples from this patient 5 months after colonisation were not available.

For patient 7 there was a peak of IgG at time zero and as well as high levels of PCT. Both levels increased again between 5 and 10 months after colonisation.

For patient 8, 5 months before colonisation levels of IgG were already high whilst those of PCT were low. PCT gave a peak after 6 months and the increase of IgG at the same time stayed high for at least 10 months.

For patient 9 both marker levels appeared to be high 4 months before the first positive *P. aeruginosa* culture and decreased thereafter.

Patient 10 showed two IgG peaks, the first one at the time of colonisation, the second 9 months later. PCT only gave a peak at 9 months.

For patient 11 both levels increased until 5 months before first colonisation and dropped at time zero. PCT levels peaked at 5 months after the first colonisation whilst the IgG levels continued to decrease.

For patient 12 both markers gave a peak at the time of colonisation but serum samples after colonisation were not available.

For patient 13 both levels were high 12 months before the first positive cough swab and decreased to reach their lowest levels at 7 months before time zero. In this case PCT gave a peak at time zero whereas levels of IgG started increasing at time zero. There was another peak level of PCT 5 months after the first colonisation.

Overall five out of 13 PCT levels and IgG titres gave similar peak levels at the time of colonisation. Three out of 13 patients gave a peak level of PCT before IgG levels could show colonisation. Four patients had a peak of IgG at time zero before PCT levels increased. Finally one patient (1) failed to show any increase of the markers before colonisation was detected by cough swabs culture.

Seven patients showed similar peaks of PCT and IgG between 5 and 10 months after the first colonisation. For five others no samples were available from 5 months onwards. For only one patient were both peaks in that period different. These peaks could be explained by the fact that *P. aeruginosa* is never eradicated from the CF lungs (Koch and Høiby, 1993) and there are periods of exacerbation of local infection where sputum counts increase dramatically.

Four patients had elevated levels of one marker (either PCT or IgG) before the first *P. aeruginosa* was cultured from the swabs. In these cases the serum markers

were clearly more effective in detecting colonisation than the cough swabs, illustrating their potential value.

Overall conclusions from this study are that a combination of both serum markers would be needed to detect accurately colonisation before the conventional microbiological methods. The study has shown that colonisation in a significant number of patients would be detected earlier by these methods than by microbiology alone. Presumably this would permit appropriate antibiotic therapy to be initiated at an earlier stage and thereby delay the progression from colonisation to full infection.

CHAPTER 4. ANTIBODY RESPONSE TO *Burkholderia cepacia*

B. cepacia poses a major threat to CF patients. In many cases, colonisation of the lungs leads to rapid progression of infection and death by 'cepacia syndrome' (Govan and Deretic, 1996). In this chapter serological methods for the early detection of *B. cepacia* colonisation were investigated. Since patients are likely to be already colonised with *P. aeruginosa* it is essential to employ antigens which are specific for *B. cepacia.* Previous studies using western blotting have shown that the major OMPs of *B. cepacia* have potential in the serodiagnosis of *B. cepacia* colonisation in patients who are already infected with *P. aeruginosa* (Lacy *et al.*, 1995). Two characteristic SDS-PAGE profiles are usually found for OMPs of *B. cepacia* from CF (Livesley *et al.*, 1998). Type B profiles contain major OMPs of 81 and 90 kDa whilst type A profiles contain OMPs of 36-39kDa, 27 kDa and 18 kDa. One CF strain of each OMP type was selected from the Aston University Microbiology Group Culture Collection (strain 5TIV, type A and 5JIV, type B).

Figure 4.1 shows the Coomassie blue stained OMP profiles obtained by SDS-PAGE for sarkosyl-prepared OMs of strains 5JIV and 5TIV.

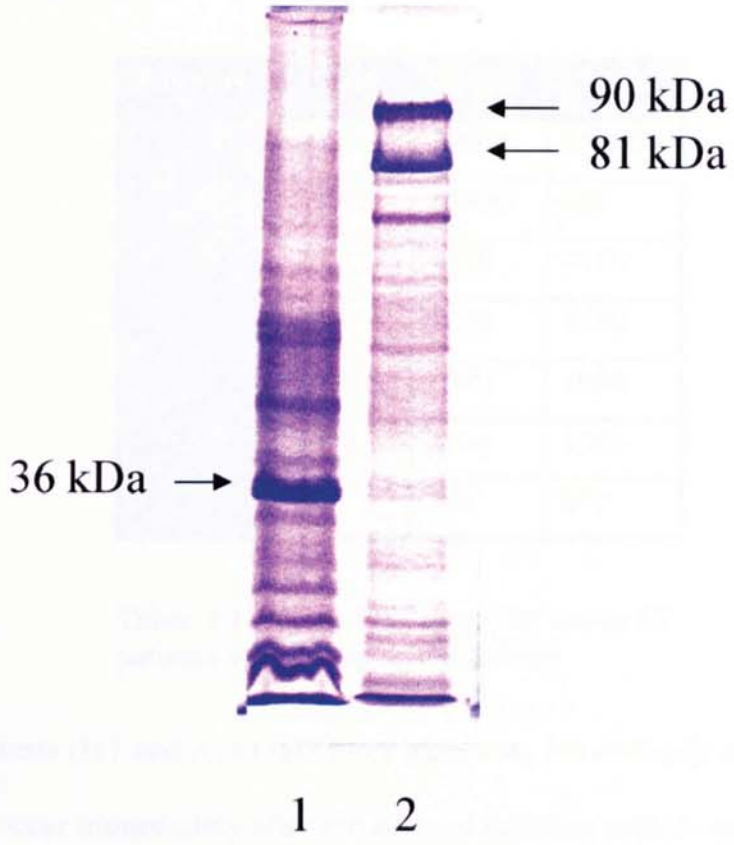


Figure 4.1 Coomassie blue-stained SDS PAGE profiles of sarkosyl OM preparations from *B. cepacia* strains 5JIV (lane 1) and 5TIV (lane 2)

The 36 kDa, 81 kDa and 90 kDa outer membrane proteins were electroeluted from SDS-PAGE gels of sarkosyl-prepared OMs (method 2.4) and used as antigens to coat ELISA plates. Plates were reacted with serum from seven CF patients with *B. cepacia* lung infection (selected from the Aston University Microbiology Research Group serum collection) and developed with anti-human IgG-peroxidase conjugate (Table 4.1).

Patients	36 kDa	90 kDa	81 kDa
O83	4000	70000	25000
G76	15000	15000	600
I17	200	<100	<100
N53	4000	1200	1500
G19	4000	1200	1000
N67	5000	2000	1500
A38	200	200	200

Table 4.1 Serum IgG titres for seven CF patients with *B. cepacia* infection

For two patients (I17 and A38) IgG titres were low, but antibody production does not always occur immediately after the first colonisation with *B. cepacia* (Lacy *et al.*, 1997). For three patients (N53, G19 and N67) IgG titres to the 36 kDa protein were higher than those to either the 90 or 81 kDa proteins. For patient O83, IgG titre to the 90 kDa protein was greater than to all the others. Overall, the 90 kDa and 81 kDa protein appeared to be good markers of *B. cepacia* colonisation but with slightly lower antibody levels than the 36 kDa protein.

Possible cross-reacting antibodies to *P. aeruginosa* were removed from four of the serum samples by incubating with PSA3 antigen and centrifugation to deposit immune complexes. The residual absorbed sera were then tested again in the ELISA using the 36 kDa protein (Table 4.2).

NAME	O83	O83+	G76	G76+	G19	G19+	A38	A38+
TITRE	12500	17500	30000	15000	4000	8000	550	700

Table 4.2 Serum IgG titres against the 36 kDa protein for four CF patients with *B. cepacia* infection before and after (+) absorption with PSA3 antigen

An unexpected slight increase in the IgG titres was obtained for three of the sera (O83, G19 and A38) and a 50 % decrease for the other sample (G76). These small variations were not thought to be significant and suggest that the antibody response detected in the ELISA was not affected by existing anti-*P. aeruginosa* IgG in the patients' sera (each patient was also infected with *P. aeruginosa*). The results therefore indicate that the patients' sera contained antibodies directed against one or more of the OM proteins eluted from the SDS-PAGE gel or to residual *B. cepacia* LPS associated with the proteins. To investigate the specificity of the antibodies, ELISA assays were carried out using serum absorbed with *B. cepacia* LPS. No reduction in titre was detected, suggesting that the patient antibody response was directed against the outer membrane proteins.

Five out of seven patients had high levels of IgG against 36 kDa protein and there was no cross-reaction with *B. cepacia* LPS and only one out of four had a cross-reaction with *P. aeruginosa*. This protein therefore has potential for exploitation in the serodiagnosis of *B. cepacia* colonisation as reported by Lacy *et al.* (1997). It is interesting to note that the 81 kDa OMP is probably an aggregate of the 36 kDa and 27 kDa OMPs. Previous studies have shown that the 80 kDa protein dissociates into components of 36 and 27 kDa after electroelution from gels and removal of LPS (Parr *et al.*, 1987). More samples would be needed to investigate the specificity of the 80 and 91 kDa proteins in detecting *B. cepacia* colonisation.

However since no more *B. cepacia* colonised patients were admitted to the Birmingham Children's Hospital during this period the study could not be continued.

CHAPTER 5. EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF POLYMYXINS IN THE TREATMENT OF *Pseudomonas aeruginosa* INFECTION IN CF

This part of the project concerned the mechanism of action of colistin E, the polymyxin antibiotic which has an important role in the treatment of *P. aeruginosa* lung infection in CF. Colomycin® (Pharmax) and Colymycin® (Parke-Davis) are the two commercially available forms of colistin E used. They are identical sulphomethate prodrug derivatives of colistin E. Colomycin® is mainly used in the UK and Europe whilst Colymycin® is used in the USA. Because samples of the active form of these drugs, i.e. colistin E sulphate, were not available, the closely related Polymyxin B sulphate was used as a reference material.

5.1 Polymyxin Resistant *Pseudomonas aeruginosa* Strains

Table 5.1 shows the MICs of twelve clinical isolates of *P. aeruginosa* obtained from Booth Hall Hospital, Manchester and the reference strain PAO1 (ATCC 15692) to colomycin and polymyxin B sulphate.

CODE	MIC colomycin (μg/ml)	MIC polymyxin B (μg/ml)
PAO1	4	0.5
C065	2	0.3
D139	4	0.5
E053	2	1
E093	2	2
E116	2	2
E124	16	4
H047	4	4
I121	2	1
I138	8	4
J024	2	2
L008	2	4
L022	2	4

Table 5.1 MICs of *P. aeruginosa* for colomycin and polymyxin B towards twelve clinical isolates and PAO1.

Only twelve of the nineteen strains obtained from Booth Hall Hospital in Manchester were confirmed by API 20NE as 99 % *P. aeruginosa*. Of these, only E124 and I138 had MICs for colomycin greater than PAO1, whereas nearly every strain had an MIC for polymyxin B greater than PAO1. This may relate to the difference in structure between colomycin and polymyxin B. Colomycin is the sulphomethate derivative of colistin E, which must hydrolyse in the growth medium to release the active form of the drug (i.e. colistin E). It is not clear when determining the MIC by the broth tube dilution method at which stage following inoculation, enough of the active form of the antibiotic would be produced by

hydrolysis to inhibit growth of the inoculum. By contrast, polymyxin B is the active form of the drug and would exert its antimicrobial action immediately the inoculum is added to the growth medium in the MIC tubes. This might explain why polymyxin B appears to be more active than colomycin. However, it is interesting to note that four of the twelve strains examined showed the same MIC to polymyxin B and colomycin.

Coomassie blue stained SDS-PAGE gels of outer membrane preparations (Figure 5.1) showed that all of the strains with colomycin MICs > 4 expressed OprH (protein H1) in their outer membrane, except E124.

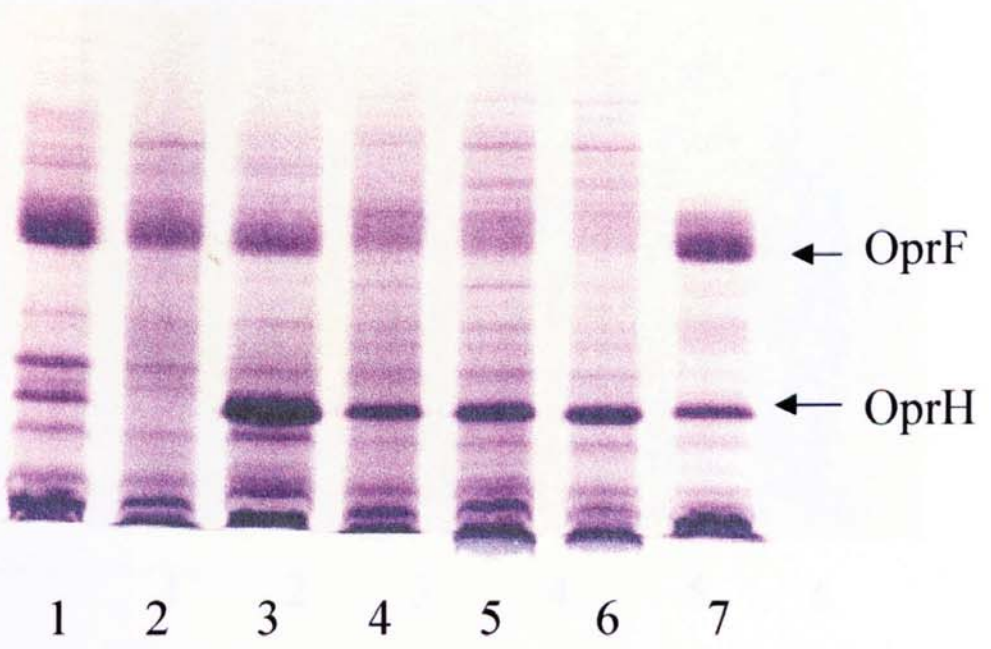


Figure 5.1 Coomassie blue stained SDS-PAGE gel of sarkosyl-prepared outer membranes of isolates of *P. aeruginosa.* Lane 1, PAO1; lane 2, E124; lane 3, I138; lane 4, D139; lane 5, JO24; lane 6, HO47; lane 7, CO65

Over-expression of OprH has been linked with polymyxin resistance in a number of studies (Nicas and Hancock, 1980). Resistance has been explained in terms of blocking of polymyxin binding sites in LPS by OprH. The absence of OprH in

strain E124 suggested a different mechanism of resistance. This was further explored by examination of LPS content of the cells by treatment of outer membranes with proteinase K, followed by separation by SDS-PAGE and silver staining (Figure 5.2). Commercial samples of purified LPS from *E. coli* serotype O111:B4 and *P. aeruginosa* serotype 10 (Sigma), together with protease K-digested outer membranes from *P. aeruginosa* PAO1 and NCTC 10554 were included as controls.

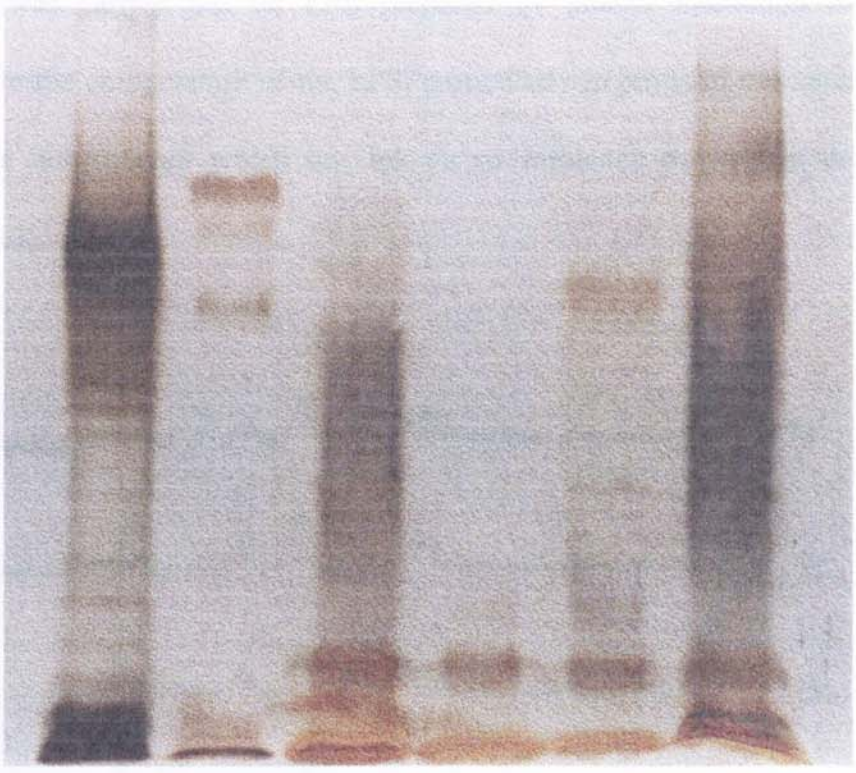


Figure 5.2 Silver-stained SDS-PAGE gel of LPS from isolates of *P. aeruginosa.* Lane 1, *E. coli* O111:B4 control; lane 2, Sigma LPS; lane 3, PAO1; Lane 4, E124; lane 5, *P. aeruginosa* NCTC 10554; lane 6, I138

The results show that strain E124 contained considerably less stained material than I138. The silver stain cannot accurately quantify the amount of LPS present because the amount of staining is dependent on the nature of the sugars present in

the LPS. However, since equivalent amounts of outer membranes for strains E124 and I138 were digested with protease K and loaded onto gels (i.e. the amounts showed in lanes 2 and 3 of Figure 5.1), the silver stain indicates that E124 contains less LPS than I138.

Taken together, these experiments suggest that the resistance of E124 is not linked to over-expression of OprH, but could be due either to a difference in the amount of LPS produced or to an alteration in its structure. Therefore, ^{31}P-NMR analysis was carried out on LPS prepared by phenol extraction to further investigate the composition of the LPS, particularly in terms of the nature of the phosphate substituents which are known to influence polymyxin sensitivity (Figure 5.3).

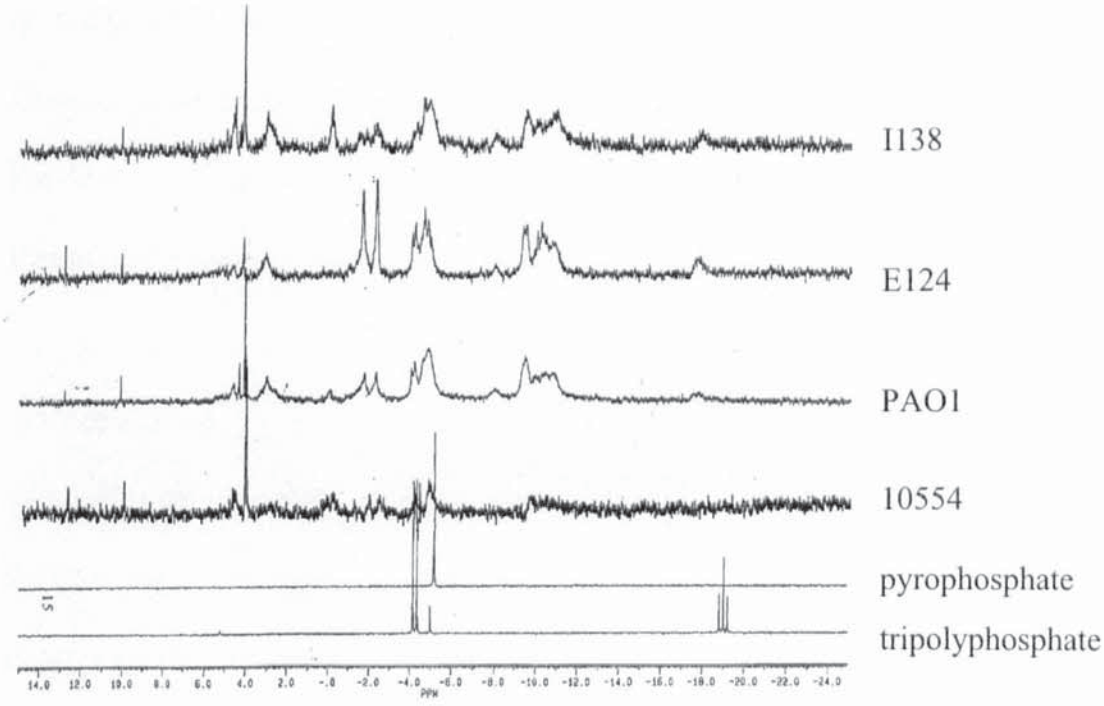


Figure 5.3 ^{31}P-NMR of phenol-extracted LPS from isolates of *P. aeruginosa*

The results show that the LPS from strains PAO1, E124 and I138 each produced several distinctive clusters of signals. The major differences between E124 and

the other two strains were seen in the 4 ppm region where the major sharp peak was significantly reduced, and in the –2.0 ppm region where the double peaks were significantly greater. An attempt to identify the peaks was made by running separate NMR spectra of authentic samples of pyrophosphate (with a single peak at –5.0 ppm) and tripolyphosphate (with double peaks at –4.0 ppm, a small peak at –5.0 ppm and a triplet at –19 ppm). The NMR spectra were standardised by an internal sample of inorganic phosphate (0 ppm). It appears that the peaks in the LPS samples around –4 to –5 ppm represent tripolyphosphate or pyrophosphate groups whilst the small peak around –17 ppm could also be derived from tripolyphosphate. However it has not been possible to account for the other major signals in terms of unsubstituted phosphate groups. They may represent LPS phosphates substituents which have other residues attached, e.g. the ethanolamine or aminoarabinose substituents which have been detected in LPS from other Gram-negative bacteria and have been shown to confer resistance to polymyxin. Previous work on the LPS from *Serratia marcescens* (Seltmann *et al.*, 1996) has identified the major phosphate signals as:

monophosphate	0 to +2 ppm
α-linked sugar phosphate	–11.9 ppm
β- linked sugar phosphate	–6.5 ppm
phosphodiester groups	0 to –3 ppm

In *S. marcescens* resistance to polymyxin was associated with the appearance of three peaks in the 0 to –3 ppm region. These represented phosphodiesters, in which the phosphate binding sites for polymyxin were modified by

4-aminoarabinose substituents. A strain which was sensitive to polymyxin lacked these peaks. It therefore appears that the resistance of the *P. aeruginosa* strain E124 could be due to a similar modification of its LPS. Further studies need to be carried out to identify the chemical nature of any modifying substituent on the LPS phosphates. This would involve acid hydrolysis of the LPS to release the phosphodiester substituent and identification by high voltage paper electrophoresis.

5.2 HPLC Studies with Colomycin® and Colymycin®

A key feature of the action of the sulphomethate prodrug form of colistin E is presumed to be hydrolysis to the fully-positively charged species *in vivo* (Barnett *et al.*, 1964). Little information is currently available on this process and its influence upon the antimicrobial action of the sulphomethate forms of colistin.

The HPLC system of Elverdam *et al.* (1981) was used to investigate the separation and stability of the preparations. This involved a reverse phase system using a C18 column with an isocratic mobile phase containing acetonitrile at 22 or 23 % (v/v). It was developed by workers at Dumex (Denmark), the manufacturers of colomycin, for separation of colistin E sulphate into the E1 and E2 components and polymyxin B sulphate into B1 and B2. The E1/E2 forms of colistin E and B1/B2 forms of polymyxin B differ only in the nature of the fatty acid substituent. For authentic polymyxin B sulphate (Aerosporin®, obtained from Wellcome) the system separated the B1 and B2 components (see Figure 5.4). However, neither the colomycin sulphomethate nor the colymycin sulphomethate preparations gave well-resolved peaks in this solvent system (Figure 5.5),

showing that the separation obtained for polymyxin B was dependent upon the absence of the sulphomethate substituents. There were minor differences in the elution profiles of the colomycin and colymycin products but the resolution was not good enough to draw definite conclusions on their relative composition. An increased acetonitrile concentration was used to obtain the separations shown in Figure 5.6 to 5.9. This produced much sharper peaks but they were only partially resolved. There was no obvious difference between the two products under these conditions.

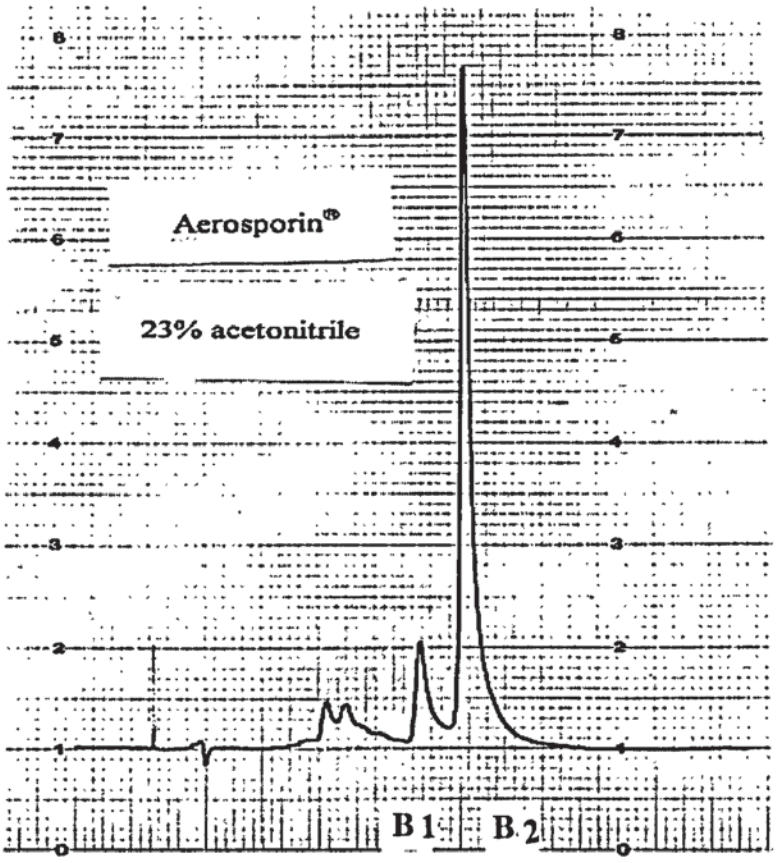


Figure 5.4 HPLC traces of Aerosporin® (polymyxin B). Reversed-phase Nucleosil 5 μm C_{18} column, 250 × 4.6 mm ID, acetonitrile/buffer (0.023 M H_3PO_4, 0.01 M acetic acid, 0.05 M Na_2SO_4 adjusted to pH 2.5 with triethylamine), acetonitrile/buffer ratio 23:77; flow rate 1 ml/min; detection 220 nm; 10 μl of 5 mg/ml solutions loaded

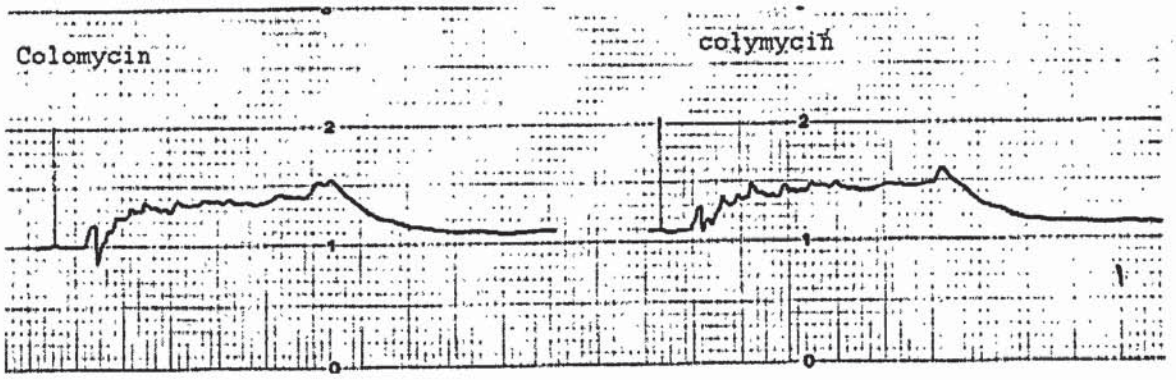


Figure 5.5 HPLC traces of Colomycin® (Colistin sulphomethate, Pharmax) and Colymycin® (Colistin sulphomethate, Parke-Davis) acetonitrile/buffer ratio 23:77

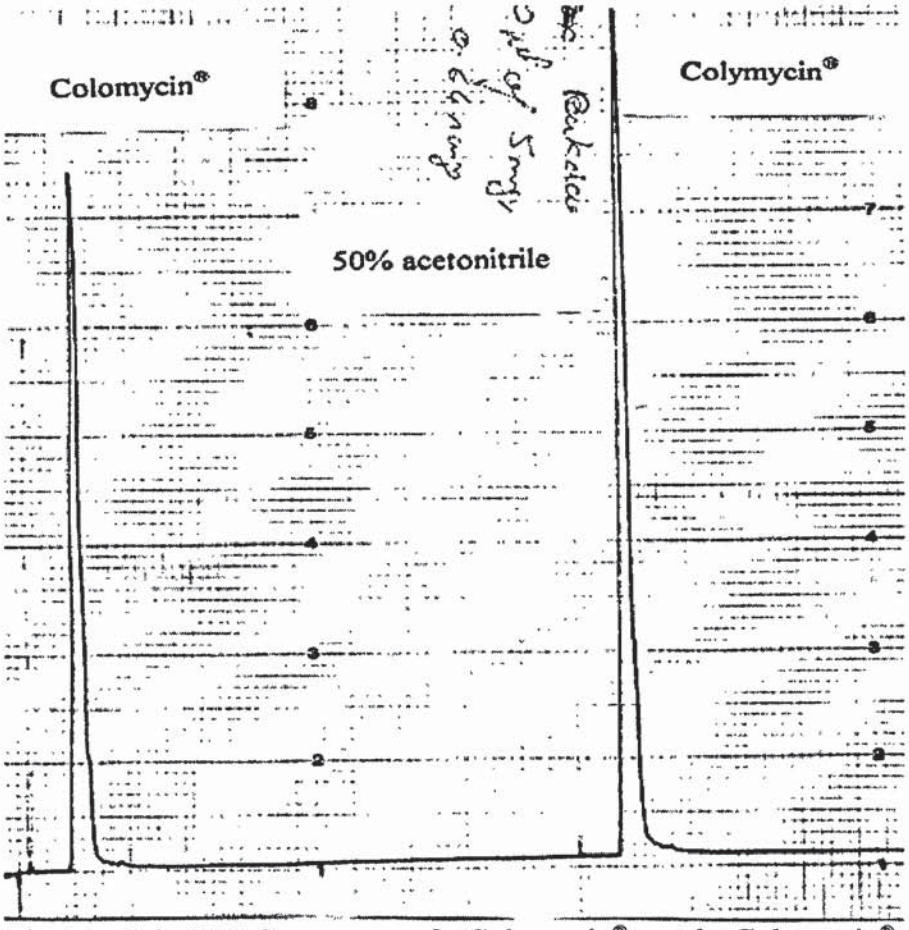


Figure 5.6 HPLC traces of Colomycin® and Colymycin®, acetonitrile/buffer ratio 50:50

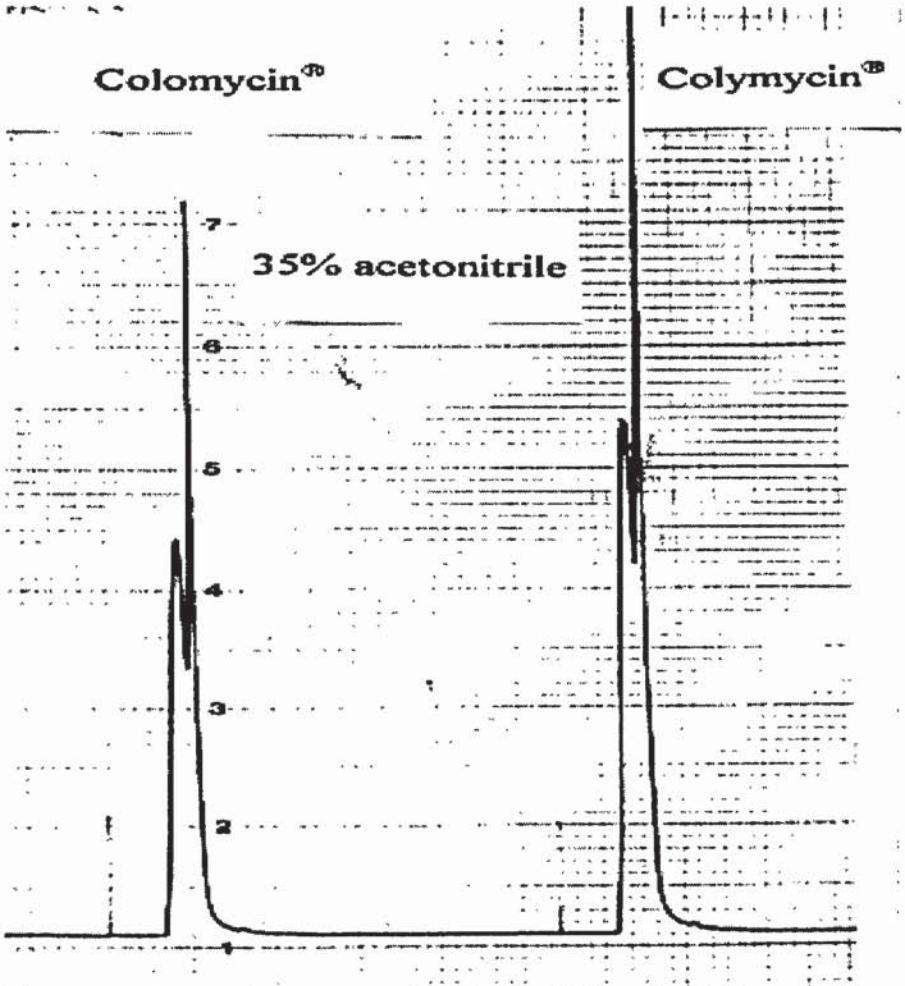


Figure 5.7 HPLC traces of Colomycin® and Colymycin®, acetonitrile/buffer ratio 35:65

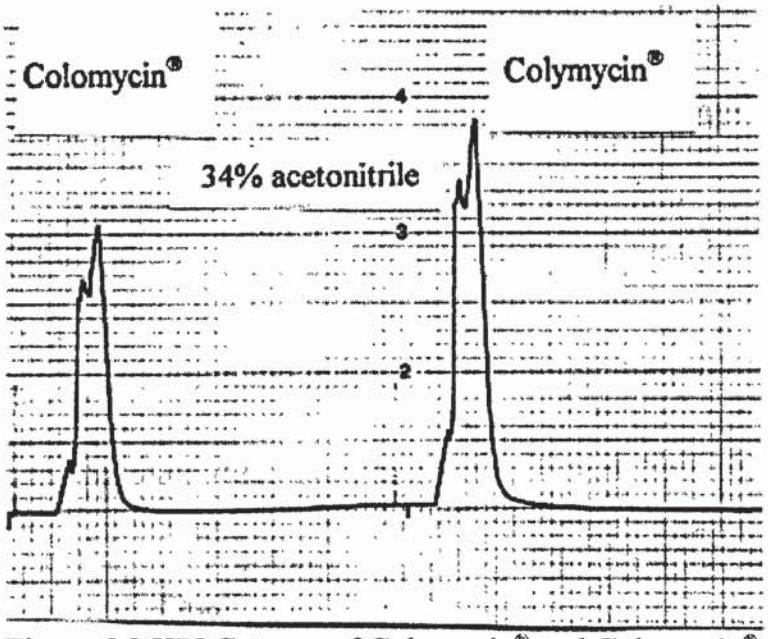


Figure 5.8 HPLC traces of Colomycin® and Colymycin®, acetonitrile/buffer 34:66

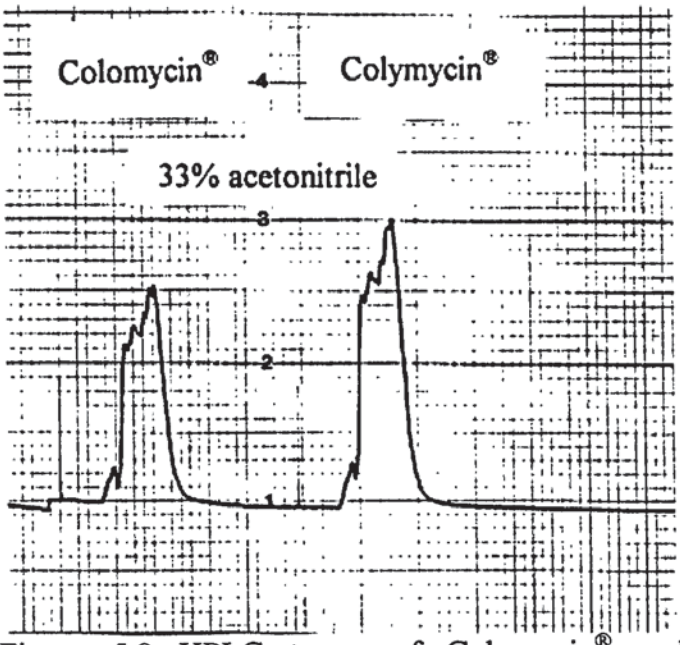


Figure 5.9 HPLC traces of Colomycin® and Colymycin®, acetonitrile/buffer 33:67

The alternative analytical methods of TLC and high voltage paper electrophoresis were used to investigate colomycin and colymycin in comparison with PMB. The time course for conversion of the sulphomethates to the active components was investigated to see if there was a difference between colomycin and colymycin.

5.3 Kill Curve for *Pseudomonas aeruginosa*

To investigate the time it takes for colomycin sulphomethate to become effective as an antimicrobial once in solution, the effect upon a growing culture of a rough strain of *P. aeruginosa* (5MV, the strain used to prepare LPS for the serological study) was determined using PMB as a control. Both compounds were dissolved in water and added to the cell suspension in nutrient broth at 12 μg/ml either immediately or after 1, 2 or 3 hours incubation at 37 °C. The action of the

antibiotics were assessed by measurement of optical density reading and a viable count. Results are shown in Figure 5.10 and 5.11.

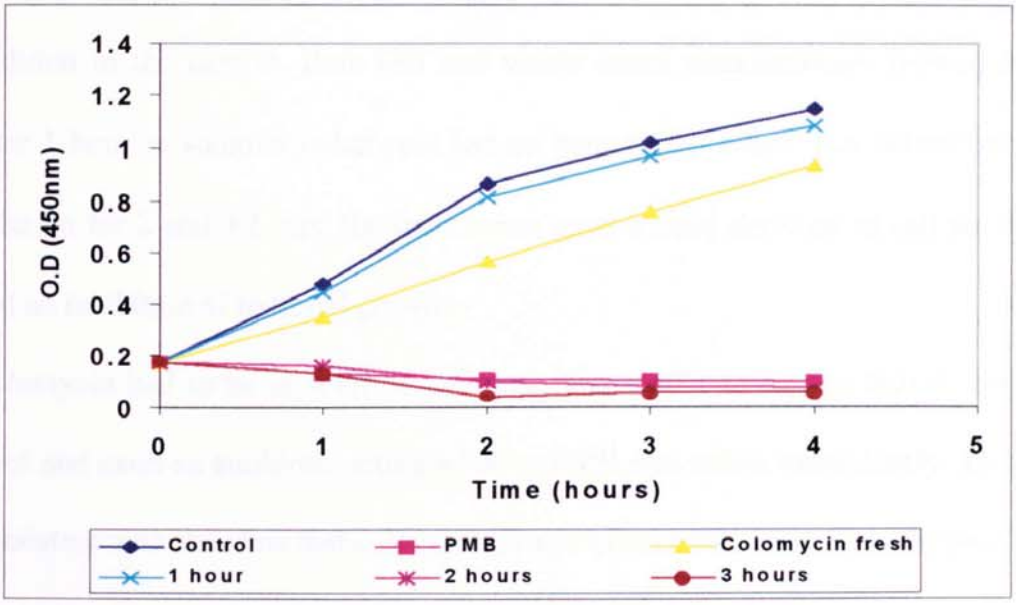


Figure 5.10 The effect of PMB and Colomycin® sulphomethate upon the OD of a grown culture of *P. aeruginosa* in nutrient broth. Colomycin® was added immediately (fresh) or 1, 2 or 3 hours after dissolving in water

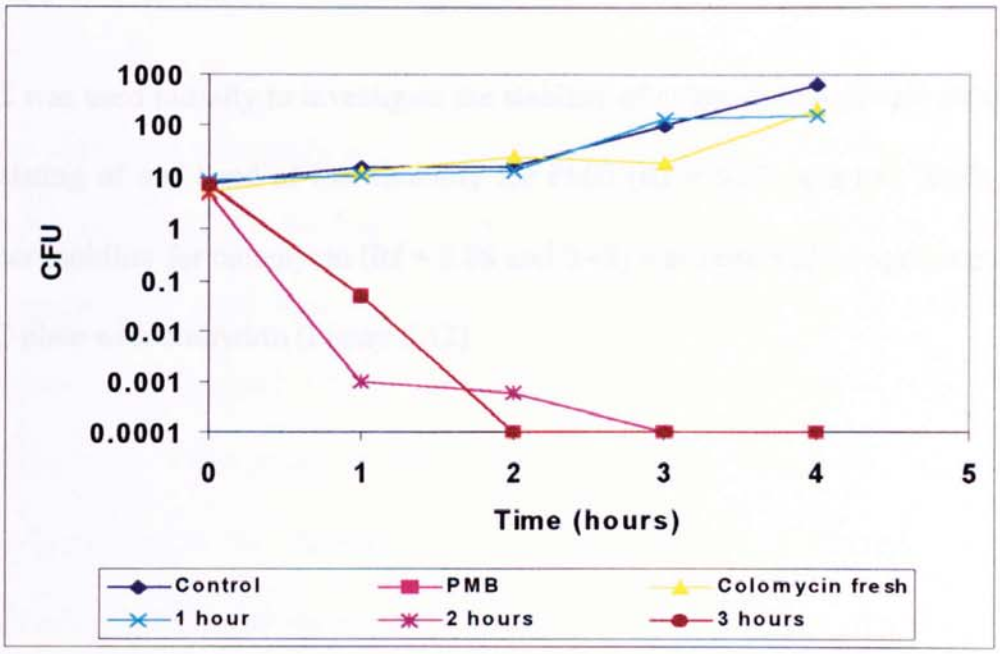


Figure 5.11 Effect of PMB and Colomycin® sulphomethate upon the viable counts of a culture of *P. aeruginosa* in nutrient broth. Colomycin® was added immediately (fresh) or 1, 2 or 3 hours after dissolving in water

PMB acted immediately, the OD and the cell viable count decreased whereas the culture inoculated with colomycin continued growing. Investigation was therefore made with colomycin solution dissolved 1, 2 and 3 hours before addition to the culture. Both OD and viable count measurements showed that after 1 hour in solution colomycin had no bactericidal effect. For colomycin in solution for 2 and 3 hours, the inoculation gave a rapid decrease in cell number and an inhibition of bacterial growth.

Colomycin had to be in solution for more than 1 hour to change into its active form and exert an antibiotic action whereas PMB was active immediately. This is consistent with the view that colomycin is a prodrug which must be hydrolysed to the active compound (colistin E) before antimicrobial activity is exerted.

5.4 TLC

TLC was used initially to investigate the stability of colomycin. A simple pattern consisting of one band of low mobility for PMB (Rf = 0.29) and two bands of higher mobility for colomycin (Rf = 0.88 and 0.48) was observed by spraying the TLC plate with ninhydrin (Figure 5.12).

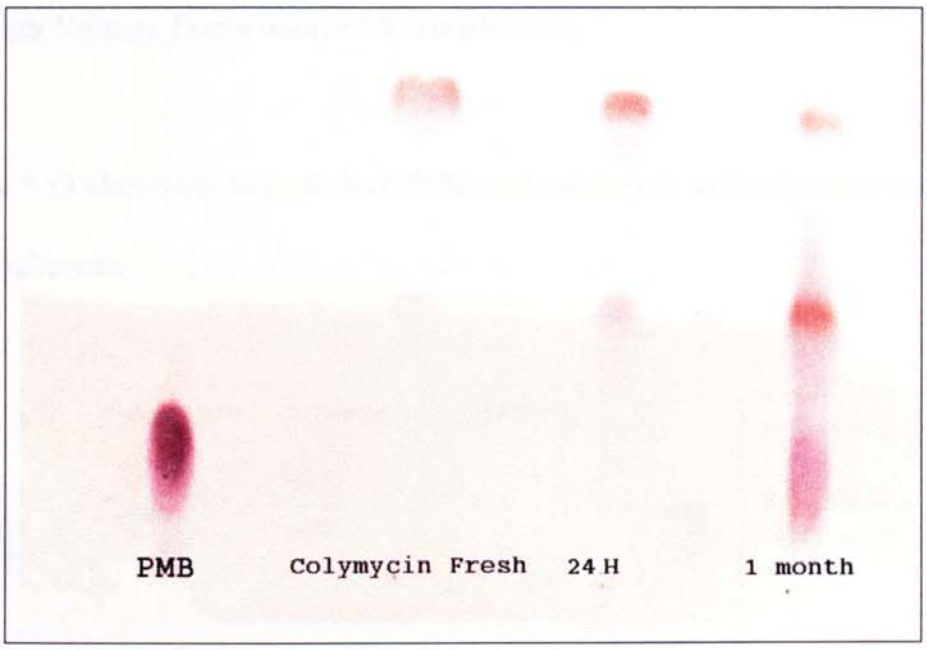


Figure 5.12 TLC separation on silica gel G plates developed with acetone: water: acetic acid: 2 N ammonia (15:5:1:2). Samples: 1, PMB; 2, colomycin freshly prepared; 3, colomycin after 24 hours at 4 °C; 4, colomycin after 1 month at 4 °C. 2 µl of 5 mg/ml solutions of each sample were applied to the plate. The plate was visualised by spraying with ninhydrin and heating at 100 °C for 5 min

When colomycin was incubated in water at 4 °C for 24 hours the relative amount of the lower band (Rf = 0.48) was slightly increased. After a month of incubation, colomycin gave three bands (Rf = 0.88, 0.48 and 0.21). The Rf 0.21 band presumably represented the positively-charged colistin E, equivalent to the Rf 0.29 band of PMB. These results show that colomycin sulphomethate was hydrolysed very slowly at 4 °C. However, it was not possible to distinguish between positively- and negatively-charged species using TLC alone. Therefore an alternative separation method, high voltage paper electrophoresis was used to investigate the charge properties of hydrolysed products of colomycin sulphomethate. This technique also enabled the interaction between colomycin and LPS to be studied.

5.5 High Voltage Performance Electrophoresis

Figure 5.13 shows the migration of PMB and colomycin and colymycin on paper electrophoresis.

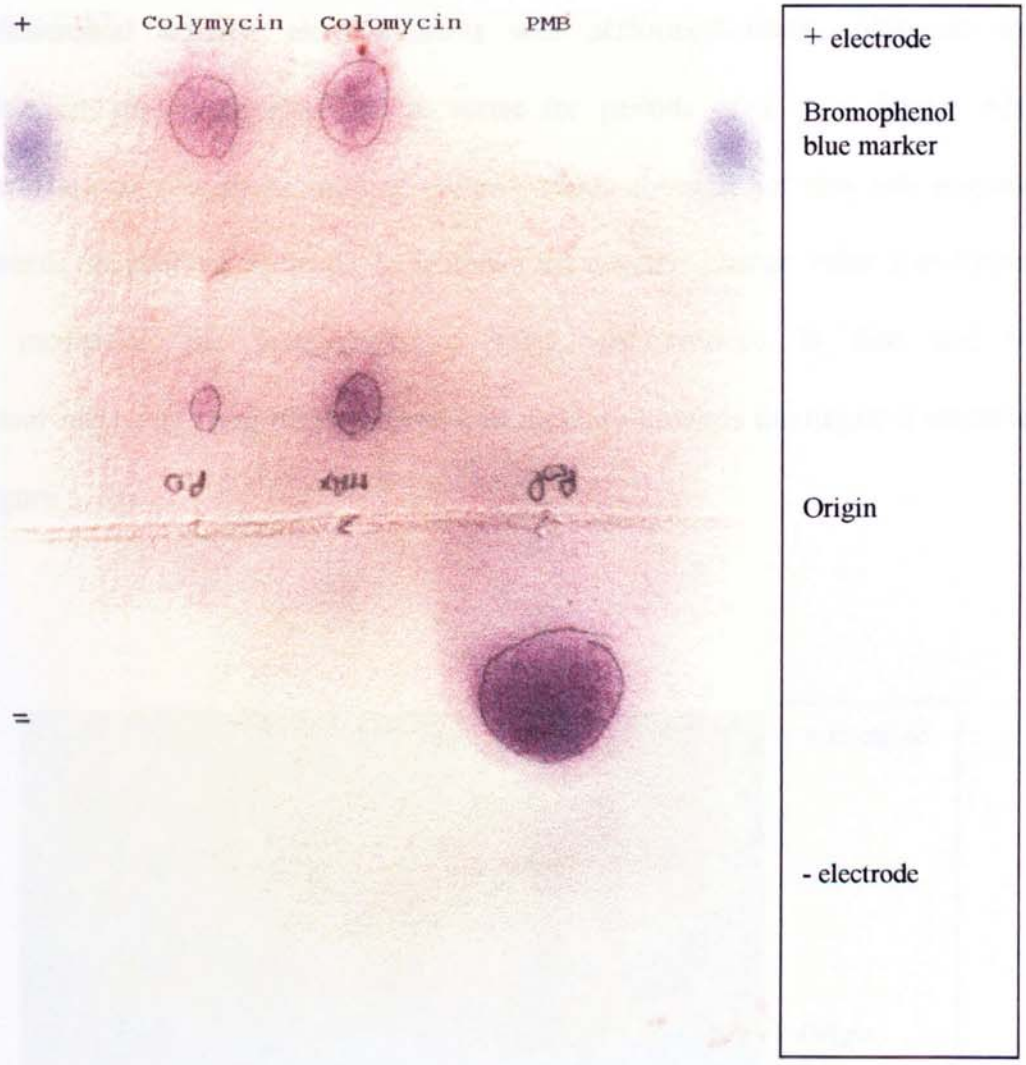


Figure 5.13 Paper electrophoresis of PMB, colomycin and colymycin. 5 µl samples of freshly-prepared 5 mg/ml samples were applied at the origin with bromophenol blue marker dye spots on either side. Electrophoresis was carried out at 300 V for 3 hours, the paper was air dried, sprayed with ninhydrin and heated at 100 °C for 5 min

During electrophoresis, PMB, the active control compound, migrated towards the negative electrode whereas colomycin and colymycin gave two distinct ninhydrin-stained bands migrating towards the positive electrode (Figure 5.13). This is consistent with PMB bearing a net positive charge and freshly-prepared colomycin and colymycin net negative charges. Since the kill curves showed that colomycin and colymycin must be in aqueous solution for more than one hour to generate antimicrobial activity, electrophoresis was performed using colomycin and colymycin previously dissolved in water for periods of 1 to 4 hours. After incubation of 1 hour the ratio of the two bands changed but they still migrated towards the positive electrode, indicating a net negative charge. After 2 to 4 hours of incubation the faster-migrating band was reduced in size and the slower-migrating band displayed reduced mobility towards the negative electrode (Figure 5.14).

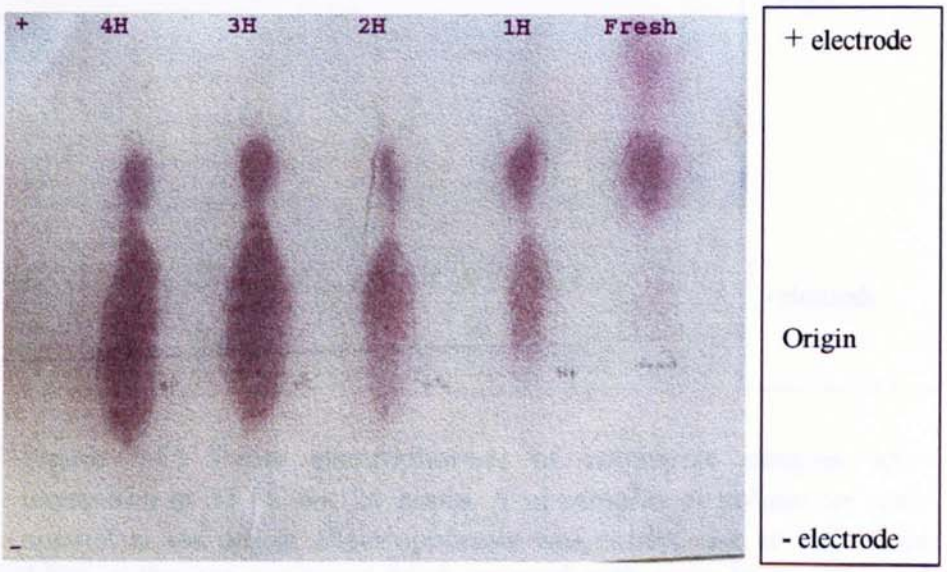


Figure 5.14 Paper electrophoresis of colomycin samples after incubation at 37 °C for the periods shown. 5 µl samples of 5 mg/ml colomycin were applied at the origin. Electrophoresis was carried out at 300 V for 3 hours, the paper was air dried, sprayed with ninhydrin and heated at 100 °C for 5 min

These results show that freshly-prepared colomycin (as the sulphomethate) slowly changes to a compound that is positively charged during incubation at 37 °C. A number of intermediate forms with gradually reducing negative charge were apparent on electrophoresis. After 24 hours in water samples of colomycin and colymycin were totally positively charged, but they did not migrate with the same mobility as PMB (Fig. 5.15). Since the antimicrobial action of colomycin was evident after 1-2 hours incubation (Fig. 5.11) these results support the findings with HPLC, which suggest that colomycin does not need to be totally converted to the positively-charged colistin to exert antibacterial action.

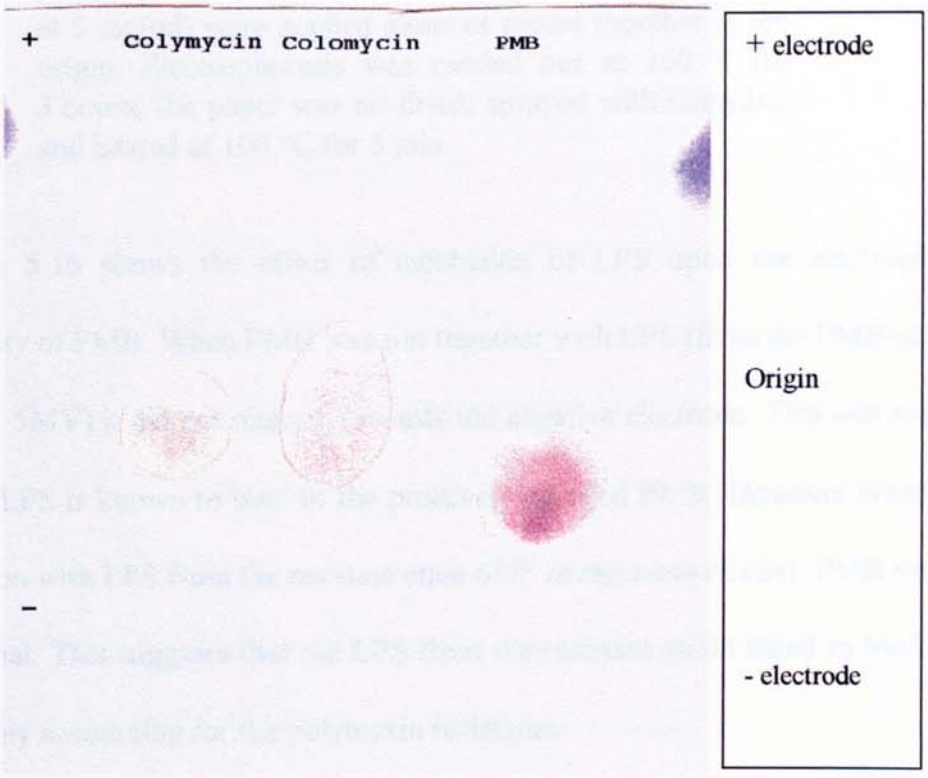


Figure 5.15 Paper electrophoresis of colomycin samples after incubation at 37 °C for 24 hours. 5 µl samples of colomycin were applied at the origin. Electrophoresis was carried out at 300 V for 3 hours, the paper was air dried, sprayed with ninhydrin and heated at 100 °C for 5 min

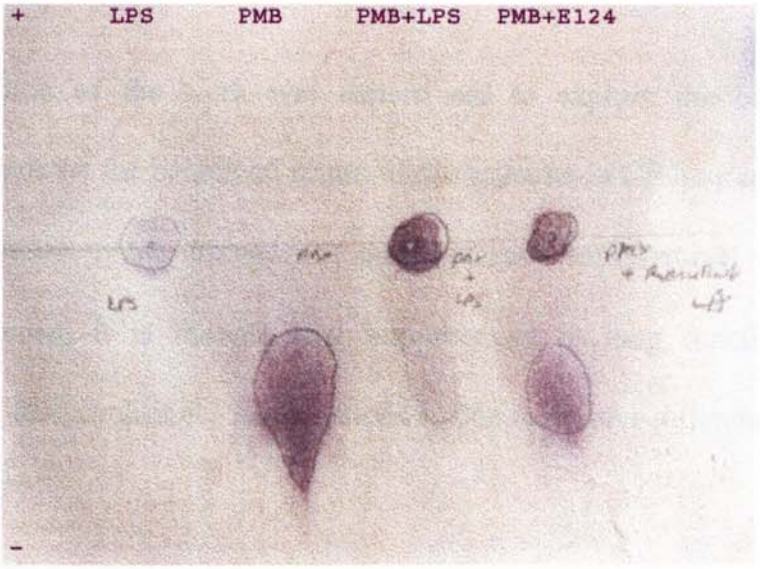


Figure 5.16 Effect of LPS on migration of PMB on paper electrophoresis. 5 µl samples of PMB and LPS from *P. aeruginosa* strain 5MV or resistant strain E124 (each at 5 mg/ml) were applied alone or mixed together at the origin. Electrophoresis was carried out at 300 V for 3 hours, the paper was air dried, sprayed with ninhydrin and heated at 100 °C for 5 min

Figure 5.16 shows the effect of incubation of LPS upon the electrophoretic mobility of PMB. When PMB was run together with LPS (from the PMB-sensitive strain, 5MV) it did not migrate towards the negative electrode. This was expected since LPS is known to bind to the positively-charged PMB. However when PMB was run with LPS from the resistant stain of *P. aeruginosa* (E124), PMB migrated as usual. This suggests that the LPS from the resistant strain failed to bind PMB, possibly accounting for the polymyxin resistance.

CHAPTER 6. ANTI-INFLAMMATORY EFFECTS OF ANTIBIOTICS

This section of the work was carried out to explore the basis of clinical observations on the beneficial action of doxycycline in CF lung infections due to *P. aeruginosa.* Since doxycycline does not have antimicrobial activity against *P. aeruginosa*, it is thought that improvement in lung function in patients receiving doxycycline for other reasons is due to an anti-inflammatory action in the lungs.

6.1 Release of IL-1β by Murine Peritoneal Macrophages

First macrophages extracted from a mouse peritoneal lavage were grown overnight in a cell culture dish where they adhere to the plastic. The supernatant was then removed and the macrophages were scraped off and resuspended in RPMI 1640 medium pre-warmed at 37 °C. Tubes containing 10^6 cells/ml were incubated at 37 °C with LPS and various drugs for different lengths of time. Samples were removed, centrifuged to deposit the cells and the supernatants analysed for the cytokines, IL-1β and TNF-α by ELISA. With this system, macrophages alone (without LPS) released as much IL-1β as those stimulated with LPS (1 mg/ml). This assay was therefore abandoned and replaced with an assay where peritoneal macrophages were incubated directly in to a 24 well plate to avoid the scraping process which could have been the cause for the mechanical stimulation of the macrophages and stimulation of IL-1β production.

Using the 24 well-plate method with adherent cells the release of IL-1β by stimulating the macrophages with increasing levels of rough LPS (range from

5 μg/ml to 1 mg/ml) was successfully investigated. Results are shown in Figure 6.1.

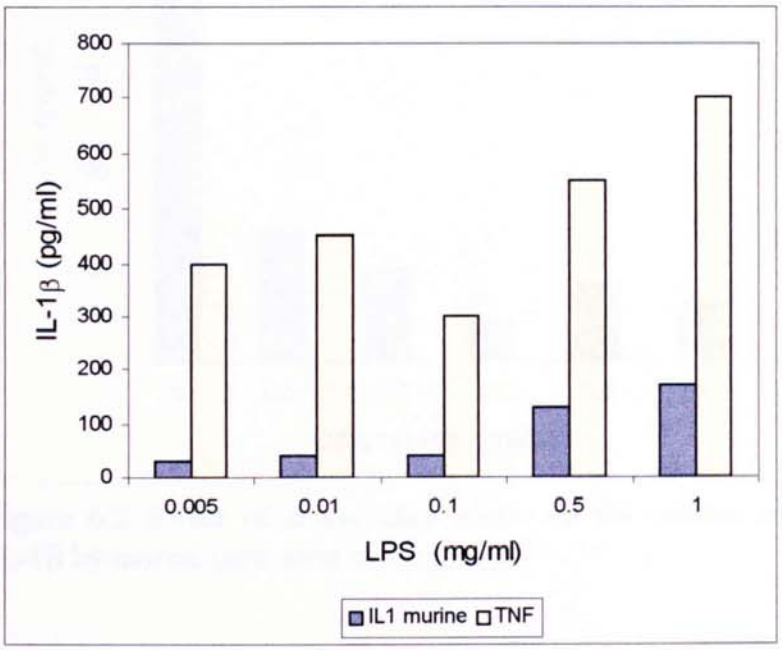


Figure 6.1 Effect of LPS alone on the release of IL-1β and TNF-α by murine peritoneal macrophages

Macrophages alone, without any stimulation by LPS, produced about 30 pg/ml of IL-1β over 5 hours, and 150 pg/ml of TNF-α. There was a good dose response to LPS, and a concentration of 0.5 mg/ml of LPS was selected for further experiments, being the lowest concentration needed to stimulate a significant release of IL-1β and TNF-α by murine macrophages.

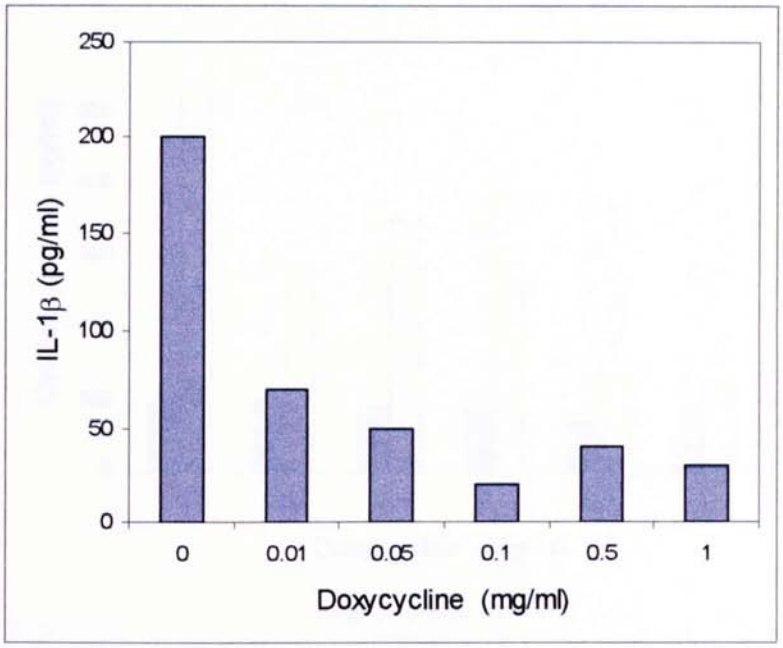


Figure 6.2 Effect of doxycycline alone on the release of IL-1β by murine peritoneal macrophages

Figure 6.2 shows the effect of doxycycline alone on the release of IL-1β by murine macrophages. The negative control was 50 pg/ml, it appears that doxycycline alone did not stimulate the release of IL-1β, and it might even inhibit it, although levels of IL-1β were too low to draw a conclusion. For TNF-α the negative control was 10 pg/ml and all concentrations of doxycycline gave results similar to 10 pg/ml (data not shown).

The effect of doxycycline on cytokine release from macrophages stimulated with 0.5 mg/ml of LPS was investigated (Figure 6.3).

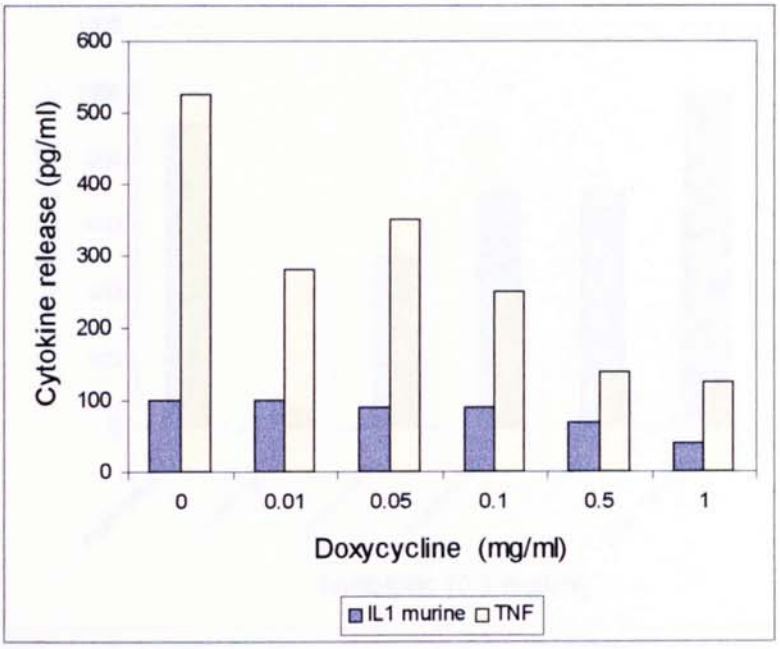


Figure 6.3 Effect of doxycycline on the release of IL-1β and TNF-α by murine peritoneal macrophages

For the murine peritoneal macrophages a concentration of doxycycline of 1 mg/ml was needed to give 50 % inhibition of the IL-1β released by LPS. At a concentration of 0.5 mg/ml it gave a 30 % inhibition. By contrast, concentrations of 1 and 0.5 mg/ml of doxycycline inhibited the release of TNF-α totally and a concentration of 0.1 mg/ml gave a 50 % inhibition. In the physiological concentration range (e.g. around 10 μg/ml), doxycycline gave a 35 to 45 % inhibition.

Figure 6.4 gives a summary of the action of a range of antibiotics which have been shown to exert anti-inflammatory effects.

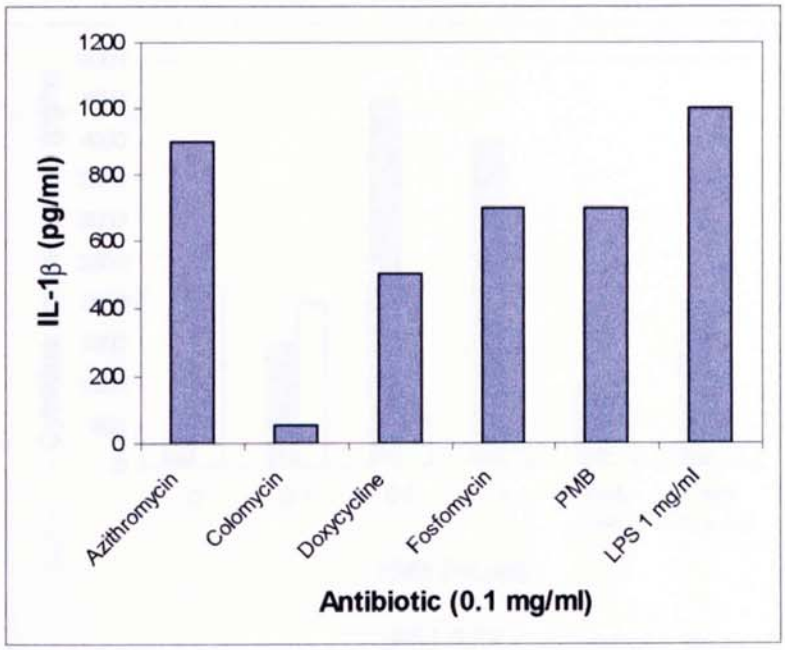


Figure 6.4 Effect of various antibiotics on the release of IL-1β by murine peritoneal macrophages

In this assay only doxycycline and colomycin gave a 50 % inhibition of the level of IL-1β at a concentration of 0.1 mg/ml. PMB and fosfomycin gave a 30 % inhibition and azithromycin only a 10 % inhibition. It is possible that the anti-inflammatory effects of azithromycin and fosfomycin do not directly involve inhibition of the release of cytokines and it seems, in this assay, that they do not act on the release of IL-1β.

PMB binds to LPS so it should therefore stop the inflammatory cascade by preventing the LPS binding to CD14 and activate the response. The effects of PMB upon LPS-stimulated cytokine release are shown in Figure 6.5.

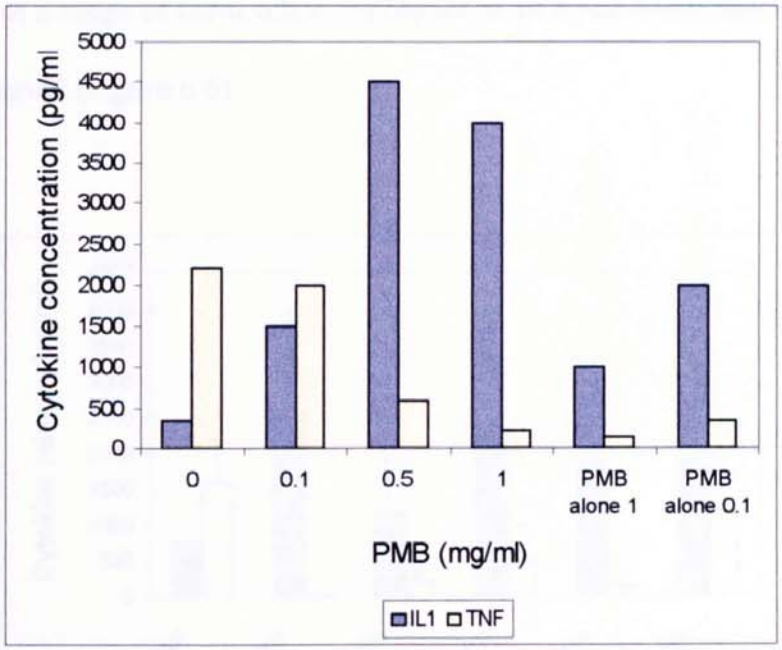


Figure 6.5 Effect of PMB on the release of IL-1β and TNF-α by murine peritoneal macrophages stimulated with LPS

Figure 6.5 shows surprising results; it appears that PMB alone (without any LPS) stimulated the release of IL-1β. The release increased further when PMB was added together with 0.5 mg/ml of LPS. Opposite results were obtained when TNF-α release was measured. There was a dose response to PMB, where higher concentrations gave a greater inhibition of LPS-stimulated TNF-α release. Even PMB alone inhibited the basic release of TNF-α by unstimulated macrophages in a dose dependent manner. This phenomenon could be due to the fact that PMB binds to the LPS, neutralising its effect upon the cells. However, this action was not observed for IL-1β, suggesting a different mechanism of action.

Fosfomycin did not give a dose response or any inhibition of IL-1β release at any concentration (results not shown). Results for TNF-α were not measured for this antibiotic.

The action of a range of tetracycline antibiotics at an equal concentration of 1 mM was investigated (Figure 6.6).

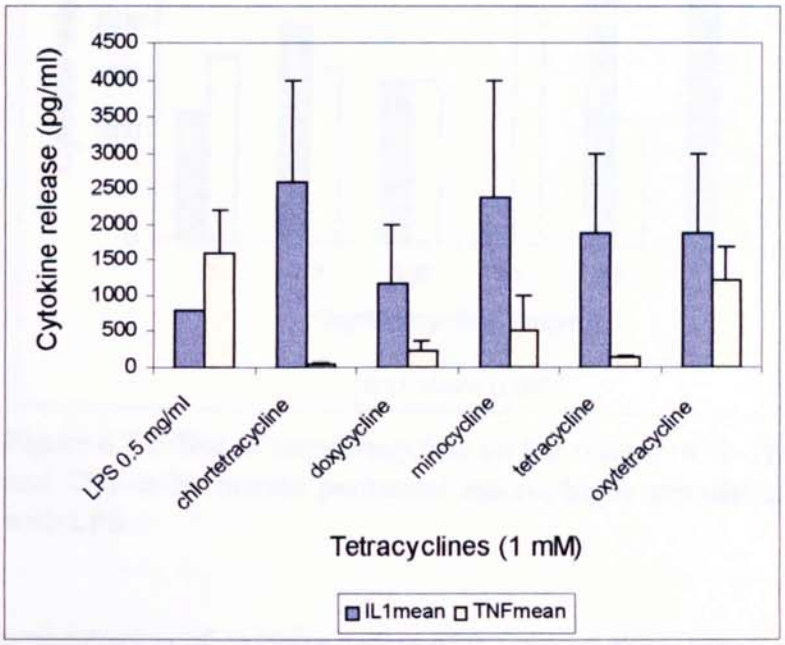


Figure 6.6 Effect of tetracyclines on the release of IL-1β and TNF-α by murine peritoneal macrophages stimulated with LPS

On mouse peritoneal macrophages only doxycycline showed an inhibition of LPS-induced IL-1β release. This could be explained by the fact that doxycycline is more lipophilic than the other tetracyclines and could therefore penetrate more easily into the macrophages (Barza, *et al.*, 1975). The action of tetracyclines on TNF-α release was once again totally different. All tetracyclines, apart from oxytetracycline, gave at least 80 % inhibition. The pathways for translation and secretion of IL-1β and TNF-α are different (Kuby, 1994) and it appears that the tetracyclines exert their effects at different sites in both pathways.

Figure 6.7 shows the effect of oxytetracycline on IL-1β levels.

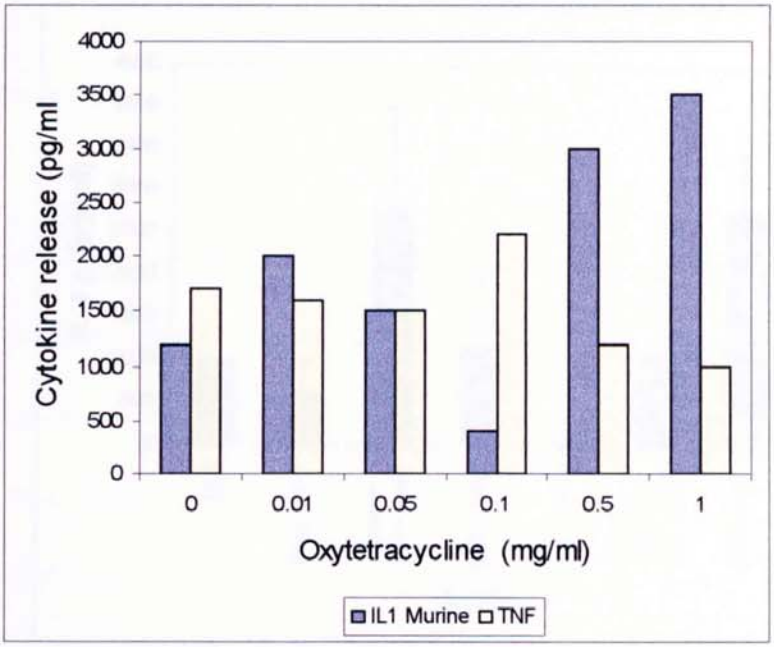


Figure 6.7 Effect of oxytetracycline on the release of IL-1β and TNF-α by murine peritoneal macrophages stimulated with LPS

Only at a concentration of oxytetracycline of 0.1 mg/ml there was an inhibition of the release of IL-1β greater than 50 %. At concentrations of 0.5 and 1 mg/ml oxytetracycline stimulated the secretion of IL-1β threefold. For TNF-α results were opposite, with 0.1 mg/ml of oxytetracycline producing a two-fold increase in TNF-α release and a concentration of 0.5 mg/ml giving a 50 % inhibition.

As tetracyclines bind divalent metal ions the effect of calcium upon the inhibition of cytokine release by doxycycline was investigated. The results are shown in Figure 6.8.

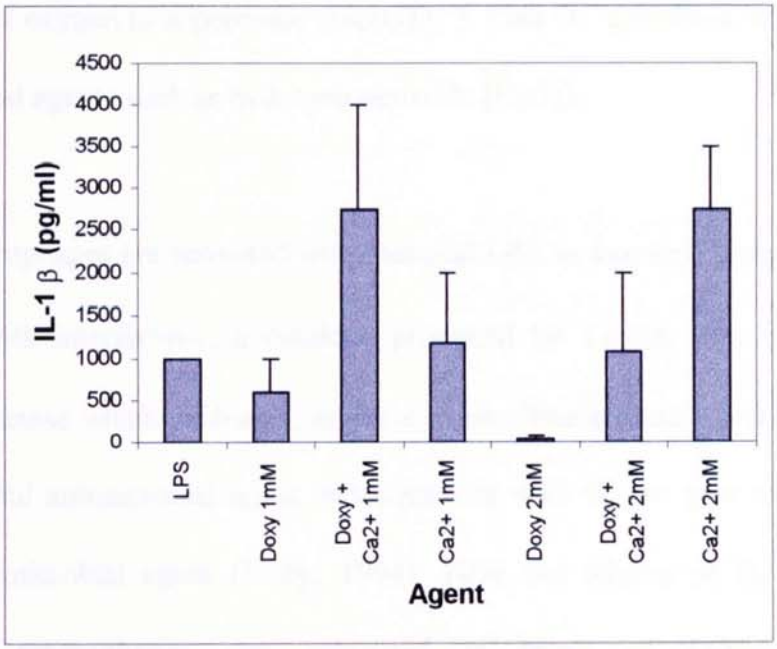


Figure 6.8 Effect of doxycycline and calcium on the release of IL-1β by murine peritoneal macrophages stimulated with LPS

Doxycycline at concentrations of 1 mM and 2 mM decreased in a dose dependent manner the release of IL-1β by murine peritoneal macrophages stimulated with 0.5 mg/ml of *P. aeruginosa* LPS (Figure 6.8). Calcium at a concentration of 1 mM did not show any significant difference in IL-1β release but, at a concentration of 2 mM, there was a three-fold increase in IL-1β production. Surprisingly doxycycline and calcium at 1mM added together to the system gave a three-fold increase in IL-1β production, whereas the same compounds added at a concentration of 2 mM did not change the amount of IL-1β produced.

6.2 Respiratory Burst of Murine Peritoneal Macrophages

Activated macrophages produce a number of reactive oxygen and nitrogen intermediates which have antimicrobial and cytotoxic activity. The oxidative burst enhances the activation of a membrane-bound oxidase which catalyses the

reduction of oxygen to superoxide anion ($O_2^{\cdot-}$). This $O_2^{\cdot-}$ generates other oxidising antimicrobial agents such as hydrogen peroxide (H_2O_2).

When macrophages are activated with bacterial LPS or muramyl dipeptide (MDP) together with interferon-γ, a cytokine produced by T-cells, they release nitric oxide synthetase which oxidises L-arginine to citrulline and nitric oxide (NO). NO is a powerful antimicrobial agent and combines with $O_2^{\cdot-}$ to give an even more potent antimicrobial agent (Kuby, 1994). Here the release of $O_2^{\cdot-}$ by murine peritoneal macrophages was measured in terms of lucigenin-enhanced chemiluminescence (respiratory burst). Figure 6.9 shows the effect of LPS, opsonised zymosan on respiratory and the action of doxycycline.

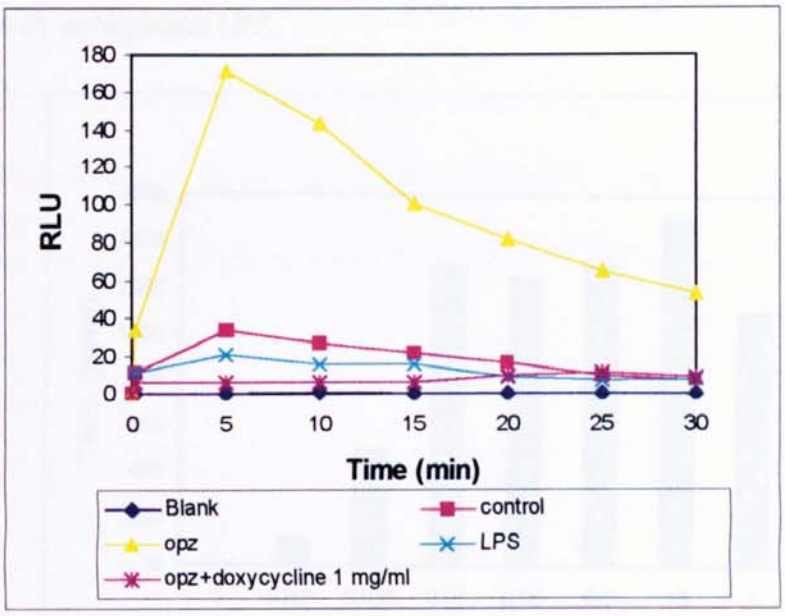


Figure 6.9 Respiratory burst of mouse peritoneal macrophages induced by LPS and opsonised zymosan and the effect of doxycycline

LPS alone produced no respiratory activity whereas opsonised zymozan (opz, serum treated yeast cells) stimulated pronounced activity over a period of 30 min with a peak level of activity after 5 min. The interesting result was that 1 mg/ml of

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these cells respond to exogenous activating agents (including LPS from *P. aeruginosa*) as well as monocytes from healthy volunteers so the action of doxycycline on CF macrophages could be similar to that shown on U937 cells (Thomassen *et al.*, 1990). Since the mouse peritoneal macrophages used in the experiments in this thesis did not respond to LPS, further work was carried out with U937 cells, a human monocyte-derived cell line. U937 cells were treated with PMA to differentiate them into a macrophage-like morphology (method 2.17.3).

6.3 Release of TNF-α and IL-1β by U937 Cells

Figures 6.11 and 6.12 show the release of cytokines from U937 cells induced by exposure to *P. aeruginosa* LPS.

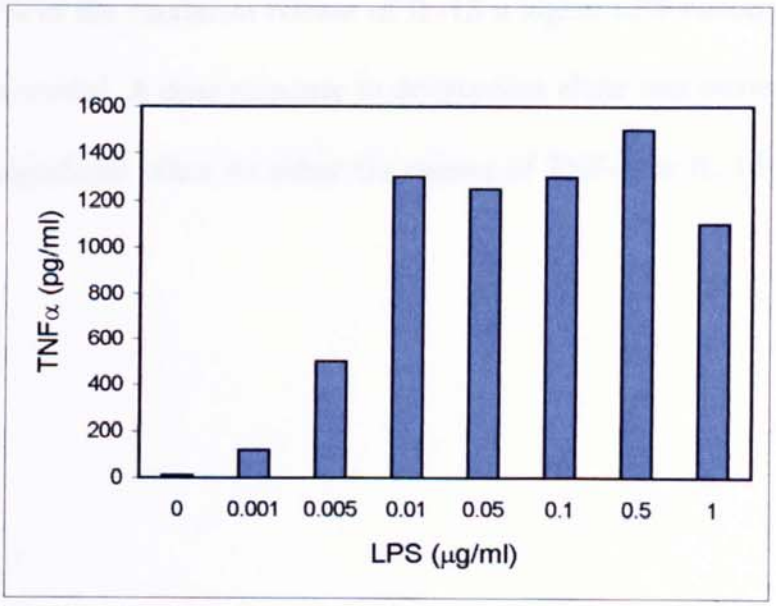

Figure 6.11 Dose response curve for release of TNF-α from U937 cells induced by LPS

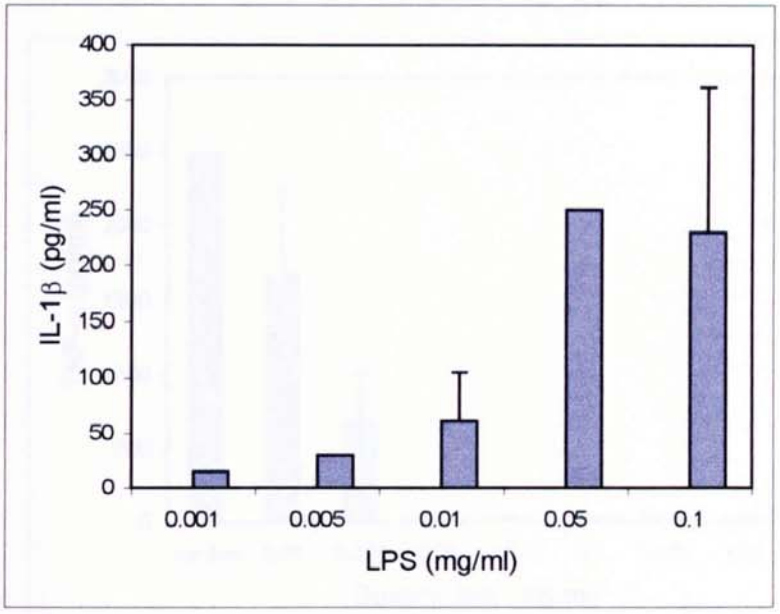

Figure 6.12 Dose response curve for release of IL1-β from U937 cells induced by LPS

From the dose response curves the concentration of LPS which gave a 50 % inhibition of maximum release of TNF-α was determined. LPS at a concentration of 5 ng/ml gave approximately 50 % of the maximum release of TNF-α. To produce a 50 % of the maximum release of IL-1β a higher LPS concentration of 0.1 mg/ml was needed. A dose response to doxycycline alone was carried out and there was no significant effect on either the release of TNF-α or IL-1β (data not shown).

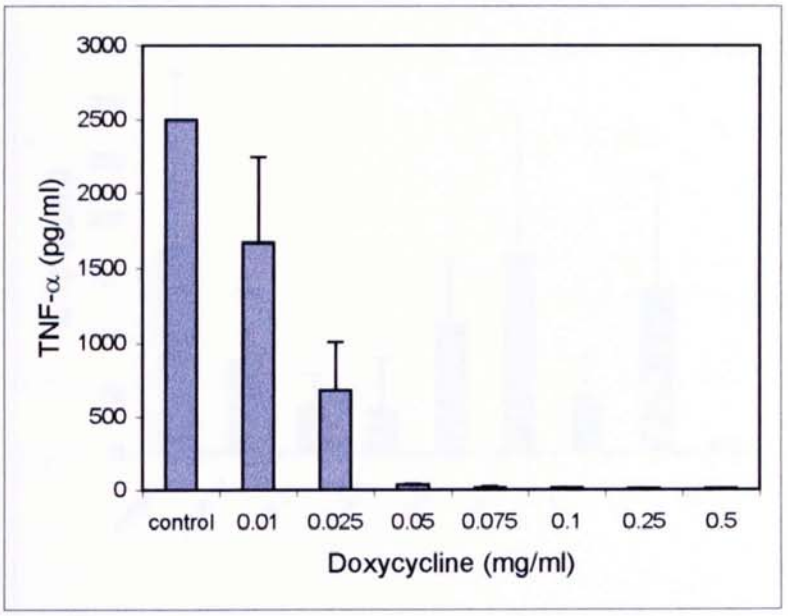

Figure 6.13 Effect of doxycycline on the release of TNF-α from U937 cells induced by 5 ng/ml of LPS

The potential dose dependant effect of doxycycline on the cytokine release was then investigated (Figures 6.13.and 6.14). Figure 6.13 shows that doxycycline had an inhibitory effect on the production of TNF-α which was dose dependent. At physiological concentrations (0.05 to 0.01 mg/ml) doxycycline gave a significant inhibition of TNF-α produced by U937 cells stimulated with 5 ng/ml of LPS.

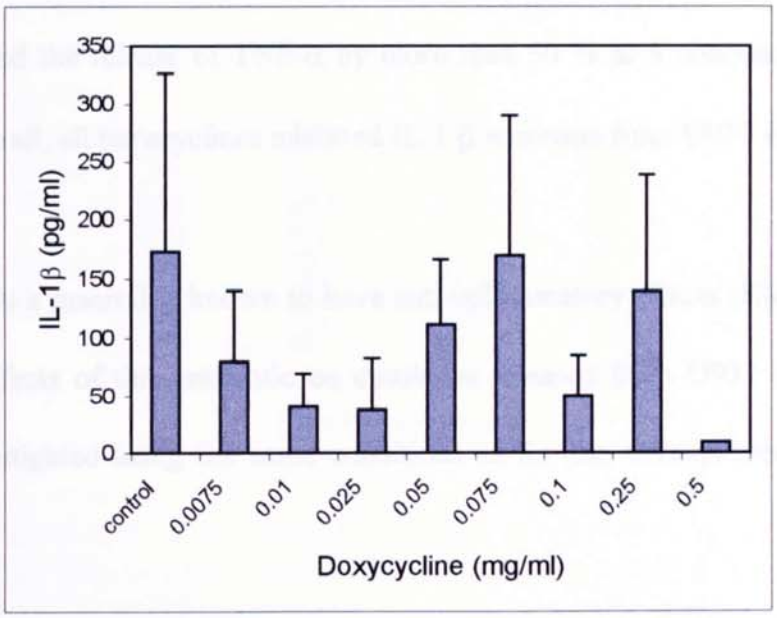

Figure 6.14 Effect of doxycycline on the release of IL1-β from U937 cells induced by 0.1 mg/ml of LPS

By contrast, the inhibitory effects of doxycycline on the IL-1β released by U937 cells stimulated with 0.1 mg/ml of LPS was not dose dependant. The greatest inhibition was obtained at concentrations of 0.025 and 0.01 mg/ml of doxycycline.

Treatment of U937 cells		**TNF-α (pg/ml)**	**IL-1β(pg/ml)**
P. aeruginosa 5 ng/ml alone		2000	200
Doxycycline	50 μg/ml	35	120
	25 μg/ml	700	40
Minocycline	50 μg/ml	10	10
	25 μg/ml	500	30
Oxytetracycline	50 μg/ml	10	8
	25 μg/ml	1100	300
Tetracycline	50 μg/ml	350	9
	25 μg/ml	1080	90
Chlortetracycline	50 μg/ml	120	15
	25 μg/ml	1800	25

Table 6.1 Summary of the effect of tetracyclines upon cytokine release from LPS induced U937 cells

All the tetracyclines were assayed at physiological concentrations of 50 and 25 μg/ml. Results are summarised in Table 6.1. It appears that minocycline is a better inhibitor of TNF-α than doxycycline, oxytetracycline had similar effects to

doxycycline whilst tetracycline and chlortetracycline were not as good inhibitors, but still reduced the release of TNF-α by more than 50 % at a concentration of 50 μg/ml. Overall, all tetracyclines inhibited IL-1 β secretion from U937 cells.

Azithromycin is a macrolide known to have anti-inflammatory effects (Khan *et al.*, 1999). The effects of this antibiotic on cytokines released from U937 cells was therefore investigated using the same conditions as for the tetracyclines (Figure 6.15).

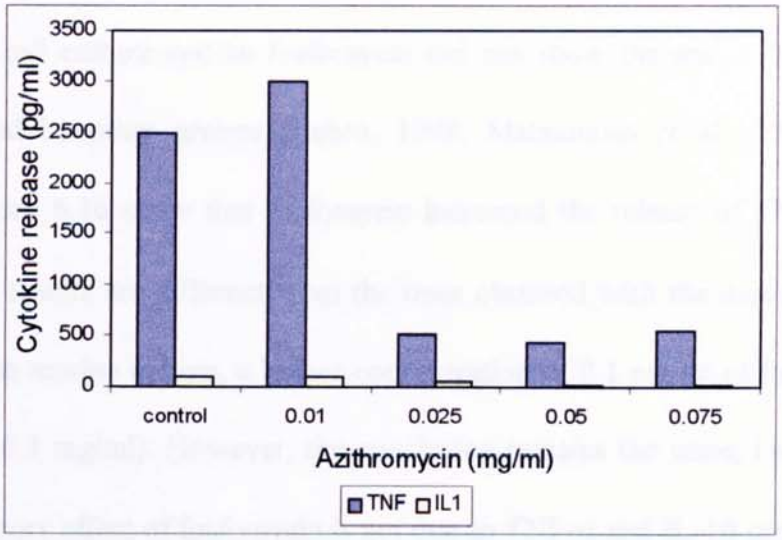

Figure 6.15 Effect of azithromycin on the release of IL-1β and TNF-α from U937 cells induced by 0.1 mg/ml and 5 ng/ml of LPS respectively

The results in Figure 6.15 show that azithromycin inhibited TNF-α and IL-1β release at concentrations from 0.075 to 0.025 mg/ml. These results contrast with those obtained from the mouse peritoneal macrophage model.

Fosfomycin is another antibiotic which has been reported to show anti-inflammatory activity. Figure 6.16 shows the effect of fosfomycin on cytokine release from U937 cells.

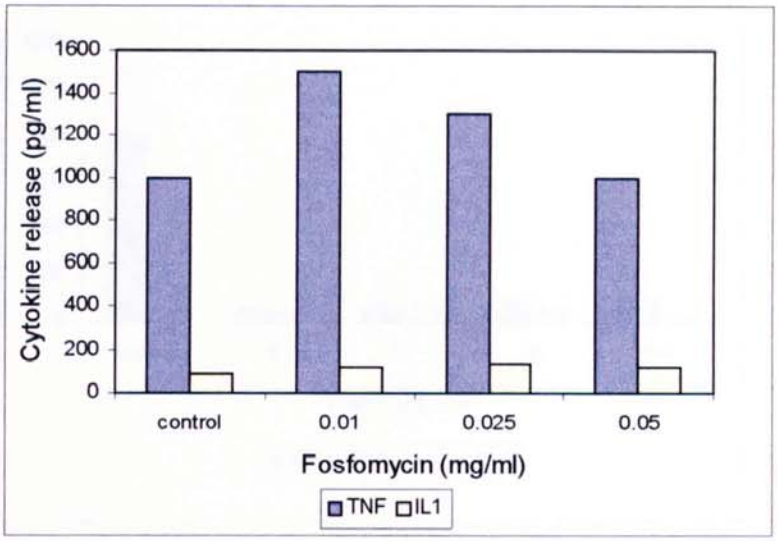

Figure 6.16 Effect of fosfomycin on the release of IL1-β and TNF-α from U937 cells induced by 0.1 mg/ml and 5 ng/ml of LPS respectively

In the U937 cell culture system fosfomycin did not show the anti-inflammatory effect reported by other groups (Labro, 1998; Matsumoto *et al.*, 1999). The results in Figure 6.16 show that fosfomycin increased the release of TNF-α and IL-1β. These results are different from the ones obtained with the mouse model, although in the murine system, a higher concentration of 0.1 mg/ml of fosfomycin was used (of 0.1 mg/ml). However, the conclusion remains the same, i.e. that the anti-inflammatory effect of fosfomycin is not due to TNF-α and IL-1β production.

Next the effect of PMB was investigated because PMB binds to LPS and might therefore exert anti-inflammatory action through neutralisation of LPS (Figure 6.17).

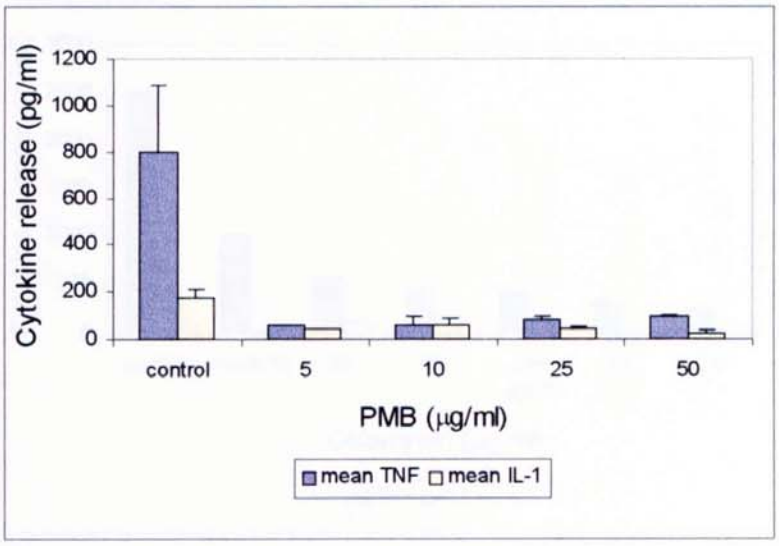

Figure 6.17 Effect of PMB on the release of IL-1β and TNF-α from U937 cells induced by 0.1 mg/ml and 5 ng/ml of LPS respectively

In the test system there was an inhibitory effect of PMB for concentrations between 50 and 5 μg/ml on the release of both TNF-α and IL-1β. PMB at these concentrations reduced the level of TNF-α by six-fold. For the release of IL-1β, the highest dose of 50 μg/ml PMB gave the greatest inhibition (70 %) while the other concentrations gave an average of 50 % inhibition. These differences could result from the fact that the concentration of LPS used to stimulate IL-1β release was 20 times higher than that of PMB so complete neutralisation would not be possible. The effect of PMB in the mouse model was totally different but the PMB-LPS ratio employed for these experiments was inverted.

To compare the effects of colymycin and PMB, fresh colymycin and colymycin incubated in water for 24 hours were added to the system. Results are shown in Figure 6.18.

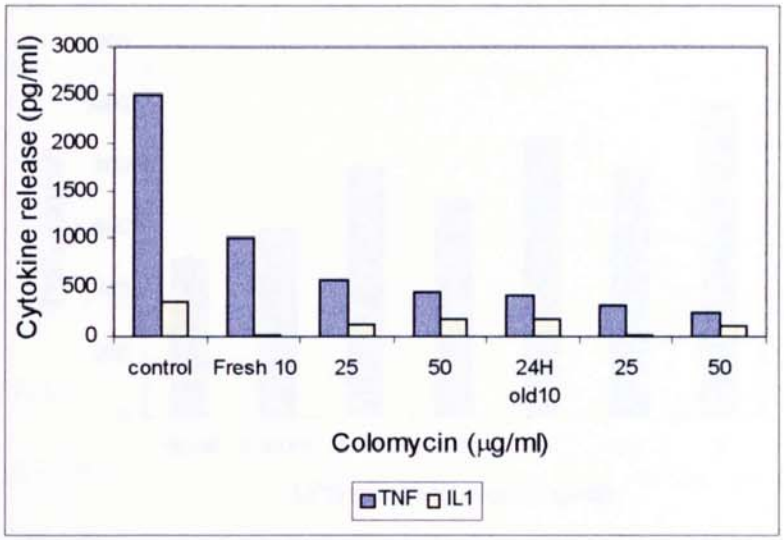

Figure 6.18 Effect of colomycin on the release of IL-1β and TNF-α from U937 cells induced by 0.1 mg/ml and 5 ng/ml of LPS respectively

The effects of freshly-prepared and 24 hour old colomycin were the same as PMB on the release of TNF-α. There was a clear dose-response for the fresh colymycin, but enhanced inhibition was observed with the 24 hour old colomycin. For all concentrations the 'old' colomycin gave at least a five-fold decrease in the TNF-α production by U937 cells stimulated with 5 ng/ml of LPS from *P. aeruginosa.* Surprisingly, the effect of colomycin on IL-1β production was different. There was an inverted dose response for fresh colomycin, the lowest concentration giving the greatest inhibition. For 'old' colomycin there was no dose response at all but all concentrations gave a 50 % inhibition of IL-1β production by U937 cells.

Since *B. cepacia* LPS is more active than LPS of *P. aeruginosa*, any inhibitory effect of doxycycline on cytokine secretion by macrophages in response to *B. cepacia* would be of clinical significance. The suitable dose for *B. cepacia* LPS was first investigated (Figure 6.19).

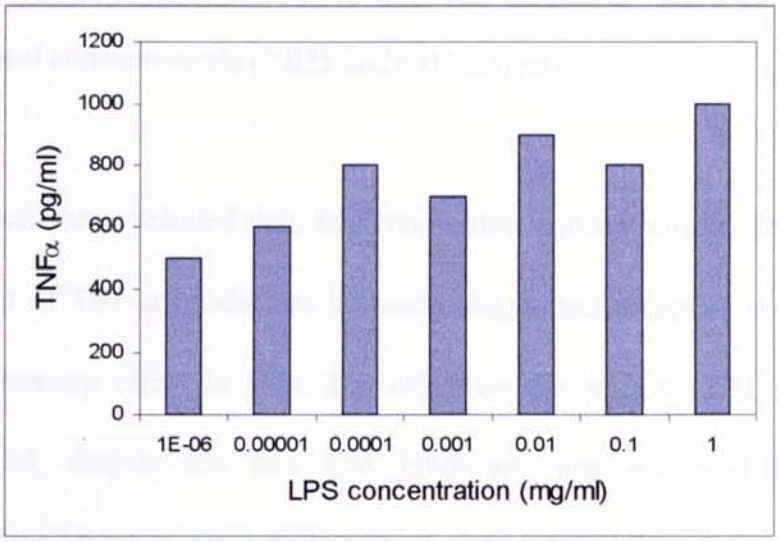

Figure 6.19 Dose response curve for release of TNF-α from U937 cells induced by LPS from *B. cepacia*

From the results in Figure 6.19, showing the dose response to *B. cepacia* LPS on the release of TNF-α, a concentration of 1 ng/ml was chosen for further experiments. The same range of concentrations was used to measure the release of IL-1β but, even at an LPS concentration of 1 mg/ml, secretion of IL-1β no higher than 35 pg/ml was achievable (data not shown).

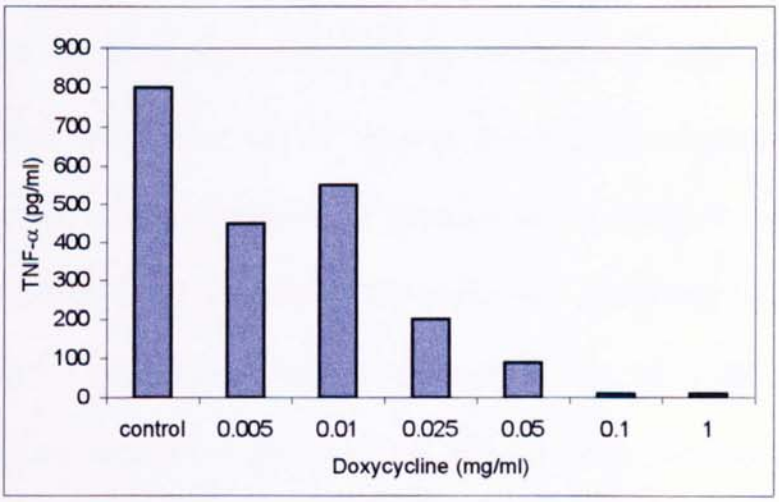

Figure 6.20 Effect of doxycycline on the release of TNF-α from U937 cells induced by LPS from *B. cepacia* (1 ng/ml)

Figure 6.20 shows the effect of doxycycline on the release of TNF-α by U937 stimulated with 1 ng/ml of *B. cepacia* LPS. There was a dose response to

doxycycline which gave a significant inhibition of TNF-α production at physiological concentrations (0.025 and 0.05 mg/ml).

Overall it can be concluded that, at physiological concentrations, doxycycline is an inhibitor of TNF-α production by macrophages and therefore could exert an anti-inflammatory effect *in vivo*. The effect on the release of IL-1β was less obvious and, despite the fact that inhibition was observed for specific concentrations of doxycycline, there was no clear pattern of results.

CHAPTER 7. DISCUSSION AND CONCLUSIONS

The work described in this thesis has provided experience with the basic methods for studying the antibody response of patients to defined bacterial antigens from *P. aeruginosa* and *B. cepacia*. Methods have also been developed for the analysis of the major antigenic components of these organisms (i.e. the outer membrane proteins and LPS). At the start of the project it was thought that *B. cepacia* would continue to pose a major threat to the health of CF patients. Whilst this is still the case, segregation policies have proved to be extremely effective in reducing the transmission of *B. cepacia* between individuals (Ledson *et al.*, 1998) and the number of cases in the UK has reduced significantly. It was therefore not anticipated that sufficient serum samples would become available to carry out a full study on the antibody response to the outer membrane antigens of this organism.

The principal objective was to support the diagnosis of colonisation of CF patients with *P. aeruginosa* and *B. cepacia*, allowing prompt and appropriate antibiotic therapy to be commenced. In Denmark serological methods have been employed for many years to monitor CF patients who are already colonised with *P. aeruginosa*. The original method devised by Høiby (1977) involved measurement of precipitins produced by crossed immunoelectrophoresis of patient serum and a mixture of whole cell antigens (equivalent to the PSA3 antigen used in this thesis). ELISA and western blotting methods were later employed as more sensitive and convenient techniques (Shand *et al.*, 1988; Pressler *et al.*, 1994). Although the PSA3 antigen provides a broad range of

whole cell *P. aeruginosa* antigens, the work described in this thesis shows that purified rough LPS from a single strain gives a more sensitive ELISA test when used for the serological detection of first colonisation by *P. aeruginosa*.

Levels of IgA in saliva were also investigated using ELISA assays based on PSA3 and purified rough LPS. In this case elevated responses were only obtained for PSA3. An increased IgA level at the time of colonisation was observed. This suggests that saliva IgA to PSA3 could be a useful marker in the detection of first colonisation, but more saliva samples are needed. The measurement of salivary IgA would be less invasive than serological methods of early diagnosis and therefore a more acceptable method of monitoring non-colonised children.

Procalcitonin is a newly-recognised potential marker for Gram-negative sepsis (Al-Nawas and Shah, 1998). Serum levels of procalcitonin were measured in patients who acquired *P. aeruginosa* during the course of the study. Overall serum levels increased at the time of the first colonisation. The results are the first demonstration of elevated levels of procalcitonin in CF and are of particular interest since lung colonisation by *P. aeruginosa* in CF does not usually progress to sepsis. The major source is the thyroid but it is also produced by mononuclear cells and other tissues in the lungs (Oberhoffer *et al.*, 1999). The mechanism which leads to elevated procalcitonin levels in CF remains to be established.

Previous studies by Lacy *et al.*, (1997) had shown that outer membrane proteins of *B. cepacia* could be used as the basis of a serological test for *B. cepacia* infection in patients who were already colonised with *P. aeruginosa*. The

methods used to prepare the outer membrane proteins involved SDS-PAGE and electroelution of protein bands from the gels. More recently, Livesley *et al.* (1998) showed that CF strains of *B. cepacia* contained two major patterns of outer membrane proteins on SDS-PAGE. One type (type A) contained major proteins of 36, 27 and 18 kDa whilst the second group (type B) contained two proteins in the 81-90 kDa range. Each of these proteins was evaluated separately in this thesis to determine which performed best in the serological detection of *B. cepacia* infection. Although only a few patient sera were available for testing, it was found that the highest levels of antibody were directed against the 36 kDa protein. This protein appears to be specific for *B. cepacia* and can therefore be used in patients who are already colonised with *P. aeruginosa.*

Nebulised colomycin, the sulphomethate prodrug form of colistin, is used in the treatment of *P. aeruginosa* infection in CF (Littlewood, 1985; Littlewood *et al.*, 2000b). The active drug, colistin sulphate cannot be administered in this way because of its toxic side effects. The sulphomethate is effective in reducing the sputum colony counts of *P. aeruginosa*, suggesting that it is rapidly converted to the active form of colistin in the lungs after aerosol administration. Very little information is available on the kinetics of this process. Using a paper electrophoresis method it was shown that the sulphomethate breaks down slowly at 37 °C. The fully positively-charged form of colistin, in which all the sulphomethate substituents have been released, could only be detected after 2 hours. In agreement with this observation, lethal activity of colomycin sulphomethate towards *P. aeruginosa* was only detected after 2 hours incubation at 37 °C.

A related part of the project concerned the role of *P. aeruginosa* LPS in CF lung infections and the potential for neutralisation of this potent endotoxin by therapeutic agents. Clinical observations have shown that the macrolide antibiotics (e.g. azithromycin) and tetracyclines (e.g. doxycycline) exert useful anti-inflammatory action in the lungs which is independent of their antimicrobial action (Khan *et al.*, 1999; Labro, 1998). Since the polymyxin antibiotics bind specifically to LPS, potential also exists for neutralisation of LPS by colomycin within the lungs. To obtain evidence that colomycin or other antibiotics with no antibactericidal activity against *P. aeruginosa* and *B. cepacia* neutralise the inflammatory action of LPS, it was necessary to devise experiments using mammalian cells which are responsive to LPS. These antibiotics were therefore investigated initially in the murine peritoneal macrophage system and in the U937 human monocyte cell line to reveal any reduction in macrophage activation stimulated by LPS. This work was carried out in collaboration with Dr A. D. Perris (Immunology Research Group, Aston University) who had developed suitable systems using macrophages derived from peritoneal lavage of mice (Griffin, 1998). The macrophages were purified by adhesion to culture plates following overnight incubation in tissue culture medium (RPMI). The stimulation of the macrophages by LPS was measured using chemoluminescence techniques as a measure of the respiratory burst. An inhibitory effect of colomycin and doxycycline at the level of the macrophage was observed. The production of cytokine being the sign of an immunological response and TNF-α and IL-1β being the first cytokines released when there is an infection and triggering the inflammation, their quantification was used to evaluate inflammation. Using the

murine peritoneal system, doxycycline had an inhibitory effect on the release on the cytokines but only levels which are not physiological (i.e. a range of 0.1-1 mg/ml doxycycline was required to produce 50 % inhibition). Also the LPS concentration needed to stimulate release of the cytokines by murine peritoneal macrophages (0.5 mg/ml) was much higher that the concentration present in colonised lungs (Schromm *et al.*, 1996).

In the U937 cell model system the stimulation of the cells with only 5 ng/ml of *P. aeruginosa* LPS enhanced the release of TNF-α. At physiologically achievable concentrations tetracyclines (1-10 μg/ml), PMB and colomycin reduced significantly the production of TNF-α. Colomycin dissolved in water 24 hours before the assay was conducted, was an even better inhibitor of TNF-α. This result is in accordance with the theory, demonstrated in this thesis, that colomycin has to release its sulphomethate substituents before becoming active.

The tetracyclines, and especially doxycycline, appear to exert their anti-inflammatory effects by preventing cytokine release. Possible mechanisms include the inhibition of the matrix metalloproteases (MMPs) required for processing pools of intracellular cytokines prior to release from the cells. For example, tetracyclines have been shown to block secretion of TNF-α by inhibition of TNF-convertase, a TNF-specific MMP (Shapira *et al.*, 1997). Doxycycline might inhibit processing of pIL-1β through inhibition of MMP-9 and MMP-2 (Singer *et al.*, 1995; Curci *et al.*, 2000). The ability of tetracyclines to chelate calcium might also play a role in inhibition of IL1 expression since elevated intracellular levels of calcium produced by calcium ionophores stimulate

processing of the precursor form (Suttles *et al.*, 1989). Tetracyclines might also block *de novo* synthesis of cytokines through inhibition of protein synthesis. Unfortunately the intracellular concentration of tetracyclines within eukaryotic cells is difficult to predict and depends upon the nature of the tetracycline (Gabler, 1991). Therefore it is not possible to correlate the observed dose-response effects of tetracyclines and cytokine release with their effects upon MMPs, intracellular calcium levels or protein synthesis inhibition.

Doxycycline has been suggested for use in the treatment of inflammatory conditions such as rheumatoid arthritis (Hanemaaijer *et al.*, 1997). The results presented in this thesis provide incentive for further studies on its use to control inflammation in CF lung infections without any associated antimicrobial activity. The mechanism of anti-inflammatory action of the tetracyclines needs to be understood so that specific anti-inflammatory drugs can be designed without any antibacterial effects which otherwise could give rise to bacterial resistance. This could prevent inflammation and therefore degradation of the CF lungs and increase life expectancy in CF patients.

As CF is an inherited disease, even if effective antibiotics against *P. aeruginosa* and anti-inflammatory drugs can prolong life, the defective CFTR gene and all its consequences will always remain. Gene therapy is therefore the only solution for curing the disease. Clinical trials are now being conducted on CF patients for the delivery of the CFTR gene by adenovirus vectors and liposomes (Flotte, 1999). The thick mucus in CF lungs prevents nanospheres from being delivered (Sanders *et al*, 2000). Production of inflammatory secretions in CF lungs fluid appears to be inhibitory to recombinant adeno-associated virus transduction (Virella-Lower et al, 2000). Even if gene therapy is the way forward there are a lot of problems

to overcome first before it can be used; until then the development of new antibiotics and anti-inflammatory compounds are vital.

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