The early detection of *Burkholderia cepacia* infection

in cystic fibrosis patients.

Thesis submitted in accordance with the requirements of

The University of Leicester

for the degree of MD

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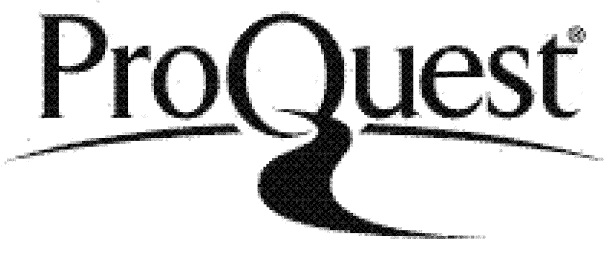
February 1996



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ABSTRACT

Burkholderia (Pseudomonas) cepacia is now recognised as an important pathogen in cystic fibrosis patients. The diagnosis of *B. cepacia* currently depends on sputum culture and isolation, using a selective medium. However, isolates are slow to grow and confusion may still occur with other organisms. There is evidence that *B. cepacia* infection may precede sputum culture by several months. Early and accurate diagnosis of *B. cepacia* is important if segregation is to prevent patient-to-patient transmission.

In an attempt to establish the use of antibody studies as diagnostic indicators of *B. cepacia* infection, the IgG reaction to *B. cepacia* outer membrane proteins and lipopolysaccharide in patients colonised with *B. cepacia* and *Pseudomonas aeruginosa*, was examined. A low-iron chemically defined medium was developed to grow clinical *B. cepacia* strains. Outer membrane was studied by SDS-PAGE and immunoblotting. A specific anti-*B. cepacia* IgG reaction to outer membrane protein and lipopolysaccharide antigen was found that was not due to cross-reactivity with *P. aeruginosa.*

The serum IgG reaction to a *B. cepacia* specific 80 kDa outer membrane protein was investigated. The 80 kDa protein is thought to be an important porin protein. The 80 kDa protein was recovered by electroelution from separated outer membrane protein. An ELISA test using the 80 kDa protein was able to distinguish between 21 cystic fibrosis patients colonised with *B. cepacia* and 21 age and sex-matched cystic fibrosis patients who were not colonised with *B. cepacia* but were colonised with *P. aeruginosa.*

Polyclonal monospecific anti-*B. cepacia* antibodies were produced by the immunisation of rabbits with the 80 kDa protein. Using this antibody *B. cepacia*-specific-immunofluorescence was demonstrated. The immunofluorescence was successfully performed on cells grown in culture but sputum samples were difficult to interpret because of excessive debris in the sputum.

These studies indicate that cystic fibrosis patients colonised with *B. cepacia* produce a *B. cepacia*-specific-antibody response. Specific *B. cepacia* antigens and antibodies can be used in the detection of *B. cepacia* infection in cystic fibrosis patients. Further work is needed to determine whether these *B. cepacia* antigens and antibodies will lead to an earlier diagnosis of infection.

To Alison, Jessica and Kristan.

ACKNOWLEDGEMENTS

The laboratory research was carried out in the department of Pharmaceutical Sciences at Aston University. I am very grateful to Professor MWR Brown who encouraged me to start this research and continued to advise me throughout my research. I would also like to acknowledge my debt to Dr AW Smith and Dr PA Lambert for their help, particularly with the laboratory work. I am grateful to all the staff at Aston University, particularly Roy Tilling and Dorothy Townley who helped me in all kinds of practical ways.

I am indebted to everyone on the cystic fibrosis team at Birmingham Children's Hospital whose enthusiasm and dedication never failed to motivate me to keep going. I am especially grateful to Dr PH Weller who advised me on the clinical aspects of the study.

I wish to also acknowledge Dr DE Stableforth and Dr EG Smith at Birmingham Heartlands Hospital, not only for allowing me to study their patients but for their advice and support. Dr D Peckham also helped by providing samples from patients at Nottingham City General.

I also wish to thank Professor Andrews of Leicester University for his help in reading through the manuscript and making many useful suggestions.

This thesis was supported by a generous grant from the Cystic Fibrosis Trust. I owe an enormous debt to all the cystic fibrosis patients and their families, too numerous to name, who helped me during the research.

Finally, special thanks to my wife and daughter, Alison and Jessica for their support throughout all the ups and downs.

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CHAPTER 1

INTRODUCTION

1.1 Cystic Fibrosis

1.1.1 *Genetics and pathophysiology of CF*

Cystic fibrosis (CF) is the commonest lethal inherited disease in Northern Europe with an incidence of 1 in 2500 live births. The CF population is increasing by 120-140 patients per year, and by the year 2000 there could be 6000 patients in the United Kingdom, with equal numbers of adult and paediatric patients (Clinical Standards Advisory Group, 1993). At present the median survival is only into the third decade (Dodge *et al*, 1993).

CF is an autosomal recessive condition carried by approximately 1 in 25 white Europeans. The cystic fibrosis gene is found in the long arm of chromosome 7 (7q31.1) and codes for the cystic fibrosis transmembrane conductance regulator protein (CFTR) (Rommens *et al*, 1989; Riordan *et al*, 1989). The DNA sequence predicts a protein sequence of approximately 170 kD composed of two homologous repeated units. Each unit is composed of six transmembrane domains and a nucleotide binding fold in which the two units are separated by a large polar R-domain. The CFTR protein is considered to be membrane bound and have a cell membrane transport function and thought to be similar to transport proteins of the P glycoprotein family (Riordan *et al*, 1989).

The major cystic fibrosis mutation among caucasians from northern Europe and north America is a three-base-pair deletion that corresponds to a single deletion of the amino acid phenylalanine, at the ΔF508 of the CFTR (Kerem *et al*, 1989). This

mutation, ΔF508, is present in approximately 70% of North European CF patients, with five other mutations accounting for a further 20% of cases (Cutting *et al*, 1992). Testing for 10 of the common mutations, including ΔF508, will detect approximately 85-90% of the CF mutations. Although several hundred other mutations have been described worldwide, many of the remaining 10% of cases are as yet untypeable. DNA analysis can be used to confirm the diagnosis in patients with an equivocal sweat test or unusual clinical features (Strong *et al,* 1991) and is invaluable in genetic counselling and antenatal diagnosis.

There appears to be some correlation between particular mutations present and the severity of the disease. However, the large spectrum of the severity associated with any given mutation makes it difficult to predict the prognosis in any one individual (Collins, 1992).

Using techniques of in situ hybridization and immunohistochemistry CFTR messenger RNA and the CFTR protein have been detected in those organs affected in CF, including the respiratory tract, gastrointestinal tract, reproductive tract, kidney and sweat glands (Collins, 1992).

The basic cellular pathophysiology in CF has been demonstrated to be altered ion transport in exocrine epithelial cells with impairment of conductance of chloride ions and reduced permeability of apical epithelial cells to chloride (Quinton and Bijman, 1983; Knowles *et al,* 1983). The CFTR protein is thought to regulate the chloride channel, by acting as the main cellular chloride channel (Bear *et al*, 1992) or an

alternative chloride channel that can regulate the main chloride channel (Ward *et al*, 1991, Egan *et al*, 1992).

CFTR cellular chloride channel can exist in an open or closed state. Transition from the closed state to the open state is mediated by a cyclic AMP-dependant protein kinase that can phosphorylate one of the many phosphorylation sites on the R domain, while ATP interacts with one of the nucleotide binding domains. ADP may exert an opposite effect by interacting with the other nucleotide-binding domain (Anderson and Welsh, 1992).

The genetic defect results in a disturbance in the movement of other ions and water across cellular membranes. The flow of water across epithelial membranes occurs in response to an osmotic gradient resulting from the active transport of sodium and chloride. CF tissues show an alteration in the hydration of the periciliary fluid of the respiratory mucus. Malfunction of chloride transport in respiratory epithelial cells results in decreased luminal chloride secretion and increased sodium resorption. Diffusion of water from the interstitial fluid into the lumen is therefore reduced resulting in relative dehydration of mucus which becomes abnormally thick and tenacious. In the lung this causes mucus plugging and reduced mucociliary clearance.

In the pancreas, viscid mucus causes duct obstruction and results in pancreatic insufficiency from inflammatory damage to exocrine cells. This leads to a reduction or absence of pancreatic enzymes, causing malabsorption and malnutrition. Diabetes mellitus occurs in approximately 10% of older patients from secondary damage to the

islets of Langerhans. The effects of chloride channel malfunction are also seen in the liver, small bowel, upper respiratory tract including the sinuses, sweat glands, and the vas deferens in the male.

1.1.2 *Clinical presentation and diagnosis*

Most patients present in infancy although the diagnosis can be made from early fetal life to adulthood. It is common for there to be a delay between the onset of symptoms and the diagnosis being established.

Approximately 12% of patients present at birth with small bowel obstruction secondary to meconium ileus or with prolonged jaundice (Kerem *et al*, 1989). The majority of patients present in infancy with a combination of respiratory symptoms and failure to thrive despite a voracious appetite. Typically, patients have abnormally loose and frequent stools due to steatorrhoea and rectal prolapse is common. However, there is a wide spectrum of disease severity and some patients with less severe respiratory disease and pancreatic sufficiency present later. Less commonly, CF can present as liver disease, nasal polyps or male infertility.

Diagnosis is confirmed by sweat test, by measuring sodium and chloride concentrations in sweat. Although defects in both sweat secretion (Sato *et al*, 1984) and the reabsorption (Quinton, 1983) of salt from secretory fluid have been described, the latter is much more pronounced. Sweat is collected on to filter paper following stimulation by pilocarpine iontophoresis. Concentrations of both ions are markedly

raised in CF. Pancreatic insufficiency is diagnosed by low faecal chymotrypsin values and the presence of fat in the stool.

1.1.3 *Lung disease*

The normal mucocilary system that cleanses bronchopulmonary epithelium of inhaled particles depends on the upward directional flow of a mucus layer positioned on the tips of cilia, which move freely in the underlying watery layer. In CF there is a net deficiency of water, which hinders the normal upward flow of the mucus "blanket". Physiological studies on respiratory epithelium of CF patients have revealed increased basal and β-agonist-stimulated reabsorption of sodium ions, together with the absence of β-agonist-stimulated secretion of chloride ions (Willumsen and Boucher, 1991). To maintain the isotonicity, water will flow passively with electrolytes and both decreased secretion and increased absorption of electrolytes will lead to dehydration of the secretions covering the respiratory epithelium. The failure of the β-adrenergic-stimulated increase in mucociliary clearance results in the failure of the normal protective mechanisms against bacterial colonisation and infection.

Persistent bacterial infections cause a chronic necrotising bronchiolitis and lung damage resulting in predisposition to chronic bacterial colonisation. A vicious cycle of infection and inflammation ensues. Further exacerbations of bronchopulmonary disease can be triggered by viral or bacterial infections.

1.1.4 *Infections of the lower respiratory tract*

The range of bacteria associated with pulmonary infection in CF are curiously

restricted. Classically, early colonisation with *Staphylococcus aureus* is followed by infection with non-typeable *Haemophilus influenza* and then "wild type" non-mucoid *Pseudomonas aeruginosa* (Hoiby, 1988).

1.1.4.1 Pulmonary infection with *Staphylococcus aureus*

Staphylococcus aureus has been identified in lower respiratory secretions as early as two months of age in a study using fibre optic bronchoscopy of infants identified prospectively by a screening programme (Armstrong *et al,* 1994). These workers demonstrated lower respiratory pathogens in 28% of infants studied using fibre optic bronchoscopy at a median age of 2 months. In 25% of cases *S. aureus* was identified.

S. aureus produces a number of virulence factors including peptidoglycan, techoic acid and protein A which can promote lung damage (Hoiby 1988). Prophylactic treatment of all newly diagnosed patients with anti-staphylococcal antibiotics, such as Flucloxacillin , has been shown to reduce the number of isolates of *S. aureus* from the respiratory tract, with fewer additional courses of antibiotics and fewer hospital admissions within the first two years of life (Weaver *et al,* 1994).

1.1.4.2 Pulmonary infection with *Haemophilus influenza*

The isolation rate of acapsulated forms of *H. influenza* has been shown to be significantly greater in patients with cystic fibrosis than in a control group with asthma (Rayner *et al,* 1990). Isolation rates for *H. influenza* rose significantly during exaccerbations in patients with CF but not in those with asthma. Routine sputum culture may fail to detect *H. influenza* where there is colonisation with *P. aeruginosa*

unless an *H. influenza* selective medium is used (Roberts *et al*, 1980). *H. influenza* produces a number of virulence factors which may cleave IgA, induce ciliary dyskinesia, release histamine and stimulate mucus secretion (Elborn *et al*, 1990). All of these may contribute to lung injury.

1.1.4.3 Pulmonary infection with *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is not pathogenic to the normal respiratory tract, but due to the defective mucociliary clearance, *P. aeruginosa* is able to infect the lung, (Hoiby and Koch, 1990). *P. aeruginosa* infection is not specific to cystic fibrosis, but children who are infected by *P. aeruginosa* often have defective immunity, burns, receiving mechanical ventilation or have a tracheostomy. *P. aeruginosa* can also occurr in children with bronchiectasis or other chronic lung disease, but is far more frequent in cystic fibrosis than in other diseases and causes much greater morbidity in cystic fibrosis.

The earliest event that occurs in the process of mucosal infection of *P. aeruginosa* is adherence to epithelial structures, either epithelial cells or mucus, via adhesins on projecting polar pili (fimbriae) or within the alginate secreted and loosely associated with the cell lipopolysaccharide (LPS) capsule (Irvin *et al,* 1989).

After adherence of *P. aeruginosa* to the epithelium, local bacterial multiplication, aided by the secretion of virulence factors, occurs if mucociliary clearance is insufficient and leads to colonisation of the lung. *P. aeruginosa* frustrates host defences by undergoing a phenotypic change, with increased expression of the genes

coding for the production of alginate (*algD*) (Martin *et al*,1993). This alteration gives the characteristic mucoid growth (Pedersen, 1992). *P. aeruginosa* persists in the respiratory tract by producing microcolonies embedded in a biofilm of alginate (Pedersen, 1992). The biofilm mode of growth protects the organism from the host defence mechanism. Occasional release of free-flowing (planktonic) bacteria may establish daughter colonies within the endobronchial tree; in time, the organism persists in the main part of the lower respiratory tract.

The transition from colonisation to infection is determined by the host response, which initially involves non-specific inflammatory components but soon includes elements of a specific immune response (Elborn and Shale, 1990). This host response detectable by a rise in leukocyte counts, acute phase reactents, systemic antibodies and cytokine production. During early infection there are minimal disease symptoms, but as the chronic infection becomes established and the host responds with production of specific antibodies, the patient gradually enters a phase of bronchopulmonary inflammation and obstruction. Abundant evidence indicates that it is not the organism but the immune-mediated inflammation that leads to tissue damage and loss of lung function (Hoiby *et al*, 1987). This inevitably leads to bronchiectasis and eventual death from Cor Pulmonale. Suppurative lung disease is usually the most serious manifestation of CF and predicts the prognosis of individual patients.

1.2 Burkholderia cepacia

1.1.2 *Introduction*

Burkholderia cepacia (*Pseudomonas cepacia*) was first described in 1950 by Burkholder as the cause of soft onion rot (Burkholder, 1950). *B. cepacia* is a catalase-positive gram-negative organism; its appearance on Gram staining is of a short oval rods with bipolar staining, the so called safety pin appearance (Sadeghi *et al*, 1994) (Fig. 1.1).

Despite previously being regarded as related to *P. aeruginosa* and a member of the genus *Pseudomonas*, *B. cepacia* is phylogenetically very different from *P. aeruginosa*. *P. aeruginosa* belongs to the *Pseudomonas* rRNA group 1 and *B. cepacia* belongs to the rRNA group 2 (Palleroni *et al*, 1973). This division has been further supported by studies based on the 16s rRNA gene sequences (Xiang Li *et al*, 1993). Therefore, *B. cepacia* along with six other pseudomonads has been transferred to the new genus *Burkholderia* (Yabuuchi *et al*, 1992). This transfer was based on 16s rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition and phenotypic characterisations.

Although *B. cepacia* is a plant pathogen, its ability to promote plant growth by antagonizing certain plant pathogens has led to its use in agriculture (Hebbar *et al*, 1992). *Burkholderia cepacia* is found in soil and water but it is not as ubiquitous as was once thought. In a study of 55 environmental sites, only 12 *B. cepacia* isolates were found (Butler *et al*, 1995). *B. cepacia* was cultured from only 1% of surfaces

obtained from homes and 4.5% from salad bars and food stores in a study by Fisher *et al* (1993). *B. cepacia* can grow in water with minimal nutritional requirements and survive in a number of disinfectants. It is therefore surprising that being so nutritionally versatile it is not as widespread as one would expect.

1.2.2 *Burkholderia cepacia* in cystic fibrosis

B. cepacia is now recognised as an important pathogen in patients with cystic fibrosis (Isles *et al* 1984; Thomassen *et al*, 1985; Editorial Lancet, 1992). The first reports of *B. cepacia* in patients with CF came from Philadelphia, USA, in 1977 (Laraya-Cuasay L *et al*, 1977). The association with an adverse outcome for some infected patients was noted. By 1982 carriage rates of 45% were reported from one Canadian clinic (Nolan *et al*, 1982), and in 1983 the same centre recognised a rapidly fatal outcome in a proportion of those who harboured the organism (Gold *et al*, 1983).

Estimates of the prevalence of *B. cepacia* in the UK vary but surveillance studies between 1986 and 1989 in the UK indicated a prevalence of 7% (Goldman *et al*, 1992). However, bacterial isolations have risen in some CF centres to equal the 40% prevalence of some North American centres (Sajjan *et al*, 1992).

B. cepacia rarely causes disease in healthy subjects. However infection of the immunocompromised host is well recognised and two cases of *B. cepacia* septicaemia in children with chronic granulomatous disease (CGD) have been reported (Lacy *et al*, 1993). Chronic granulomatous disease is characterised by recurrent life threatening infections with catalase-positive bacteria as a consequence of defective neutrophil

function. *B. cepacia* accounts for 5% of all infections in CGD (Gallin *et al,* 1983). Neutrophil function in CF patients colonised with *B. cepacia* was found to be normal (Muhdi *et al,* 1994). The ability of *B. cepacia* to survive intracellularly in CGD raises the possibility that this might be possible in the CF lung. Nosocomial acquisition can also occur from contaminated equipment (Pallent *et al,* 1983).

Environmental strains of *B. cepacia* are usually susceptible to multiple antibiotics whereas strains from patients with CF are frequently multiresistant (Prince *et al,* 1985). Even when in-vitro sensitivity is observed, the clinical response is poor, although Peckham *et al* (1994) showed no statistical difference in the response to intravenous antibiotics between patients colonised with *B. cepacia* and patients colonised with *P. aeruginosa. B. cepacia* is often sensitive to carbapenems such as imipenem and meropenem although carbapenamases have now been isolated from *B. cepacia* (Simpson, 1993).

1.2.3 *Virulence factors associated with* B. cepacia

The virulence factors of *B. cepacia* have been reviewed by Nelson *et al* (1994). Although the mucoid phenotype of *B. cepacia* may be observed under appropriate culture conditions (Sage *et al,* 1990) biochemical characterization of the exopolysaccharide indicates that it is not alginate (Nelson *et al,* 1994). The absence of alginate biosynthesis in *B. cepacia* is supported by PCR studies which showed no evidence of the *alg*D gene, encoding the key enzyme mannose dehydrogenase, in ten strains of *B. cepacia* (Nelson *et al,* 1994).

Some strains of *B. cepacia* synthesise iron-chelating siderophores including pyochelin, cepabactin and azurechelin (Sokol, 1986; Bukovits *et al,* 1882; Sokol *et al,* 1992; Meyer *et al,* 1989).

B. cepacia exhibits variable degrees of binding to human respiratory mucin (Sajjan *et al,* 1992). Mucins from CF patients bind specifically to *B. cepacia,* but not to *P. aeruginosa.* Mucin from non-CF patients do not bind *B. cepacia* (Sajjan *et al,* 1992). Electron microscopy studies have shown that approximately 60% of *B. cepacia* strains express peritrichous fimbriae (Kuehn *et al,* 1992). Other *B. cepacia* strains possess polar fimbriae, similar to those expressed by *P. aeruginosa* (Saiman *et al,* 1990).

CF isolates of *B. cepacia* can produce putative virulence factors such as proteases, lipases (McKevitt and Wood, 1984) and haemolysin (Gessner and Mortensen, 1990). Protease activity was detected in 88% of isolates examined (McKevitt and Wood, 1984). The protease was subsequently purified by McKevitt *et al* (1989) and was found to be a 34 kDa metalloprotease. This was found to cause bronchopneumonia when instilled intratracheally in rats (Mckevitt *et al,* 1989). This 34 kDa protease appeared to be immunologically related to *P. aeruginosa* elastase, a key virulence factor associated with *P. aeruginosa* colonisation in CF *(Kooi et al,* 1994). B. cepacia also produces a 40 kDa extracelluar protease (Kooi *et al,* 1994).

B. cepacia lipopolysaccharide antigen (LPS) has been shown to have endotoxic activity and the capacity to induce a high level of tumour necrosis factor (TNF) (Shaw *et al,* 1995). *B. cepacia* LPS, on a weight-for-weight basis, induces approximately nine times as much tumour necrosis factor (TNF) compared to *P.*

aeruginosa LPS. Although there are likely to be many other triggers and mechanisms whereby immune-mediated lung damage might occur, this might suggest that *B. cepacia* has a greater potential than *P. aeruginosa* for sustained immune-mediated damage in the lung.

1.2.4 *Prognosis following acquisition of* B. cepacia

Some CF patients remain stable after acquisition of *B. cepacia*, but many show a progressive deterioration. A small group of CF patients have a systemic infection with recurrent fever, the "*B. cepacia* syndrome". The isolation of *B. cepacia* from blood cultures accompanied by clinical evidence of systemic infection provides further evidence of a pathogenic role for this organism (Lewin *et al*, 1990). In some patients deterioration is rapid, leading to death within weeks of *B. cepacia* infection (Tablen *et al*, 1985, Tablen *et al*, 1987).

In a retrospective study of the patterns of *B. cepacia* pulmonary infection at a major CF centre, all patients who had normal or mild lung disease at the outset of infection remained stable, whereas six of the 28 patients with severe disease remained stable, three of whom were only transiently infected with *B. cepacia* (Taylor *et al*, 1993).

A Canadian centre for lung transplantation reported a much higher morbidity and mortality post transplant for patients colonised with *B. cepacia* due to post operative *B. cepacia* septicaemia (Snell *et al*, 1993). It was suggested that patients colonised with *B. cepacia* should not be considered for lung transplantation. However, experience from the transplant centres in the UK has differed from the Canadian

experience, with no significant increased risk for those patients colonised with *B. cepacia* (Egan *et al,* 1995).

1.2.5 *Immune response to* P. aeruginosa *and* B. cepacia

Cystic fibrosis patients not previously exposed to *P. aeruginosa* lack a detectable immune response to the organism, however, on subsequent infection a rapid response is mounted against surface components (Doring and Hoiby, 1983; Hancock *et al,* 1984; Shand *et al,* 1988). Several studies have demonstrated an interval of several months between when sputum cultures become positive for *P. aeruginosa* and when the humoral response occurs (Doring and Hoiby, 1983; Fomsgaard *et al,* 1988). This delay may indicate a sluggish immune response to mucosally presented antigen. However, rises in serum antibodies to *P. aeruginosa* LPS before detection in the sputum have been reported (Brett *et al,* 1987). This could represent sampling error (in which samples obtained do not accurately reflect the colonisation of the lower respiratory tract), cross-reactivity between antigens of *P. aeruginosa* and other microbes, or assay differences (Shand *et al,* 1988).

High concentrations of specific serum IgG anti-*P. aeruginosa* antibodies are positively correlated with both the aggressive course of chronic *P. aeruginosa* infection and poor prognosis (Hoiby and Koch, 1990). The characteristic chronic persistence of *P. aeruginosa* in CF lungs is attributed to the failure of this immune response to opsonise the bacteria effectively. Moreover, attempts at opsonophagocytosis are frustrated by conversion from the lipopolysaccharide (LPS) smooth state to the LPS-rough state and production of mucoid alginate (Hoiby and

Koch, 1990). Indeed, Pier *et al* (1987) have demonstrated the presence of antibody to *P. aeruginosa* mucoid alginate in older patients not colonised with *P. aeruginosa*, suggesting that the specific lack of this antibody may result in the chronic colonisation observed in other CF patients.

In most patients with cystic fibrosis, hyperimmunoglobulinaemia G and A develops (Moss, 1987). Despite the vigour of the antibody response to *P. aeruginosa* in CF, only a minor proportion of the hypergammaglobulinaemia is caused by specific antibody formation (Hoiby and Hertz, 1981). Antibodies to *P. aeruginosa* antigens rise during episodes of pulmonary exacerbation and fall in response to antibiotic therapy, which is supportive of the pathogenic role attributed to *P. aeruginosa* (Brett *et al,* 1987).

B. cepacia infection occurs frequently in patients already colonised with *P. aeruginosa,* in whom high levels of anti-*P. aeruginosa* serum antibodies exist. Studies by Aronoff and Stern (1988) have shown that anti-*B. cepacia* antibodies can be detected in the serum of *P. aeruginosa*-infected patients not culture positive for *B. cepacia*. Longitudinal studies indicate that antibodies against the 27 and 36 kDa *B. cepacia* outer membrane proteins (OMPs) can be detected up to four years prior to positive sputum culture of *B. cepacia* (Aronoff *et al*, 1991). Another longitudinal study demonstrated that the titre of *B. cepacia* LPS core antigen antibodies are higher in patients colonized with *B. cepacia* and the increase in antibody titre sometimes precedes positive sputum culture by several months (Nelson *et al*, 1993). Therefore, at least in these patients, this raises the question of whether *B. cepacia* colonisation

occurred despite the presence of specific anti-OMP and anti-core LPS antibodies. Previous exposure to *B. cepacia,* not resulting in a positive sputum culture for *B. cepacia* may have occurred, resulting in an antibody response, without persistence of the organism in the lungs. Alternatively exposure to *B. cepacia* may have resulted in undetected infection that persisted either at low levels within the lungs, or possibly at another site, such as the upper respiratory tract or sinuses, undetected by sputum culture.

1.2.6 *Transmission of* B. cepacia *from patient to patient*

There is growing evidence that transmission of *B. cepacia* can occur by patient-to-patient contact (Smith *et al*, 1992; Smith *et al*, 1993; Govan *et al*, 1993). Segregation of patients in hospital and clinic, and advising patients to avoid social contact, has resulted in a reduction in the number of new cases in some CF centres (Thomassen *et al*, 1986). Segregation of *B. cepacia*-positive and *B. cepacia*-negative in patients and out patients has been practised at the Birmingham and Nottingham CF centres since 1989 and 1991, respectively.

Lipuma *et al* (1994) documented two patients that had undetected pulmonary colonisation of *B. cepacia* 12 and 24 months respectively after their contact with a CF patient colonised with *B. cepacia* with the same PCR-ribotype analysis. The cause of this inapparent transmission may be due to low density of *B. cepacia* in the pulmonary secretions preventing positive culture using routine culture methods.

Alternatively there may be sites that sequester the organism for a period and act as

a reservoir for subsequent more complete colonisation. A change in specific host or pathogen factors might occur over time to allow an increase in bacterial density to detectable levels. It is not clear whether these patients from whom *B. cepacia* cannot be detected, who subsequently become colonised, pose a risk to those who are not colonised.

Steinbach *et al* (1994) failed to demonstrate any patient-to-patient transmission of *B. cepacia*. They detected 22 patients colonised with *B. cepacia.* All the patients were found to have different strains as determined by pulsed-field gel electrophoresis. However, it has been suggested that not all strains are equally transmissible and that some CF centres may not harbour a "highly transmissible" strain (Govan, 1995).

Early identification of *B. cepacia* colonisation is essential if segregation of colonised and non colonised patients is to be practised, and transmission thus prevented. In the future early identification may be important if effective antibiotics are found to treat *B. cepacia* and to prevent the rapid onset "*B. cepacia* syndrome".

1.2.7 *Early detection of* B. cepacia

The diagnosis of *B. cepacia* currently depends on sputum culture and isolation using a selective medium such as MAST, UK (Fig. 1.4) and biochemical identification *eg* API 20 NE System (Gilligan *et al*, 1985). *B. cepacia* was first isolated in Nottingham CF patients in 1991 after the introduction of the *B. cepacia*-specific culture medium; this medium has been in use in Birmingham since 1989. Some clinical isolates may take up to seven days to grow, and confusion may occur with *Xanthomonas*

maltophilia (Burdge *et al,* 1995), *Pseudomonas acidovorans* (Govan and Nelson, 1992) and *Burkholderia gladioli* (Simpson *et al,* 1994). These organisms may be mistaken for *B. cepacia* on culture and biochemical identification leading to unnecessary segregation of the patient and distress for the patient.

Further confirmation of all *B. cepacia* isolates by ribotyping is recommended (Doring and Schaffer, 1993). However, even this technique may not always differentiate *B. cepacia* from other organisms. Indeed Simpson *et al* (1994) have postulated that some strains may be a hybrid of *B. cepacia* and *B. gladioli.*

Serological diagnosis may have a role in providing an early indication of *B. cepacia* infection in three different circumstances. Firstly, serological diagnosis may give a result within 24 hours whereas culture may take up to seven days. Secondly, detection of antibodies to *B. cepacia* may occur at an early stage of the infection possibly preceding positive-sputum culture by many months. Thirdly, once an organism has been cultured that is thought to be *B. cepacia,* confirmation by a serological test would avoid further delay involved with sending the organism to a reference laboratory for ribotyping.

Furthermore, diagnosis may be facilitated in younger patients who are unable to expectorate sputum. The predictive value of throat swab and gagged-sputum cultures compared with that of culture of bronchial secretions is limited (Ramsey *et al,* 1991). Serological tests have been used in children who cannot expectorate sputum to detect *P. aeruginosa* infection (Cordon *et al,* 1992) and *B. cepacia* infection may also be

detected in younger children using a serological test for *B. cepacia.*

Detection of a raised antibody response to *B. cepacia,* whether it be in serum or sputum, is only indirect evidence of infection and uncertainty may remain as to whether the antibody response is to a previous infection or to current infection. Detection of antibodies may represent infection of the upper respiratory tract rather than lower respiratory tract infection. A method to detect antigen rather than antibody would be preferable. The safety-pin morphology of *B. cepacia* (Fig. 1.3) as seen on Gram stain had a positive predictive value of 84% in a recent study (Sadeghi *et al,* 1994). This result was obtained in a centre where up to 40% of CF cultures were positive for *B. cepacia.* Greater sensitivity and specificity may be possible by utilising this characteristic morphology in combination with an immunofluorescence technique. This could be facilitated by producing a polyclonal or possibly monoclonal antibody raised against a specific *B. cepacia* antigen. Polyclonal or monoclonal antibodies could also be employed to detect antigen in sputum.

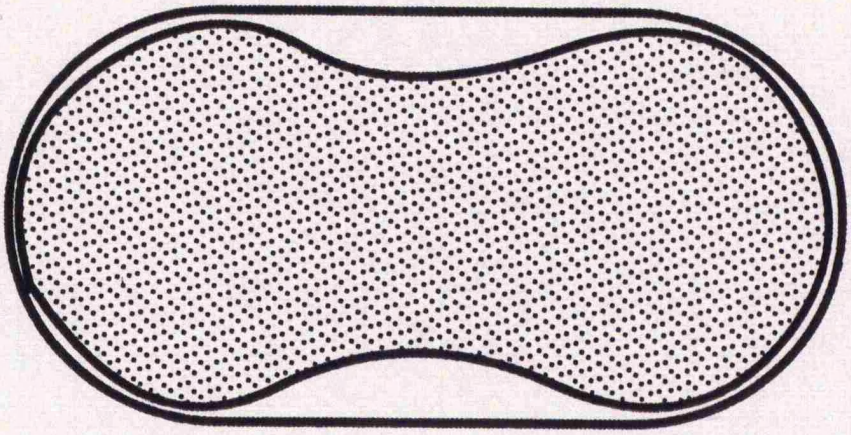

Figure 1.1. Diagrammatic representation of the Gram stain appearance of *B. cepacia*, showing the bipolar staining; the so-called safety pin appearance.

Figure 1.2. *B. cepacia* cultured on the selective medium, MAST (UK), showing characteristic pink colour.

1.3 Aims and objectives

The aim of this thesis is to study the immune response to *B. cepacia* in patients with CF. This will facilitate development of serological tests for *B. cepacia* in patients with CF. It is hoped that these tests will be useful in the early diagnosis of *B. cepacia* infection, firstly by detecting patients who are sputum-culture-negative for *B. cepacia,* but who have *B. cepacia* in the lower respiratory tract, and secondly to develop a test that could be performed within 24 hours that would determine whether a patient was infected with *B. cepacia* before the sputum culture result that might take up to seven days. These aims will be accomplished by the following steps:

1) The development of an in vitro medium for *B. cepacia* allowing expression of antigens also expressed in vivo.

2) To study the immune response to *B. cepacia* in CF patients colonised with *B. cepacia* and to study cross-reactivity with *P. aeruginosa* which is likely to be also present in the CF lung.

3) Using a *B. cepacia*-specific antigen to develop an ELISA-based serological assay of the IgG response to *B. cepacia.*

4) Using the same *B. cepacia*-specific antigen to produce rabbit polyclonal antibodies. These will then be used to develop an *B. cepacia* specific immunofluorescence test.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 Preparation of outer membranes

2.1.1 *Bacterial strains and growth conditions*

Sputum from patients with cystic fibrosis was routinely cultured for *P. aeruginosa* on MacConkey agar (Unipath, Basingstoke). Sputum was also inoculated onto *B. cepacia*-selective medium (MAST, UK; Gilligan *et al*, 1985) incorporating ticarcillin (1000 mg/l) and polymixin B (30 000 units/l). Agar plates were incubated for 48 hours at 37°C and then for five days at room temperature. *B. cepacia* was identified by colonial appearance, API 20NE strips (bioMerieux, Marcy l'Etoile, France), positive oxidase reaction, and resistance to polymyxin B (Pitt and Govan, 1994).

Nine clinical isolates of *B. cepacia* and *P. aeruginosa* were collected from nine adult CF patients at The Heartlands Hospital, Birmingham (Table 2.1, strains 1-9) and confirmed to be *B. cepacia* as above. Eight of the nine strains were ribotyped at the Central Public Health Laboratory (Rabkin *et al*, 1989). Six of the nine were further typed by the pulsed field gelelectrophoresis with the clamped homogenous electric field technique (CHEF typing, Anderson *et al*, 1991) at the Central Public Health Laboratory. The results are summarised in Table 2.1. A non-clinical isolate of *B. cepacia* (NCTC 10661) was also studied.

B. cepacia and *P. aeruginosa* strains were stored in nutrient broth supplemented with 15% v/v glycerol at -70°C and cultured in either a modified iron-supplemented chemically-defined medium (CDM+Fe) or a low-iron chemically defined medium (CDM-Fe) consisting of: 40 mM glucose; 0.62 mM KCl; 40 mM $(NH_4)_2SO_4$; 0.4 mM

$MgSO_4$; 50 mM 3-(N-morpholino) propane sulphonic acid (pH 7.4) supplemented with 0.1% casamino acids (Difco); 0.02mM $FeSO_4.7H_20$ was added to the CDM+Fe. Bacteria were grown to early stationary phase (A_{470} 0.9) in an orbital shaking incubator at 37°C, harvested by centrifugation at 10 000 g and washed once with 0.85% saline. For LPS isolation, strains were grown in tryptone soy broth (TSB, Oxoid) to an optical density A_{470} of 5.

2.1.2 *Measurement of optical density*

This spectrophotometric method was used to measure changes in cell concentration during bacterial growth. It utilises the ability of bacterial cells to scatter light . At relatively low concentrations the light scattered by a bacterial cell suspension is directly proportional to the concentration of the cells in suspension. The relationship is expressed by the Beer-Lambert law:

$$OD \propto \log I_0/\log I$$

where Io is the intensity of the incident light, and I is the intensity of the emergent light provided that the light pathway is constant. The relationship obeys the Beer-Lambert law up to an OD of approximately 0.3. Above this absorbency the OD does not increase proportionally due to secondary light scattering. If the suspensions were diluted to an absorbance less than 0.3 proportionality was restored. Measurements were made at a wavelength of 470nm (A_{470}) to minimise absorption by bacterial metabolic products. A sample of growth medium was retained for use as a blank and as a diluent for optical density measurements. An optical density of 1 at A_{470} indicates

a concentration of approximately 10^9 cells per ml for *P. aeruginosa* and *B. cepacia* per ml (Anwar, 1981), although different growth conditions may change this relationship.

2.1.3 *Growth measurements*

Growth of *B. cepacia* (NCTC 10661) in CDM+Fe and CDM-Fe was followed by measuring changes in the optical density of the culture with time. Flasks containing medium prewarmed to 37°C, were inoculated with cells from an overnight culture, grown in the same medium, to give an OD_{470} nm of approximately 0.01. Each flask was incubated at 37°C in an orbital shaker. Samples were removed at appropriate time intervals, diluted where necessary, and the A_{470} measured. Undiluted samples were returned to the flask; diluted samples were discarded.

2.1.4 *Preparation of outer membranes-Sarkosyl method*

Outer membranes (OMs) of clinical strains of *B. cepacia* and *P. aeruginosa* were prepared by the method of Filip *et al,* (1973). The washed bacterial pellet was suspended in 20 ml distilled water and broken by passage through a french pressure cell (Aminco). Unbroken cells were removed by centrifugation at 5 000 g for 5 min. Sarkosyl (sodium N-lauroyl sarcosinate, Sigma) was added to the supernate to 2% (w/v). After 1 h at room temperature the mixture was centrifuged at 40 000 g for 40 mins. The OM pellets were washed in distilled water and stored at -20°C.

2.1.5 *Lowry protein assay*

The protein content of OM preparations was determined by the method of Lowry *et al* (1951). Briefly, OM samples (50μl) were made up to 0.5 ml with double distilled water. An equal volume of 0.5 M NaOH was added to each sample and heated to 100°C for 10 mins. A solution (2.5 ml) comprised of 24 ml of 5% w/v Na_2CO_3 and 1 ml of 0.5% w/v $CuSO_4.5H_2O$ in Na Tartrate, was added to each protein sample. Folin and Coicalteu's phenol reagent (0.5 ml) (Sigma) was added and left for 30 mins. The absorbance at 750 nm was measured. A standard curve was constructed using bovine serum albumen.

2.1.6 *Preparation of purified lipopolysaccharide from* B. cepacia

LPS was prepared by the hot phenol extraction method of Westphal and Jann (1965). Cells were harvested by centrifugation, suspended in 300 mM Tris.Cl (pH 8.0), broken by sonication and incubated with DNase (0.125 mg/ml, Sigma, EC number 3.1.21.1) and RNase (0.125 mg/ml, Sigma, EC 3.1.27.5) at 37°C for 1 h. Proteinase K (Sigma, EC 3.4.21.14) was added to 0.125 mg/ml and incubated at 37°C overnight.

The mixture was heated to 80°C and extracted with an equal volume of phenol (80% w/v). After separation of the two phases by centrifugation 10 000 g for 25 min, the aqueous phase was retained. The phenol phase was re-extracted with an equal volume of water. EDTA was added to 1 mM to the pooled aqueous phase, which was dialysed against water overnight. Magnesium sulphate was added to the dialysate to 10 mM.

The LPS was recovered by centrifugation at 10^5g for 4 h. The pellet was freeze-dried and stored at -20°C.

2.1.7 *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

OM preparations were electrophoresed according to the method of Lugtenberg *et al* (1975) on 6% acrylamide stacking gels and 12% w/v acrylamide running gels. Each lane was loaded with approximately 2.5 μg protein.

For visualisation of LPS, OMs were heated to 100°C in SDS 2.5% w/v and 2-mercaptoethanol 2% w/v, cooled and then incubated with an equal volume of proteinase K (1 mg/ml in SDS 2.5% w/v and 2-mercaptoethanol 2% w/v) for 60 min at 60°C prior to electrophoresis.

Separated outer membrane proteins (OMPs) were stained with Coomassie Blue R-250 0.1% w/v in methanol:acetic acid:water 50:10:40. LPS gels were silver stained according to the method of Tsai and Frasch (1982).

2.1.8 *Immunoblotting*

Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose (NC) using a Mini Transblot cell (Bio Rad), by a modification of the method of Towbin *et al*, (1979). LPS separated by SDS-PAGE was also electroblotted onto NC by the method of Sturm *et al*, (1984).

After transfer, the NC paper was incubated at room temperature in 10 mM Tris (Tris [hydroxymethyl] amino methane, Sigma) in 0.85% saline (Tris-buffered saline [TBS]) with 0.1% v/v Tween 20 (TBS-Tween) for 1 h to saturate non-specific binding sites and then incubated overnight at 4°C with patient's serum diluted 1:50 in TBS-Tween. The NC membrane was washed thoroughly with TBS and incubated for 2 h at 37°C with protein A-peroxidase (Sigma) 0.5 μg/ml in TBS-Tween.

After incubation the NC membrane was thoroughly washed again. The antibody binding sites were visualised by reaction with 3.5 mM H_2O_2 and 0.5 mM 4-chloronaphthol in 10 mM Tris.Cl [pH 7.4]. Replicate blots were stained with amido black 1% w/v in methanol 10% v/v and acetic acid 7% v/v to show qualitative transfer of proteins.

Table 2.1. Ribotype, CHEF (pulsed field gel electrophoresis with the clamped homogenous electric field technique, Anderson *et al*, 1991) type and LPS type from *B. cepacia* strains isolated from cystic fibrosis patients."nd" indicates test not done.

Strain	Ribotype	CHEF type	LPS type
1	A	I	smooth
2	A	I	rough
3	D	IV	smooth
4	A	III	nd
5	A	III	rough
6	D	V	nd
7	nd	nd	rough
8	A	III	rough
9	A	nd	rough

2.2 Isolation and Purification of outer membrane proteins

2.2.1 *Bacterial strain and growth conditions*

A lipopolysaccharide rough clinical isolate of *B. cepacia* was collected from an adult CF patient at The Heartlands Hospital, Birmingham (strain 2, see table 2.1). The *B.cepacia* strain was known to be a ribotype common to many cystic fibrosis centres throughout the UK (ribotype A).

The strain was cultured in both iron-depleted and iron-supplemented chemically-defined medium (CDM+Fe and CDM-Fe). The OMs were prepared as in section 2.1.4.

2.2.2 *Preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

OM preparations (750 μg protein in a single well spanning the whole cell) were electrophoresed on 12% w/v acrylamide gels in a Protean II xi Vertical Electrophoresis cell (Bio-Rad, 15cm x 15cm x 1mm). Electrophoresis was performed at 50 V overnight until the dye front had reached the bottom.

A narrow strip was cut from each side of the gel and stained rapidly with Coomassie blue to locate the protein bands. The required OM protein band was excised by comparing with molecular markers and the stained edges of the gel.

2.2.3 *Electroelution*

The excised gel segments were cut up into small pieces. Electroelution was performed in an electroeluter (Model 422, Bio-Rad) at 8mA per tube for 4 h (Harrington, 1982). The electroeluted protein was stored at -20°C. The presence of electroeluted protein was confirmed by further SDS-PAGE. Immmunoblotting was performed on proteins separated by SDS-PAGE (see section 2.1.7).

2.3 ELISA

2.3.1 *Bicinchoninic acid protein assay*

The bicinchoninic acid (BCA) protein assay was performed to measure the concentration of electroeluted protein. This assay is a development of the method of Lowry *et al* (1951) by Smith *et al* (1985) and is based on formation of a protein-copper complex is formed. The purple reaction product is water soluble enabling spectrophotometric measurements. It is more sensitive than the Lowry method and can detect protein at concentrations as low as 10 μg/ml. It can be performed in a microplate effectively reducing the sample volume to 10 μl.

One volume (10 μl) of sample or BSA standard (20 to 200 μg/ml) was added to 20 volumes (200 μl) of freshly prepared reagent (see composition below) in a microtitre plate. Colour development proceeded at 60°C for 1 h and the plates were allowed to cool at room temperature before absorbance was measured at 550 nm (Anthos Reader 2 000). A standard curve of BSA was constructed.

The BCA protein reagent was prepared by mixing 50 volumes of reagent A with one volume of reagent B. This solution was freshly prepared. Reagent A consisted of an aqueous solution of 1% w/v BCA (disodium salt), 2% w/v $Na_2CO_3.H_2O$, 0.16% w/v disodium tartarate, 0.4% w/v NaOH, and 0.95% w/v $NaHCO_3$ and pH adjusted to 11.25. Reagent B consisted of 4% w/v $CuSO_4.5H_2O$ in double distilled water.

2.3.2 *ELISA of the purified protein*

Immulon 2 microELISA plates (Dynatech, USA) were coated overnight with 100 μl of the selected protein at 1.5 μg/ml in 50 Mm-carbonate/bicarbonate buffer, Ph 9.6. Plates were washed in isotonic phosphate buffered saline (0.14 M Na Cl, 2.7 Mm Kcl, 1.5 Mm KH_2PO_4, 8.1 Mm Na_2HPO_4 [PBS]) and then blocked for 4 h at 37°C with PBS containing 0.1% (w/v) BSA and 0.1% Tween 20 (PBSTB).

The plates were incubated at 37°C for 1 h with 100 μl volumes of patient serum, diluted 1:50 and doubling dilutions in PBSTB. Plates were washed three times in PBS and 100 μl of 0.25 μg/ml protein A peroxidase conjugate (Sigma) in PBSTB were added to each well and incubated at 37°C for 1 h.

Plates were washed and developed with 100 μl 0.0058% (w/v) 3,3',5,5'-tetra methylbenzidene in 0.1M-acetate buffer, pH 5.2 until a colour reaction developed after 5-10 min; the reaction was stopped with 50 μl 2M sulphuric acid. The A_{450} was measured using an Anthos 2001 reader (Labtec).

2.3.3 *Calculations for ELISA*

On each plate a control serum was used with high absorbance (A) reading, from a *B. cepacia* positive patient (patient with strain 3, see Table 2.1) was used as a reference. The same high A serum was used on each plate. To allow direct comparison between different assays the A readings were multiplied by the reference high A reading divided by the high A result for that assay and the value expressed in ELISA units (EU).

Data analysis was performed as follows; because the data when plotted was not normally distributed the results were expressed as arithmetic means and ranges. A paired nonparametric test was performed using the Wilcoxon signed rank test and Welch's alternative t test (Instat, GraphPad Software, San Diego, USA). A p value of < 0.05 was regarded as statistically significant.

2.4 Preparation of serum

2.4.1 *Collection of sera*

Blood was taken by venepuncture and allowed to clot at 37°C for 2 h. After centrifugation at 2 000 g for 10 min, the serum was collected and stored at -20°C. Serum samples from a total of 25 CF patients colonised with *B. cepacia* were obtained. Nine patients were from Birmingham Heartlands Hospital and sixteen were from Nottingham City General Hospital.

2.4.2 *Immunoblotting*

B. cepacia and *P. aeruginosa* strains and sera from nine colonised patients from Birmingham Heartlands hospital (strains 1-9) were studied in detail by immunoblotting. In addition immunoblotting was performed on sera from five *B. cepacia* colonised patients from Nottingham City General Hospital and compared with five age and sex-matched patients colonised with *P. aeruginosa.*

2.4.3 *ELISA studies of electroeluted protein*

For the ELISA studies sera from 21 patients colonised with *B. cepacia* were age and sex-matched with a CF control from whom *B. cepacia* had never been isolated. Two further *B. cepacia* colonised male patients (age 27.8 and 27.1 yr) were studied but not matched. Serum from a further forty-seven non-matched CF patients colonised with *P. aeruginosa* and from 50 non-CF non-matched control patients (samples taken prior to elective surgery) were collected. The *B. cepacia* colonised patients were all co-colonised with *P. aeruginosa* and had been colonised with *B. cepacia* for 2 to 51

months (mean 11 months). The *B. cepacia* negative groups from both centres had all been colonised with *P. aeruginosa* for greater than 6 months.

2.4.4 *Preadsorption of serum for immunoblotting*

Serum was preadsorbed with whole cells of *B. cepacia* and *P. aeruginosa* by a method outlined by Hughes *et al* (1992). Cells were grown overnight in 200 ml CDM-Fe to early stationary phase (A_{470} 0.9) in an orbital shaking incubator at 37°C and harvested by centrifugation at 10 000 *g* and resuspended in 10 ml 0.85% saline. One ml of this suspension was pelleted by centrifugation at 13 000 *g* for 2 min. Serum (0.5 ml) was added to the pellet of cells, the cells were resuspended and the mixture was incubated at 37°C for 15 min with gentle shaking. The serum-cell mixture was centrifuged at 13 000 *g* to remove the cells, and the serum was transferred to another cell pellet from 1 ml of resuspended cells, mixed and incubated as before. Each serum sample was adsorbed six times with cell pellets. Preadsorption with LPS was performed in a similar manner using 0.1 ml of LPS (10 mg/ml in water).

2.4.5 *Preadsorption of serum for ELISA*

Serum (0.4 ml) from a *B. cepacia* positive patient was diluted 1 in 1000 with PBSTB and was mixed with 0.1 ml of the 80 kDa protein (0.3 mg/ml) and incubated overnight at 4°C. Immune complexes were separated by centrifugation at 13 000 g.for 5 min Preadsorption with *B. cepacia* LPS was performed in a similar manner using 0.1 ml of LPS (10 mg/ml in water). LPS was prepared as described previously.

2.5 Production of polyclonal antibodies to a purified protein

2.5.1 *Immunisation of rabbits*

Two rabbits were obtained for producing polyclonal antibodies, following the acquisition of a project licence (New Zealand Whites, 42-70 days old, female, weight approximately 1.5 kg, Charles River UK Ltd, Kent).

Purified OM protein was obtained as previously described (section 2.2). The protein concentration determined by the Bicinchoninic acid assay (see section 2.3.1) was 130 μg/ml.

The rabbits were immunised with 0.25 ml (32.5 μg) of the OM protein mixed with 0.25 ml Freund's Complete adjuvant by subcutaneous injection in the mid line posteriorly at the level of the four to sixth thoracic vertebrae (week one). Seven days later a further immunisation of 32.5 μg of 80 kDa protein was given subcutaneously, but on this and all subsequent occasions, 0.25 ml of Freund's Incomplete adjuvant was given. A further nine weekly immunisations were administered to the rabbits.

Preimmunisation blood, plus samples at six and nine weeks were taken from the ear vein from both rabbits.

The rabbits were sacrificed 77 days after the first innoculation. This was performed after anaesthesia with nitrous oxide and Halothane. The rabbits were exsanguinated and the blood allowed to clot at 37°C for 2 h. After centrifugation at 2000 g for 10

min, the serum was collected and stored at -20°C.

2.5.2 *Immunoblotting of polyclonal rabbit serum*

Immunoblots of *B. cepacia* and *P. aeruginosa* OMs and rough type LPS were probed with preimmunisation and postimmunisation sera as previously described.

2.6 Immunofluorescence microscopy using polyclonal antibodies

2.6.1 *Immunofluorescence microscopy of* B. cepacia *and* P. aeruginosa *cultures*

The technique of fluorescence microscopy used a fluorescent-labelled antibody to demonstrate the presence of the 80 kDa protein on the surface of *B. cepacia* (Aitchison, 1992). Clinical isolates were grown overnight in tryptone soya broth (Oxoid) to early stationary phase (A_{470} 0.9) in an orbital shaking incubator at 37°C, harvested by centrifuging at 10 000 *g* and washed once with 0.85% saline and resuspended in 0.85% saline. Cells were taken and placed in wells on a PTFE-coated microscope slide (12 x 10 μl wells per slide, Hendley Essex). The bacterial film was dried quickly and fixed with acetone.

The cells were then labelled with fluorescein indirectly by the following method. Rabbit serum, both pre and post immunisation with the purified protein was diluted 1:200 in PBS (pH 7.6). This dilution had been previously determined by titration experiments. Samples of the diluted serum were pipetted onto the fixed wells of the Hendley-Essex slides.

The slides were incubated at 37°C for 30 min in humid chambers. Excess serum was removed, taking care that cross-contamination did not occur. The slides were washed by gentle agitation in two changes of PBS for a total of 10 min, followed by a final rinse in distilled water. Excess liquid was blotted from around the wells with tissue, taking care not to disturb the antigen film.

Purified fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G, heavy and light chain specific (Cal Biochem), 1mg/ml diluted 1:100 in PBS containing 2% v/v Tween 80 and 0.01% v/v Evans blue counter stain (Sigma), was then added to each well and the slides were incubated at 37°C for 30 mins in humid chamber. After incubation the slides were washed in PBS, rinsed in distilled water and dried as above. Slides were mounted in buffered glycerol PBS pH 8.5 (one volume of PBS and nine volumes of "AnalR" grade glycerol). A cover slip was used and the slide was examined as soon as possible under oil immersion at x 1000 magnification using a fluorescence microscope. Slides were compared with the Gram stain appearance of organisms from the same culture.

2.6.2 *Immunofluorescence of sputum*

Sputum from CF patients was collected when they attended outpatients or were admitted to hospital. The sputum was put into sterile universal containers and immediately frozen to -20°C. The sputum was left at room temperature for 1 hr and 0.5 ml removed to a 1.5 ml microcentrifuge tube. An equal volume of freshly prepared 2% v/v N-acetyl-L-cysteine was added to the sputum and agitated for 10 min.

Sputasol was then added and the sputum was agitated for a further 10 min.
Alternatively neat β-mercaptoethanol (100μl) was added and agitated for 10 min. The 1.5 ml microcentrifuge tube was centrifuged for 30 s at 6500 *g*. The supernate was then removed and washed in 1 ml PBS and centrifuged at 6500 *g* for 30 s. This was repeated twice with the final spin at 13 000 *g* for 10 min.

The small pellet of cells was resuspended in 0.5 ml PBS. Ten μl of the suspension was put into a well on the Hendley-Essex slide and dried with acetone as described above. The rest of the procedure was as described in section 2.6.1. Slides were again compared with Gram stain appearances of the sputum.

CHAPTER 3

DEVELOPMENT OF AN IN VITRO MEDIUM FOR B. CEPACIA AND THE EFFECT OF IRON DEPLETION ON GROWTH AND ON SURFACE ANTIGENS OF B. CEPACIA

3.1 Introduction

Previous studies have shown that the outer membrane protein (Brown *et al*, 1984) and antigenic profiles (Anwar *et al*, 1984) of *P. aeruginosa* strains grown in an iron-depleted medium are similar to those from cells recovered directly from the CF lung. Anwar *et al* (1983) have previously characterized the outer membrane of *B. cepacia* using a similar iron-depleted-chemically-defined medium. Iron depletion induced the synthesis of an OM protein with apparent molecular weight of 66 kDa.

B. cepacia has been shown to produce at least three siderophores including pyochelin (Sokol, 1986; Bukovits *et al,* 1982), cepabactin (Meyer *et al*, 1989), and azurechelin (Sokol *et al*, 1992). The ability to produce pyochelin has been suggested as a possible virulence factor (Sokol and Woods, 1988). Pyochelin may increase the ability of *B. cepacia* to disseminate through the lung and thus induce a greater inflammatory response (Sokol and Woods, 1988). Cepabactin strongly chelates Fe III and facilitates iron translocation (Meyer *et al*, 1989). Azurechelin has been identified in 88% of *B. cepacia* strains isolated in the respiratory tract (Sokol *et al*, 1992). Production of siderophores enables bacteria to compete for iron with the host iron binding proteins including transferrin and lactoferrin and enables bacteria to establish and maintain infection in the lung. In view of the importance of growth in an iron-limited environment clinical strains of *B. cepacia* were characterised when grown in an iron-limited chemically-defined medium.

3.2 Growth characteristics of *B. cepacia* grown in iron-supplemented and low-iron depleted media.

Flasks containing iron supplemented (CDM+Fe) and low-iron chemically defined medium (CDM-Fe), prewarmed to 37°C, were inoculated with the cells from an overnight culture of *B. cepacia* NCTC 10661 strain grown in low-iron chemically defined medim (CDM-Fe) and growth monitored over 48 hr (see 2.1.3).

The growth curves are shown in Fig. 3.1. Bacterial growth was in a batch culture (that is bacteria incubated in a closed culture with a single batch of medium) and followed a growth curve with three phases (lag, exponential, and stationary). The growth curve was not continued long enough to see the characteristic decay phase of the growth curve.

The initial optical density prior to incubation of the CDM+Fe was higher than the CDM-Fe because of the iron in the solution. There was a long lag phase with no growth over the first 150 min (this time period is not shown in Figure 3.1). As the bacteria were grown over night in CDM-Fe prior to innoculation into the CDM+Fe and CDM-Fe at the start of the growth curves, the bacteria were well adapted to the chemically defined media. However, the bacteria inoculated from the overnight culture were likely to be in stationary phase and would, therefore, need to synthesise new components, essential cofactors and ribosomes before growth could begin.

The condition of cells prior to inoculation is known to effect the length of the lag

phase, particularly if the cells are old or refrigerated prior to inoculation. However in this study cells were taken straight from the overnight culture and therefore the long lag phase cannot be attributed to their age or condition.

The growth rates for the CDM+Fe and CDM-Fe were similar but the yield from the CDM+Fe was much greater. The effect of iron supplementation on the shape and size of the bacteria and hence on the optical density was not investigated, but this was unlikely to have altered the results significantly. The growth curves confirmed that *B. cepacia* growth in this medium is limited by iron availability. There was a long lag phase followed by an exponential or log phase and finally a stationary phase.

3.3 Effect on growth of clinical strains of *B. cepacia* after supplementation of chemically defined media with Casamino acids

3.3.1 *Clinical Isolates*

All the clinical isolates of *B. cepacia* showed low yield in the chemically defined medium, but this could be improved by addition of Casamino acids (Difco, Bacto Vitamin Assay Casamino Acids). Casamino acids are made from hydrolysed casein. It is free from vitamins and contains no iron (Difco Manual, Ninth Edition, page 267-8). Different concentrations of casamino acids (0.0001% w/v to 0.1% w/v) were added to iron supplemented and low-iron chemically-defined media. Cells from a clinical strain (Strain 1) were grown overnight as previously described in the different media.

The results are summarised in Table 3.1. The A_{470} for the clinical strain grown in CDM+Fe with 0.1% casamino acids was 6.8 but without casamino acids was only 0.37. The A_{470} for the clinical strain grown in CDM-Fe with 0.1% casamino acids was 1.3 but without casamino acids was 0.21. The optimum concentration of casamino acids for the maximum yield at 24 hr was found to be between 0.05% to 0.1%. This experiment however does not differentiate different growth rates between the different concentrations of casamino acids.

3.3.2 *Laboratory strain*

Growth of *B. cepacia* NCTC 10661 strain was improved by the addition of casamino acids to the low-iron culture, but not the iron supplemented culture. The A_{470} at 24

hr was measured as above for the following *B. cepacia* NCTC 10661 strain cultures: CDM+Fe with 0.1% w/v casamino acids the A_{470} was 13 but without casamino acids was 12.7, CDM-Fe with 0.1% w/v casamino acids the A_{470} was 2.1 but without casamino acids was 1.0.

The laboratory strain appeared to have similar yields when the CDM+Fe was supplemented with casamino acids. There was a greater difference in yield if the CDM-Fe was supplemented with casamino acids. This in contrast to the clinical strains where both the low-iron and the iron supplemented cultures gave greater yields with the addition of casamino acids.

Table 3.1 Clinical isolate (strain I) grown in iron supplemented and low-iron chemically defined media at different concentrations of casamino acids (w/v).

Cas-amino acids	0.1%	0.05%	0.01%	0.005%	0.001%	0.0001%	0
A_{470} at 24 hr, Fe+	6.8	7.0	3.6	2.6	0.6	0.4	0.4
A_{470} at 24 hr, Fe-	1.3	1.0	0.6	0.5	0.3	0.2	0.2

3.4 Effect of low-iron on outer membrane proteins and LPS

3.4.1 *SDS-PAGE*

When grown in the CDM+Fe *B. cepacia* (NCTC 10661) expressed six major OMPs; 106 kDa, 90 kDa, 80 kDa, 52 kDa, 36 kDa, 30 kDa. When grown in the CDM-Fe with 0.1% w/v casamino acid *B. cepacia* expressed seven major OMPs (NCTC 10661, bands at 113 kDa, 95 kDa, 80 kDa, 66 kDa, 42 kDa, 29 kDa, and 20 kDa; clinical strain 1 [Table 2.1] bands at 80 kDa, 75 kDa, 66 kDa, 39 kDa, 20 kDa and weak bands at 113 kDa, 111 kDa: Fig. 3.2, lanes 2 and 3).

A clinical strain of *P. aeruginosa,* from the same patient as the *B. cepacia* strain 1, expressed the characteristic outer membrane profile after growth in CDM-Fe with 0.1% w/v casamino acids (Brown *et al,* 1984) including the major porin, protein F at 39 kDa, proteins H/L at 21 and 20 kDa and two high molecular weight iron-regulated proteins at 80 and 72 kDa (Fig. 3.2, lane 4).

3.4.2 *Silver stained SDS-PAGE*

Figure 3.3 shows a silver-stained SDS-PAGE gel of outer membranes from four clinical *B. cepacia* isolates after treatment with proteinase K. All strains possessed Lipid A, but only two strains expressed smooth-type LPS (Fig 3.3, lane 1 and 2), indicated by O-polysaccharide bands of increasing molecular weight.

Seven of the nine independently isolated strains of *B. cepacia* (strains 1-3, 5 and 7-9) were studied to determine their O-antigen status. Five of these isolates were

identified as the ribotype strain thought to be common to many CF centres in the UK (Table 2.1). Only strains 1 and 3 had smooth type LPS, the other strains were all R-type LPS.

3.4.3 *Immunoblotting*

Immunoblots were prepared from the OM of a clinical strain of *B. cepacia* (strain 2) grown in CDM+Fe and CDM-Fe. The immunoblot was probed with serum from the patient colonized with this strain of *B. cepacia*. The immune reaction to the OMs of *B. cepacia* grown in CDM+Fe and CDM-Fe were compared.

There was detectable immune reaction to the higher molecular weight bands present on the SDS-PAGE of the CDM+Fe OM (106 kDa and 90 kDa) or the CDM-Fe OM (113.5 kDa, 111.6 kDa, 83.0 kDa). A strong band at 66 kDa appeared on both immunoblots and appeared to be an immune reaction to an OMP on the CDM-Fe SDS-PAGE, which was not present on the CDM+Fe SDS-PAGE. There was a further strong immune reaction at the 32.5 kDa site which again appeared to be an immune reaction to a faint band on the CDM+Fe equivalent SDS-PAGE and the same band on the CDM-Fe gel. (Immunoblotting results for *B. cepacia* and *P. aeruginosa* will be given in greater detail in chapter 4).

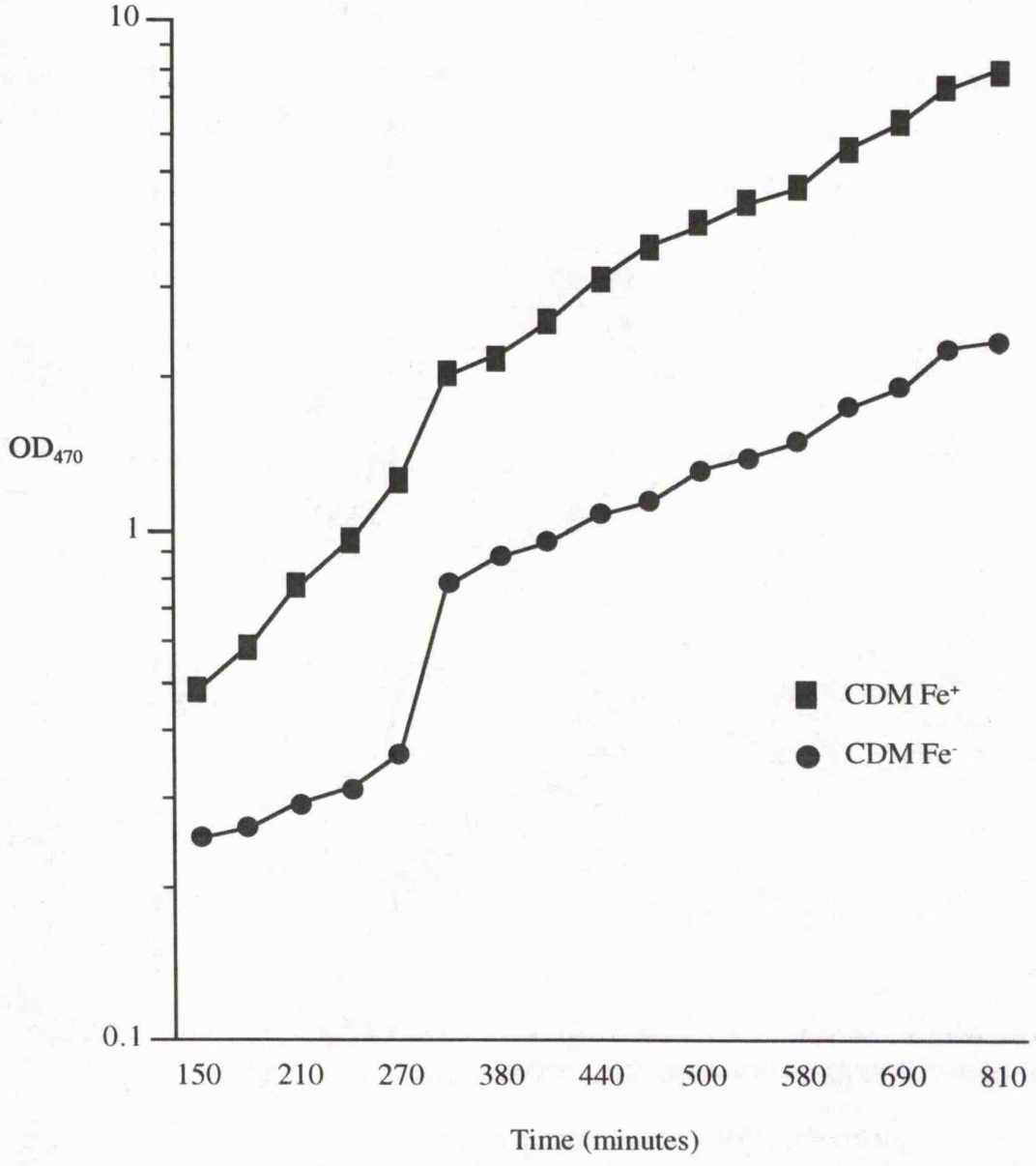

Figure 3.1. Growth curve of *B. cepacia* grown in an iron depleted and iron supplemented chemically defined media.

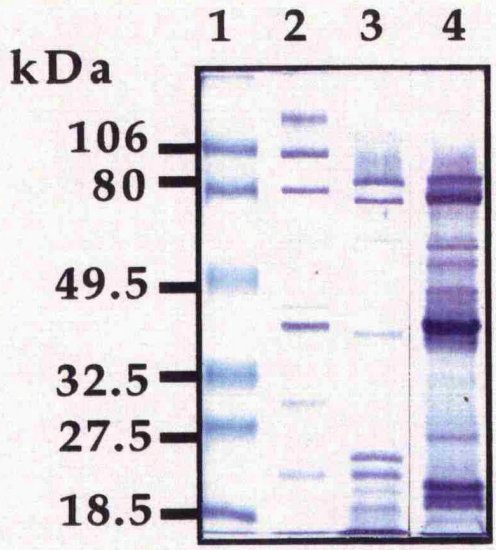

Figure 3.2. SDS-PAGE analysis of outer membrane proteins of *B. cepacia* NCTC 10661 (lane 2) and representative clinical isolates of *B. cepacia* (lane 3) and *P. aeruginosa* (lane 4) after growth in an iron-depleted medium. Molecular weight markers (lane 1).

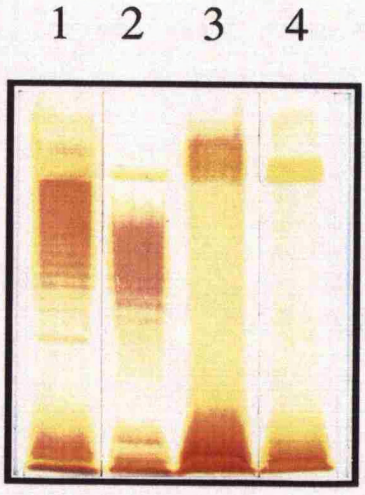

Figure 3.3. Silver-stained SDS-PAGE showing LPS profile from four clinical *B. cepacia* isolates. Isolate from patient 1 (Lane 1), patient 3 (Lane 2), patient 2 (Lane 3), and patient 7 (Lane 4).

3.5 Discussion

3.5.1 *Growth curve and chemically defined medium*

The strains were grown in an iron-supplemented and low-iron chemically-defined medium which had been developed for *P. aeruginosa* (Brown *et al*, 1984). Previous studies have shown that the outer membrane protein (Brown *et al*, 1984) and antigenic profiles (Anwar *et al*, 1984) of *P. aeruginosa* strains grown in the low-iron medium are similar to those recovered directly from the CF lung. This iron supplemented and low-iron chemically defined medium was also used to characterize the outer membranes of *B. cepacia* (Anwar *et al*, 1983). Despite the ability of some *B. cepacia* strains to grow in some disinfectants (Holmes B, 1986), the clinical strains proved difficult to grow in this study under laboratory conditions. Supplementation of the chemically defined medium with casamino acids increased the yield of bacteria after 24 h, especially among the clinical strains. The yield of bacteria after 24 h growth was still much greater in the iron supplemented medium after the addition of the casamino acids suggesting that there was still iron limitation.

The requirement for casamino acids by the clinical isolates suggested an auxotrophy for one or more amino acids. However, if this were so one would expect no growth; whereas in this study very low yields of bacteria were still obtained if grown for 24 hr in the chemically defined medium. There may have been contamination of the chemically defined medium with protein, or the culture may have contained a mixture of auxotrophic and prototrophic isolates. This may have arisen if the auxotrophic isolate was repeatedly grown in a chemically defined medium without amino acids

and the isolate reverted back to the prototrophic form. This assumes the genotype had not changed and the relevant gene had been inactive, rather than lost, in the auxotrophic isolate.

The development of auxotrophy by clinical strains has previously been reported for *P. aeruginosa* (Barth and Pitt, 1995; Taylor *et al*, 1992; Taylor *et al*, 1993). Specific compounds must be provided in the medium to support these variants. These nutritionally deficient strains have been shown to be confined to CF and bronchiectasis.

Auxotrophy has also been described for isolates of *Staphylococcus aureus* from CF. Gilligan *et al* (1987) found *S. aureus* auxotrophy for thymidine in 20 of 95 patients with CF. These strains were invariably resistant to trimethoprim-sulphamethoxazole. They had atypical colonial morphologies and failed to grow on routine isolation media.

Barth and Pitt (1995) found that 40 isolates of *B. cepacia* from 38 CF patients out of a total of 89 isolates from 81 CF patients were auxotrophic. In contrast all 29 of the isolates from non-CF (clinical and environmental) sources were prototrophic. Addition of a pool of amino acids to the minimal medium (containing glucose and mineral salts only) was sufficient to promote growth of all the CF auxotrophic isolates. Phenylalanine, tyrosine, cysteine, methionine, and histidine alone were sufficient to promote growth by the majority of the nutritionally deficient *B. cepacia* isolates. Some of the CF strains grew weakly if the minimal medium was supplemented with

one of the following amino acids; tyrosine, phenylalanine, methionine or cysteine. Extracts of sputum from the CF patients when added to the minimal medium promoted growth in 29 of the auxotrophic *B. cepacia* isolates. Auxotrophic and prototrophic isolates from the same CF patient exhibited conserved genotype.

Sputum extracts have previously been shown to support the growth of mucoid *P. aeruginosa* (Ohman and Chakrabarty, 1982). Ohman and Chakrabarty (1982) also demonstrated that CF sputum also contains amino acids and small peptides in relatively high concentrations. Therefore, it is likely that auxotrophic strains are selected from the prototrophic population and maintained in the nutritionally rich environment of the CF airway.

3.5.2 *Immune reaction to outer membrane antigens*

Immunoglobulin G antibodies to *B. cepacia* OM antigens from cells grown in CDM+Fe and CDM-Fe media were demonstrated. As previously reported, synthesis of an OM protein with apparent molecular weight of 66 kDa was induced when *B. cepacia* (NCTC 10661) was grown under low-iron conditions in a chemically defined medium without casamino acids (Anwar *et al*, 1983). An immune reaction to this protein was detected on immunoblotting. It was not apparent when the cells were grown in an iron supplemented medium. The iron regulated outer membrane proteins (IROMPs) produced an immune reaction on the immunoblot of the CDM+Fe OMPs despite there being no visible band on the gel. Small concentrations of IROMPs present but not detectable on the gel, were amplified by the immunoblot. The IROMPs are thus present in small amounts even in an iron rich environment. It is

likely that this iron-regulated outer membrane protein is involved in the transportation of iron across the outer membrane and may, therefore, influence the growth rate of *B. cepacia* in the relatively iron poor environment of the CF lung.

In a recent study Burnie *et al* (1995) grew a clinical strain of *B. cepacia* in a low-iron medium, composed of tryptone soya broth in the presence of an iron chelator $\alpha\alpha$-dipyridyl. Growth in this medium induced two new antigenic bands of 21 kDa and 19 kDa. Antibody against the 19 kDa band was specific to patients colonised with *B. cepacia*. Its presence, however, did not correlate with a good prognosis. Antibody against the band at the 21 kDa was present in both controls and patients with CF and the presence of IgM antibody against the 21 kDa protein seemed to occur more frequently in those with milder CF lung disease.

In conclusion *B. cepacia* will grow in a chemically defined low-iron environment. Clinical strains required supplementation with casamino acids to improve the growth of organisms. In the clinical strain studied the OMP profile was altered by growth in a low-iron medium with the appearance of an OM protein with an apparent molecular weight of 66 kDa.

CHAPTER 4

SERUM IgG REACTION TO BURKHOLDERIA CEPACIA OUTER MEMBRANE ANTIGENS IN CYSTIC FIBROSIS: ASSESSMENT OF CROSS REACTIVITY WITH PSEUDOMONAS AERUGINOSA.

4.1 Introduction

B. cepacia infection often occurs in patients who are already colonised with *P. aeruginosa* (Thomassen *et al*, 1985) in whom high levels of anti-*P. aeruginosa* serum antibodies exist (Aronoff and Stern, 1988). Previous studies have demonstrated that anti-*B. cepacia* antibodies can be detected in the serum of *P. aeruginosa*-infected patients not culture proven to be infected with *B. cepacia* (Aronoff *et al*, 1991). In order to assess the degree of cross-reactivity between *B. cepacia* and *P. aeruginosa,* the immune reaction to both *B. cepacia* and *P. aeruginosa* outer membrane components was studied. The non cell-mediated immune reaction from patients colonised with both *P. aeruginosa* and *B. cepacia* was compared that of patients colonised with *P. aeruginosa* alone. Serum from patients colonised with both *P. aeruginosa* and *B. cepacia* was preadsorbed with *B. cepacia* or *P. aeruginosa* whole cells and the immune reaction to the outer membrane components was studied before and after preadsorption.

4.2 Results

4.2.1 *Immunoblotting*

The nine Birmingham Heartlands Hospital patients studied had a mean age of 23.3 years (range 10.2 to 29.5 years) and had been colonised with *B. cepacia* for a mean time of 13 months (range 4 to 28 months) and *P. aeruginosa* for a mean time of 36.5 months (range 18 to 89 months). The *B. cepacia*-negative patients from both centres had all been colonised with *P. aeruginosa* for greater than 6 months. Sera from the six patients colonised with both *B. cepacia* and *P. aeruginosa* selected for study in greater detail had IgG antibodies reactive against both *B. cepacia* and *P. aeruginosa* OMPs and representative immunoblots using the isolate and serum from patient 1 are shown in Figs. 4.1 and 4.2, respectively.

Figure 4.3 (lane 1) shows an LPS immunoblot of *B. cepacia* from patient 1 probed with serum from the same patient demonstrating a strong immune reaction to *B. cepacia* LPS, particularly against the O-chains of repeating polysaccharide units.

The IgG reaction to the *B. cepacia* OM (strain 9) from the five patients from Nottingham City General hospital colonised with both *B. cepacia* and *P. aeruginosa* (mean age 24.5 years, range 22.3-29.6 years, Fig 4.4, lane 1-5) was studied. The immunoblot had a similar pattern to the immunoblot of patients from Birmingham Heartlands Hospital. This reaction was compared with the immune reaction of five-age and sex-matched patients colonised with *P. aeruginosa* alone (mean age 22.7 years, range 19.6-25.9 years, Fig 4.4, lane 6-10). Two of the non-*B. cepacia*

colonised patients' sera did not react with the *B. cepacia* OM (Fig 4.4, lane 6 and 10, approximately 10% reaction only), whereas three non-*B. cepacia* colonised patients' sera showed a reaction comparable with the reaction of a *B. cepacia* colonised patient, although the bands were less distinct (Fig 4.4, lanes 7-9). There was no reaction to *B. cepacia* OM by sera from either a non-colonised CF patient (not shown) ~~and~~ or a non-CF patient (Fig 4.4, lane 11).

4.2.2 *Immunoblotting with preadsorbed serum*

Attempts were made to determine if the immune reaction against *B. cepacia* OM antigens was specific to that organism or attributable to cross-reactivity of anti-*P. aeruginosa* antibodies. Patient sera were adsorbed with *B. cepacia* or *P. aeruginosa* whole cells. In addition, sera were also adsorbed with *B. cepacia* LPS, to distinguish between a genuine anti-OMP reaction and one due to co-migrating LPS.

Serum was preadsorbed with *B. cepacia* whole cells, *P. aeruginosa*, or *B. cepacia* LPS for two to six cycles. Preliminary experiments indicated that six cycles of preadsorption were sufficient for optimum immunoadsorption. No further adsorption was detected after the fifth cycle.

Serum preadsorbed with *P. aeruginosa* diminished the immune reaction to *P. aeruginosa* OMP (intensity of the bands were reduced by approximately 60-70%) with some antigens still appearing as intense bands (Fig 4.2, lane 4), whilst not affecting the immune reaction to *B. cepacia* OMPs (Fig 4.1, lane 3). Serum preadsorbed with *B. cepacia* had no effect on the reaction to *P. aeruginosa* (Fig 4.1, lane 3). The

immune reaction to the major *B. cepacia* OMPs was not diminished significantly (Fig 4.1, lane 4), but there was a reduction in the minor bands which was attributed to LPS O-antigen. This was confirmed by preadsorbing serum with purified LPS from *B. cepacia* (Fig 4.1, lane 5).

The contribution of the anti-LPS reaction to the total serum immune reaction was confirmed by probing *B. cepacia* LPS blots with serum preadsorbed with *P. aeruginosa* and *B. cepacia* whole cells. Preadsorbing with *P. aeruginosa* (Fig 4.3, lane 3) had no affect on the immune reaction, whereas it was abolished when *B. cepacia* whole cells were used (Fig 4.3, lane 2).

Serum from a patient colonised with an R-type LPS *B. cepacia* strain (patient 5) showed no O-antigen reaction to LPS from the colonising strain, but did demonstrate a strong reaction to *B. cepacia* smooth LPS from patient 1, who was colonised with *B. cepacia* of smooth type LPS. This was not diminished by preadsorption with his own colonising strains of *B. cepacia* and *P. aeruginosa*. Serum IgG reactions to R-type LPS and smooth type LPS are summarised in Table 4.1.

Table 4.1 Serum IgG reaction to two *B. cepacia* strains with smooth type LPS. Immunoblots of proteinase-K treated OMs from the two strains were probed with serum from five patients colonised with *B. cepacia* of different LPS types.

Patient's serum	LPS type	Strain 1 (smooth LPS)	Strain 3 (smooth LPS)
1	smooth	Positive	Positive
2	rough	Positive	Negative
3	smooth	Positive	Positive
5	rough	Positive	Negative
7	rough	Positive	Positive

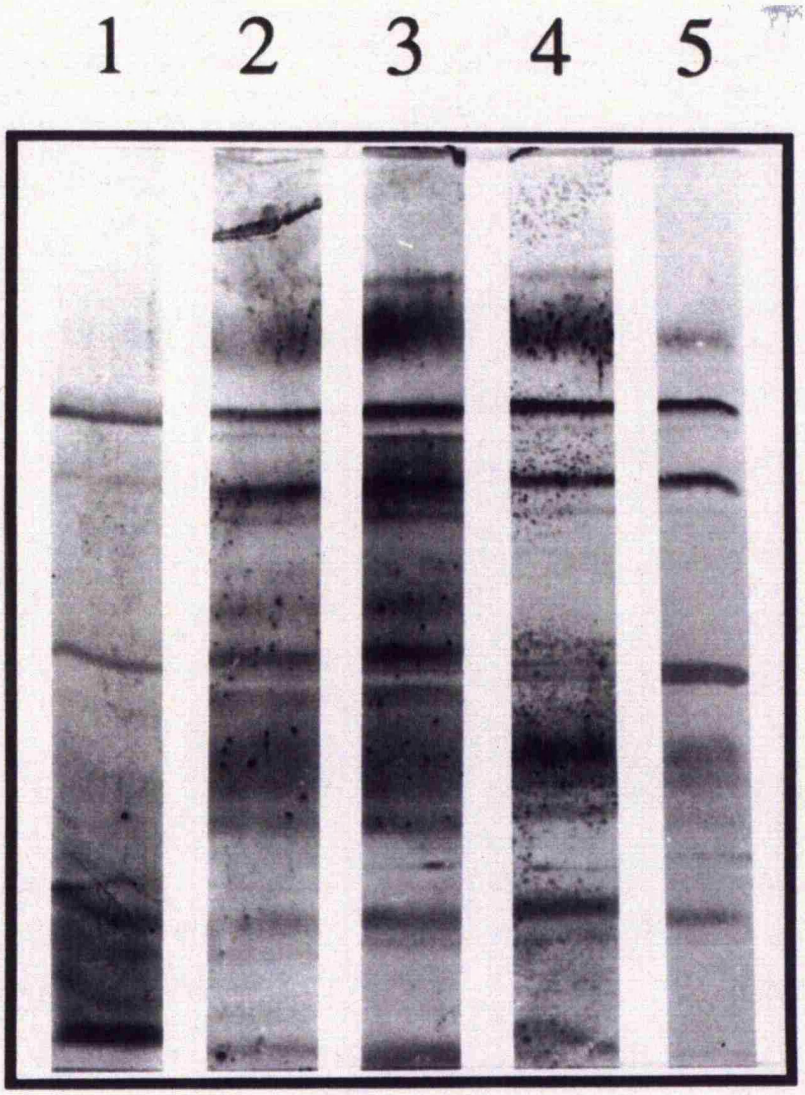

Figure 4.1. Immunoblot of *B. cepacia* outer membranes from patient 1. Lane 1, OMP's visualised with amido-black; Lane 2, serum from patient 1; Lane 3 and 4, serum from patient 1 preadsorbed with *P. aeruginosa* and *B. cepacia* whole cells, respectively; Lane 5, serum from patient 1 preadsorbed with *B. cepacia* LPS.

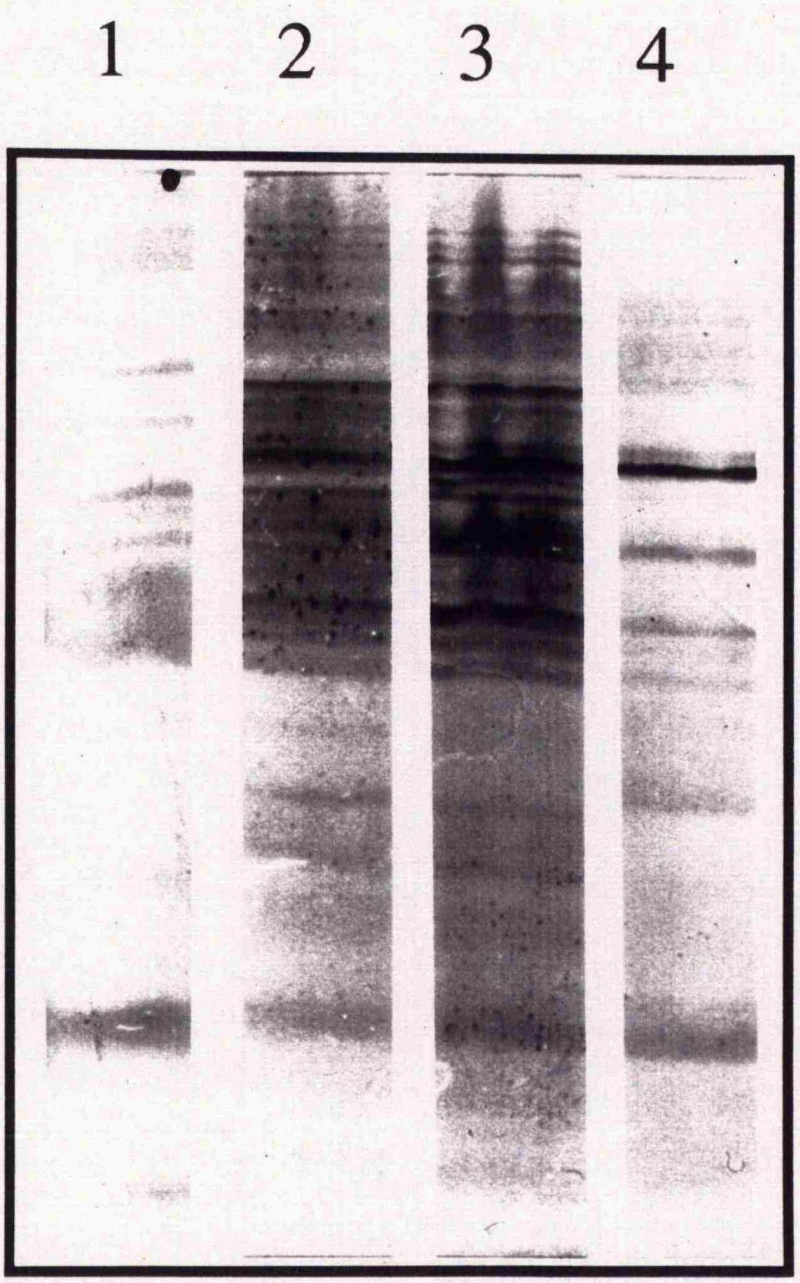

Figure 4.2. Immunoblot of *P. aeruginosa* outer membranes from patient 1. Lane 1, OMP's visualised with amido-black; Lane 2, serum from patient 1; lane 3 and 4, serum from patient 1 preadsorbed with *B. cepacia* and *P. aeruginosa* whole cells, respectively.

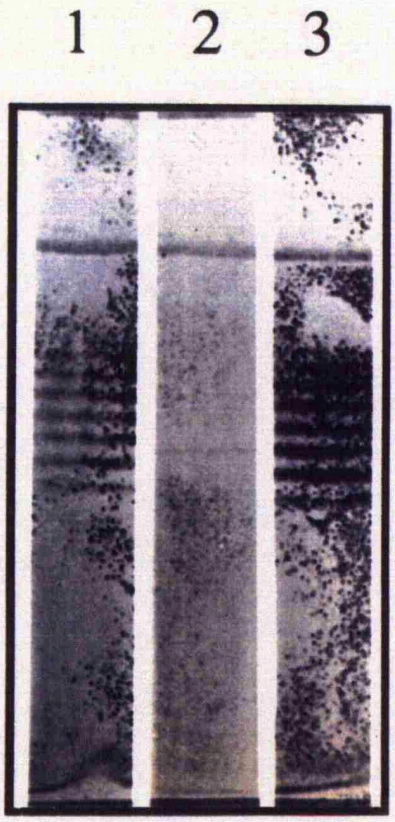

Figure 4.3. Immunoblot of *B. cepacia* LPS from patient 1. Lane 1, serum from patient 1; Lane 2 and 3, serum from patient 1 preadsorbed with *B. cepacia* and *P. aeruginosa* whole cells, respectively.

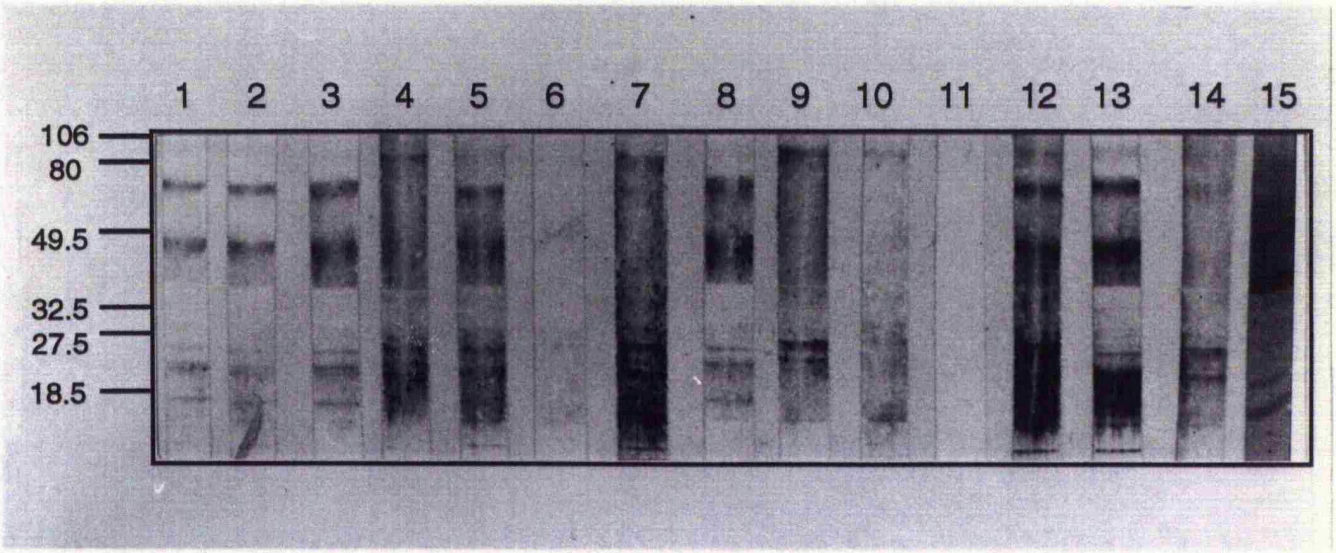

Figure 4.4. Immunoblot of *B. cepacia* outer membranes from patient 9. Lanes 1-5, patients from Nottingham City General colonised with *B. cepacia* and *P. aeruginosa*; lanes 6-10, patients from Nottingham City General colonised with *P. aeruginosa* only; lane 11, non CF patient; lane 12, serum from patient 9; lane 13 and 14, serum from patient 9 preadsorbed with *P. aeruginosa* and *B. cepacia* respectively; lane 15, outer membrane proteins stained with amido-black.

4.3 Discussion

Immunoglobulin G antibodies to outer membrane antigens of *B. cepacia* were demonstrated in patients colonised with *B. cepacia* and *P. aeruginosa*. Extensive pre-adsorption with *P. aeruginosa* did not diminish the reaction to *B. cepacia*. Serum adsorption experiments with *P. aeruginosa* whole cells did not affect the reaction directed against *B. cepacia* outer membrane proteins but reduced considerably the reaction to *P. aeruginosa*, the intensity of the bands on the immunoblot appearing to be reduced to 30-40% compared with the bands on the immunoblots probed with un-adsorbed serum.

The method for preadsorbing the serum was thought to be efficient for the following reasons: the method has previously been verified by Hughes *et al* (1992); 0.5 ml of serum was incubated with approximately 10^{10} whole (cells grown to an A_{470} of 1, would have 10^9 cells per ml; this was concentrated by a factor of ten resulting in 10^{10} cells); and finally, the process was repeated six times.

Whereas pre-adsorption with *P. aeruginosa* did not diminish the reaction to *B. cepacia,* the reaction to *B. cepacia* O-antigen LPS could be readily removed by adsorption of serum either with *B. cepacia* whole cells or purified LPS. The reaction to *B. cepacia* OMP was not reduced by the pre-adsorption of serum by either *P. aeruginosa* or *B. cepacia*. It is not clear why we were unable to adsorb anti-*B. cepacia* OMP antibodies from serum using whole cells. However, complete adsorption of anti-LPS antibodies indicates that the reaction is directed against protein epitopes and not co-migrating LPS (Poxton *et al*, 1987). The failure to adsorb anti-

OMP antibodies using either smooth- or rough-type LPS *B. cepacia* whole cells suggests that it is not due to O-antigen LPS preventing interaction between antibodies and proteins at the cell surface, previously postulated as a possible mechanism of bacterial defence in the presence of anti-*B. cepacia* antibodies (Aronoff and Stern 1988, Van der Lay 1986).

However, it cannot be ruled out that the epitopes are not surface exposed in intact cells, and this would also account for the observation that there was a remaining antibody reaction to *P. aeruginosa* after adsorption with *P. aeruginosa* whole cells. If this is the case then it could be that these hidden epitopes could give cross-reactive immune reactions.

In the present study, two out of the seven strains in which LPS type was determined had smooth LPS. In the study by Mckevitt and Woods (1984) 22 strains were found that possessed smooth LPS and 26 strains that possessed R-type LPS. The epidemic strain (CF5610) from the Edinburgh and Manchester CF clinics which is ribotype A, appeared to be exclusively R-Type LPS (Govan *et al,* 1993; Nelson *et al,* 1993). Indeed it has never been associated with a smooth type LPS even in newly colonised patients (Butler *et al*, 1994). In this study within the ribotype A group there was one smooth type LPS and four R-Type LPS, the other smooth type LPS striain was ribotype D (Table 2.1).

Three patients colonised with a *B. cepacia* R-type LPS strain had serum IgG antibodies against smooth type LPS, although in two of the three patients the reaction

was against only one of two smooth strains tested (Table 3.2). Adsorption of serum with the patient's colonising rough *B. cepacia* strain did not diminish the reaction. Although preliminary, this may suggest that *B. cepacia* undergoes a transition from smooth to R-type LPS in the CF lung. If the *B. cepacia* isolate was initially smooth type LPS transient growth of smooth isolates may have occurred without being observed by culture, especially as examination of colonial appearance may not distinguish smooth and rough types. This may explain why the epidemic strain (CF5610) appeared to be exclusively R-Type (Govan *et al*, 1993) whereas Mckevitt and Woods (1984) found a high proportion of smooth LPS strains.

The clinical importance of LPS type amongst colonising strains is unclear. *B. cepacia* infection is sometimes accompanied by fever and bacteraemia (Rosenstein and Hall, 1980). Bacteraemia due to *P. aeruginosa* lung infection is rare in patients with CF. *P.aeruginosa* lung infection in CF is almost exclusively due to the R-type LPS, which is much more sensitive to the lytic action of serum than the smooth type LPS (Hoiby and Koch, 1990) and therefore bacteraemia is rare in CF compared to other non CF patients who *P. aeruginosa* infection where bacteraemia is common. Patients with a *B. cepacia* smooth type LPS strain may be more susceptible to *B. cepacia* bacteraemia, however, fever and bacteraemia are not limited to patients with the smooth type LPS alone. Anwar *et al* (1984) reported that the nutrient conditions under which a single strain with smooth type LPS was grown had a profound effect on the killing by serum factors and polymorphonuclear leukocytes. Butler *et al* (1994) looked at the resistance of *B. cepacia* to the lytic action of serum and found the major determinant to be possession of a complete O-side chain. The clinical observation that

rough forms of *B. cepacia* are associated with bacteraemia in CF, suggests that the in vivo characteristics of the organism are different from those observed in vitro, or that *B. cepacia* resists serum killing by an as yet unknown mechanism.

Serum adsorption experiments with *P. aeruginosa* whole cells did not affect the reaction directed against *B. cepacia* outer membrane proteins but reduced considerably the reaction to *P. aeruginosa*, the intensity of the bands on the immunoblot appearing to be reduced to 30-40% compared with the bands on the immunoblots probed with un-adsorbed serum. Again, these data may suggest that the immune reaction to *B. cepacia* is not attributable to cross-reactivity with *P. aeruginosa* .

In this study the IgG reaction to *B. cepacia* OM antigens of five patients sputum-culture-negative for *B. cepacia* has been investigated; three of these patients had anti-*B. cepacia* antibodies. Using ELISA studies, Nelson *et al* (1993) detected IgG antibodies directed against *B. cepacia* whole cell antigens in serum from both *B. cepacia*-colonised and non-colonised CF patients. Focusing on the reaction to *B. cepacia* core LPS, these workers found that a significant component of the reaction was specific and could not be reduced by adsorption with *P. aeruginosa* whole cells. Burnie *et al* (1994) also found anti-*B. cepacia* antibodies in eight out of 22 patients with negative sputum culture for *B. cepacia.* Other workers have detected anti-*B. cepacia* antibodies in some patients up to two years prior to sputum-culture-proven colonisation (Aronoff *et al,* 1991). Although some *B. cepacia* OM components may be cross-antigenic with *P. aeruginosa*, our immunoblotting experiments suggest that

there are some specific *B. cepacia* antigens.

The marked phylogenetic difference between *B. cepacia* and *P. aeruginosa* would suggest that the OM antigens are likely to have at least partially differing epitopes. However it is well recognised that markedly different bacteria can have very similar antigens. Within the *B. cepacia* species there is marked variation in the OMPs, as demonstrated by the difference between *B. cepacia* NCTC 10661 and the clinical strain (Fig 3.2, lane 1 and 2). This may reflect the diverse phenotypes of *B. cepacia*, evident even if the same strain is grown from the same patient, and from the same site (Larsen *et al*, 1993). The diversity of the phenotype of the same strain appears to be differential gene expression, although there may also be marked heterogeneity of the genotype within the species. For a serological test to be sensitive a large number of antigens would need to be included if the majority of phenotypes and genotypes were to be detected. However, by using a large number of antigens there is increased risk of cross-reactivity, and thus reduced specificity. Alternatively, if one or two antigens were selected that were known to be conserved in the different phenotypes and genotypes, specificity and sensitivity would be possible.

In this study possible cross-reactivity between *B. cepacia* and *P. aeruginosa* has been examined. However, the cross-reaction between *B. cepacia* and *B. gladioli*, *P. acidovorans,* or *Xanthomonas maltophilia* has not been studied. Clinical isolates of these four organisms have all been mis-identified as *B. cepacia* despite gowth on a selective medium (Burdge *et al*, 1995; Govan and Nelson, 1992; Simpson *et al*, 1994). It would therefore be important to assess possible cross-reactions before

selecting an antigen or antigens to be used in a serological test.

As first noted by Aronoff *et al* (1991), apparent *B. cepacia* colonisation of the CF lung occurs despite the presence of anti-*B. cepacia* antibody. This study supports the specificity of some of these antibodies in patients already colonised with *P. aeruginosa,* although cross-reactive immune reactions by hidden epitopes could not be excluded in this study. Immunoblotting of a patient's serum alone and without consideration of other immune reactions may give a false impression of the patient's ability to control *B. cepacia* infection. These antibodies may represent previous exposure to *B. cepacia* without colonisation, and may have a protective role in preventing infection. However, at present it is still highly speculative to assign a function to these antibodies, but if interpreted cautiously, they may have diagnostic value.

CHAPTER 5

SERUM IgG REACTION TO B. CEPACIA-SPECIFIC OUTER MEMBRANE ANTIGENS: ASSESSMENT BY ELISA

5.1 Introduction

Immunoblotting, as described in Chapter 4, provided a qualitative assessment of the immune reaction to *B. cepacia* OM antigens. To assess further the immune reaction in a quantitative manner, an ELISA test was developed. A single protein was chosen as this represents a more defined antigenic preparation than a system based on whole cells or whole outer membrane. A single protein ELISA assay is a well established technique that could easily be performed in clinical laboratories. This test could then be used in conjunction with sputum culture. It could provide a result within 24 hours, in contrast to sputum culture, that may take four days or longer. As a quantitive test, a rise in antibody level may also provide further evidence of infection, especially if serial serum samples are taken over a period of time. Early identification *B. cepacia* infection is important if segregation of colonised and non-colonised patients is to be practised most effectively.

5.2 Results

5.2.1 *Patients*

Serum samples were collected as described in section 2.4.3. 21 patients colonised with *B. cepacia* were age and sex-matched with 21 patients colonised with a CF control from whom *B. cepacia* had never been isolated. Subsequent analysis showed that the two groups were comparable for FEV_1, FVC, weight and height (Table 5.1).

5.2.2 *SDS-PAGE and immunoblotting of the 80 kDa protein*

The electroeluted 80 kDa protein was composed mainly of the 36 kDa component; there remained some intact 80 kDa aggregate but no 27 kDa protein could be detected (Fig 5.1). Immunoblotting of this protein resulted in a reaction at the 36 kDa site and a weak reaction to material migrating at 80 kDa, possibly because there was less protein present after dissociation.

5.2.3 *ELISA*

The *B. cepacia* colonised patients showed a high EU (median 1.40, range 0.37-1.88, 95% CI 1.21-1.54), whereas the *B. cepacia*-negative patients showed a lower EU (median 0.54, range 0.34-0.92, 95% CI 0.50-0.63 [Welch's alternative t-test $P < 0.0001$]) (Fig 5.2). Two *B. cepacia* positive patients not matched with *B. cepacia* negative patients gave EU results of 0.77 and 1.02.

A further 43 *B. cepacia* non-colonised patients showed a low EU (mean 0.56, range

0.23-0.92, CI 0.19-0.92). Fifty non CF control patients had very low EU values (median 0.20, range 0.11-0.39, 95% CI 0.11-0.39).

Inter-assay and intra-assay variation was determined from 96 and five measurements respectively. The inter-assay coefficient of variation was 6.0% and that for the intra-assay variation was 4.9%.

Nineteen out of 23 *B. cepacia*-positive patients gave EU values greater than 1.0, 2/23 *B. cepacia*-positive patients' values were just below 1.0 (0.95 and 0.96); 2/23 *B. cepacia*-positive patients had low results (0.77 and 0.37). 20/21 patients ***B.cepacia*** negative patients had low EU values. However, one *B. cepacia*-negative patient had an intermediate value (0.92) (Fig. 5.2). Using a cut-off value of EU 1.0 to distinguish between *B. cepacia*-colonised and non-colonised patients the test would have a specificity of 100%, a sensitivity of 83%, and a negative predictive value of 84% using data from the age and sex-matched patients.

Table 5.1. Clinical details of the *B. cepacia* positive and negative patients. Values are means (range).

	B. cepacia positive	*B. cepacia* negative	*p**
sex	13 males 8 females	13 males 8 females	
age (years)	22.9 (12.3-29.7)	22.4 (12.5-31.8)	0.626
weight (Kg)	51.6 (28.9-70.0)	49.6 (28.0-76.3)	0.305
height (metres)	1.64 (1.36-1.81)	1.60 (1.43-1.86)	0.076
mean % predicted FEV_1	37.5 (14-95)	41.3 (16-86)	0.251
mean % predicted FVC	50.7 (16-103)	58.7 (26-111)	0.251

* Wilcoxon signed rank test for paired nonparametric data (two tailed p value). The age data was analyzed to see if matching of patients had been effective for age and was found to have a nonparametric Spearman correlation coefficient of 0.79 and a one-tailed p value of < 0.0001, and was thus well paired.

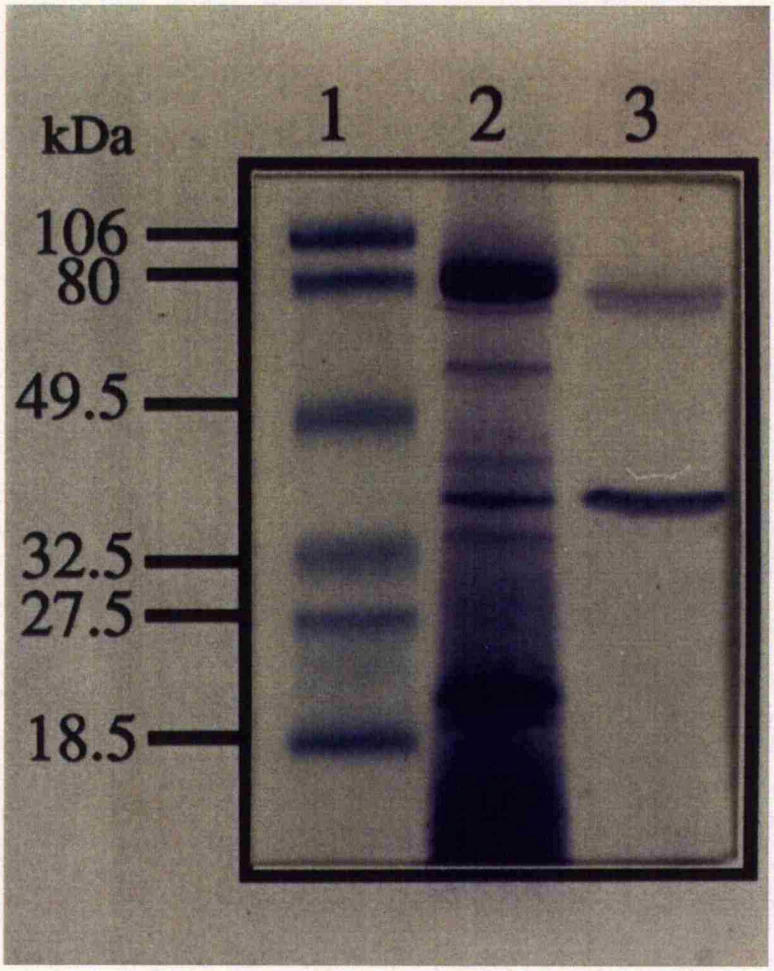

Figure 5.1. SDS-Page analysis of the whole outer membrane of *B. cepacia* (Lane 2) and the electroeluted proteins from the 80 kDa outer membrane band that had been excised from the preparative gel (Lane 3). Molecular weight markers (Lane 1).

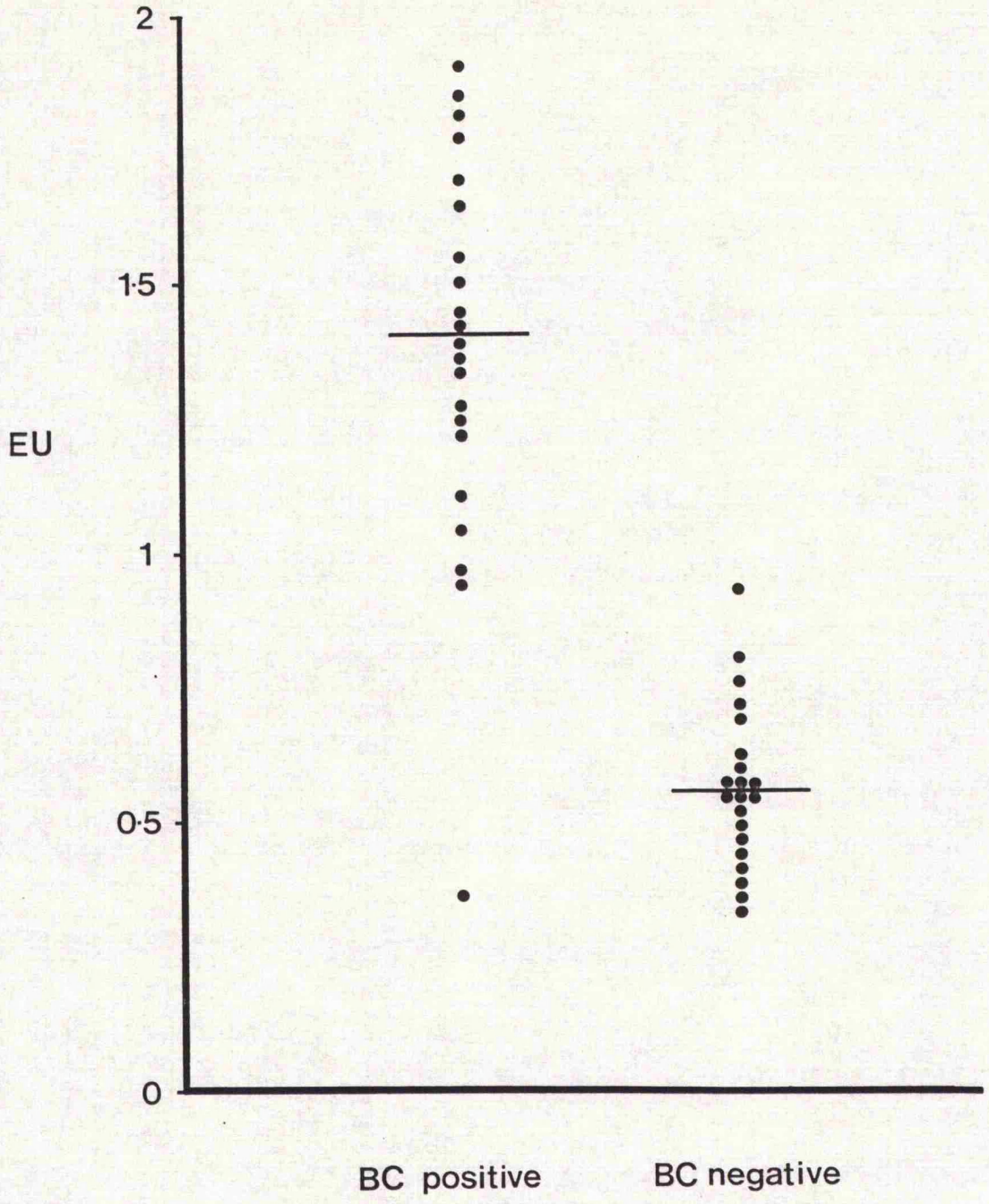

Figure 5.2. IgG response to the 80kDa antigen in patients colonised with both *Burkholderia cepacia* and *Pseudomonas aeruginosa* (positves), patients colonised with *Pseudomonas aeruginosa* alone (negatives). IgG measured in ELISA units at a serum dilution of 1:200.

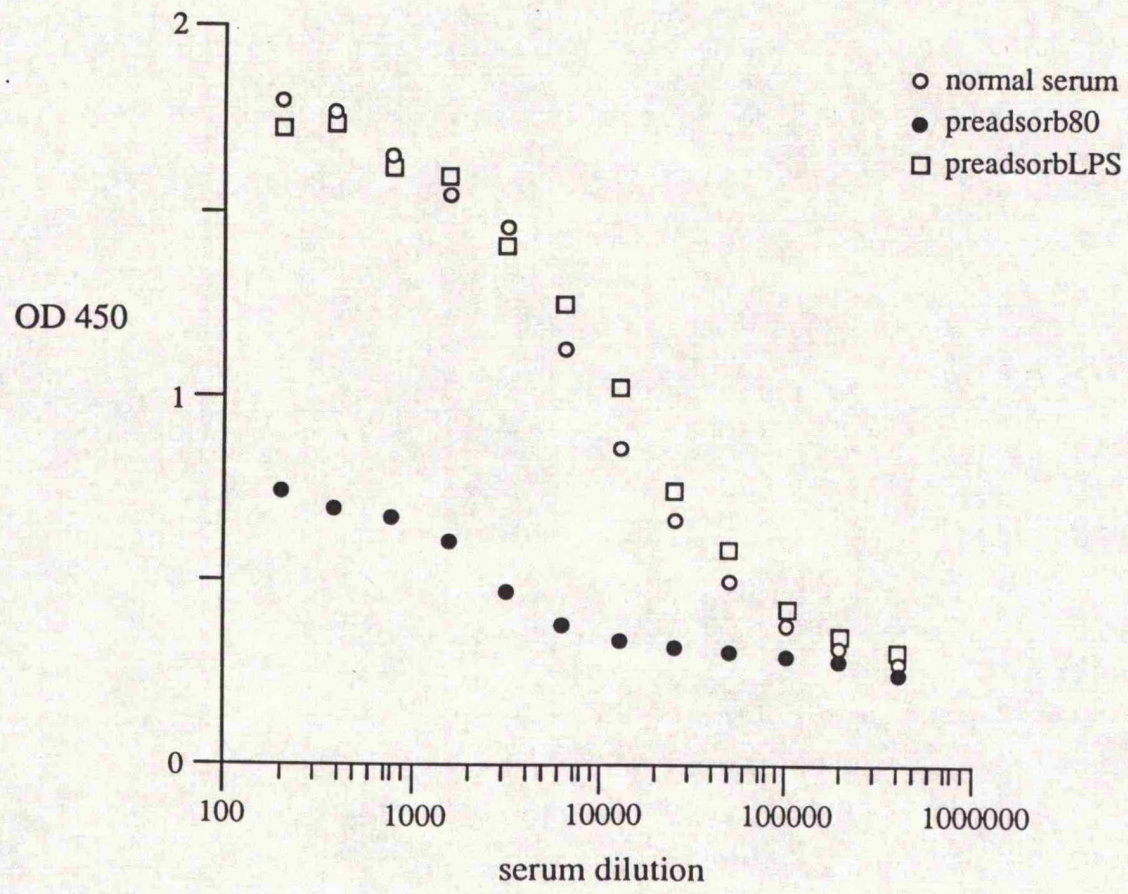

Figure 5.3. IgG response to 80 kDa antigen before and after the serum was preadsorbed with the 80 kDa and LPS antigens.

5.3 Discussion

The immune reaction to the *B. cepacia* 80 kDa outer membrane protein was studied. A single protein was chosen as it is a more defined antigenic preparation than a system based on whole cells or whole outer membrane. The 80 kDa OMP was selected as a *B. cepacia* specific antigen after initial SDS-PAGE studies and on the basis of previous immunoblotting studies (see chapter 3, 3.4.1, Aronoff and Stern, 1991; Gotoh *et al,* 1994). The 80 kDa OMP is composed of 36 kDa and the 27 kDa OMP subunits and these are thought to be specific for *B. cepacia* (Aronoff *et al,* 1991).

Parr *et al* (1987), purified the 80 kDa protein by gel filtration of proteins obtained from residues following SDS extraction of the outer membranes isolated by sucrose density gradient fractionation of *B. cepacia* whole cell isolates. They found that the protein has a molecular weight of 80 kDa on SDS-PAGE but is composed of a major subunit of 36 and a minor subunit of 27 kDa (Parr *et al,* 1987). Their results also suggested that the 80 kDa protein was a peptidoglycan-associated protein.

To improve recovery of this porin protein Gotoh *et al,* (1994), used total membranes (cell envelopes) and lithium dodecyl sulphate (LDS) instead of SDS to prevent crystallisation during chromatography. Using this protein Gotoh *et al,* (1994) detected by SDS-PAGE a 140 kDa protein in addition to the 80 kDa protein and the subunits, the 36 and 27 kDa proteins. They purified the 36 kDa and 27 kDa subunits separately from the 80 kDa protein. Formation of the 80 kDa from a mixture of the purified 36

and 27 kDa subunits suggested that the 36 and 27 kDa subunits were not proteolytic fragments. The purified proteins were used for the production of murine anti-sera directed against each protein independently. Immunoblot assays with both murine anti-sera showed no cross reaction between the 36 and 27 kDa proteins, further supporting the conclusion that the 27 kDa protein is not a proteolytic fragment of the 36 kDa protein, and that the 36 and 27 kDa proteins are immunologically distinct components of the 80 kDa protein.

Gotoh *et al*, (1994) also found that the 140 kDa and not the 80 kDa protein occurred in the protein preparation released from crude peptidoglycan and that the 140 kDa protein could be dissociated into the 80 kDa, which could then further dissociate in to the 36 and 27 kDa proteins. They suggested that the 140 kDa protein was composed of three molecules of 36 kDa protein and one or two molecules of the 27 kDa protein, but did not propose what the composition of the 80 kDa might be.

To determine the molecular weight of a trimer of the 36 kDa protein, Gotoh *et al*, (1994) compared the mobility of the 36 kDa protein with the mobility of *Escherichia coli* porin protein OmpF which is a 36 kDa trimer with a total molecular weight of 108 kDa. They found that after SDS-PAGE of the purified OmpF protein solubilised at 25°C for 10 $\overset{mins}{m}$ they detected a band at approximately 72 kDa; the purified *B. cepacia* 36 kDa also appeared as a band at 72 kDa under identical conditions. The authors therefore concluded that the 36 kDa trimer that appears as a band at 72 kDa actually has a molecular weight similar to the OmpF protein, of 108 kDa.

The 80 kDa protein is thought to be an important porin molecule (Parr *et al,* 1987; Gotoh *et al,* 1994). As a porin protein it is likely to be surface exposed and involved in early antigen reaction. The low permeability of *B. cepacia* outer membrane to nitrocefin is thought to be due to the small size of the 80 kDa porin protein, contributing to the beta-lactam resistance of *B. cepacia* (Parr *et al,* 1987; Aronoff, 1988; Gotoh *et al,* 1994). Furthermore, in some clinical strains, failure to express the 27 kDa and reduced amounts of the 36 kDa polypeptide has been associated with increased resistance of *B. cepacia* to beta-lactam antibiotics (Aronoff, 1988). In this study, the 27 kDa was not detected, suggesting that it was not expressed in this strain or that it was present in low levels. This is consistent with the fact that the clinical strains studied were resistant to beta-lactams but MICs were not measured.

By using electroeluted OM protein it was hoped that any residual LPS from the outer membrane protein would be removed, as LPS can associate tightly with protein and would elicit its own immune reaction (Parr *et al,* 1986). Preadsorption of serum with purified LPS antigen did not diminish the immune reaction to OM protein, whereas preadsorption with the 80 kDa protein resulted in a significant reduction in the immune reaction confirming that the reaction was specific to the 80 kDa protein and not to LPS or non specific antigens (Fig 5.3). It can therefore be concluded that the antibody reaction is directed toward the 80 kDa antigen alone.

Aronoff and Stern (1988) have previously shown raised IgG antibody titres to *B. cepacia* whole outer membranes in patients colonised with *B. cepacia*. There was, however, considerable overlap with non-colonised patients. Nelson *et al* (1993) have

reported raised IgG titres to a *B. cepacia*-specific lipopolysaccharide (LPS) core determinant. They found that an ELISA based on core LPS was better at distinguishing between *B. cepacia*-positive patients and *B. cepacia*-negative patients than whole cell ELISA. Three out of the nine *B. cepacia* colonised patients they studied were not colonised with *P. aeruginosa*. Longitudinal analysis of their serum IgG reaction indicated that the rate of increase of the levels of anti-*B. cepacia* antibodies varied from patient to patient, but in some cases a two-to-four-fold rise in titre preceded the first positive sputum culture for *B. cepacia* by several months.

The 21 *B. cepacia* patients studied were matched as closely as possible with *B. cepacia*-negative patients (Table 5.1). Mean FEV_1 and FVC were slightly lower in the *B. cepacia* group but this was not significant and therefore, it is unlikely that the increased antibody reaction seen in the *B. cepacia* patients is a reflection of the increased severity of the lung disease of the *B. cepacia* patients. Despite a wide age range of patients, and patients from two centres, the raised EUs were remarkably uniform for the *B. cepacia*-colonised patients.

The diagnosis of *B. cepacia* currently depends on initial isolation using a selective medium such as MAST, UK, and biochemical identification *eg* API 20 NE System, and further confirmation by ribotyping. Serological diagnosis may have a role in providing an early diagnosis of *B. cepacia* infection prior to confirmation by culture. An ELISA could be performed within 24 hours, whereas culture may take between four and seven days; ribotyping may take longer and is currently only performed at a small number of centres. Diagnosis may be facilitated in younger patients who are

unable to expectorate sputum. Colonisation with *P. aeruginosa* is thought to occur in these young children as determined by serum antibodies to *P. aeruginosa* (Cordon *et al*, 1993) and the same process is likely to occur with *B. cepacia* infection.

Previous studies suggest that a serum IgG reaction to *B. cepacia* antigens is present prior to colonisation and sometimes a rise in titre precedes or accompanies the first isolation of *B. cepacia* (Aronoff *et al*, 1991 and Nelson *et al*, 1993). In this study, a *B. cepacia*-positive patient with a low EU had an initial isolation of *B. cepacia* only 2 months prior to the serum sample, and a later rise in the antibody reaction may well occur. In contrast, the *B. cepacia*-sputum culture-negative patients who had intermediate EU values may grow *B. cepacia* at a later date.

Longitudinal studies of IgG reaction to *B. cepacia* OM proteins using immunoblotting have shown that, although low serum levels of IgG against the 27- and 36-kDa outer membrane are detectable prior to colonisation, there are increased levels after acquisition of *B. cepacia* (Aronoff *et al*, 1991). Bernie *et al* (1995) detected a serum IgM reaction at the 30 kDa site, thought to be equivalent to the 27 kDa porin protein, was more likely to be present in those patients not colonised with *B. cepacia* and those patients colonised with *B. cepacia* who did not deteriorate or die. They suggest that the presence of this antibody against the 27 kDa protein carries a good prognosis but does not protect against colonisation.

Longitudinal serum samples, and samples from other CF centres, are needed to assess further the diagnostic applicability and usefulness of this 80 kDa antibody assay for

the detection of *B.cepacia* in patients with CF. Studies of the two subunits of the 80 kDa protein (36 and 27 kDa) are also needed.

CHAPTER 6

PRODUCTION OF POLYCLONAL MONOSPECIFIC B. CEPACIA ANTIBODY AND ITS APPLICATIONS.

6.1 Introduction

In this chapter the production of polyclonal monospecific anti-*B. cepacia* antibodies is presented. Serum from patients colonised with *B. cepacia* was used as a source of anti-*B. cepacia* antibody in chapter 3, 4 and 5. Polyclonal monospecific anti-*B. cepacia* antibodies could have many potential applications. The main advantage of a monospecific antibody is that it would be unlikely to cross-react with *P. aeruginosa*, whereas serum from patients colonised with *B. cepacia* is likely to have high levels of anti-*P. aeruginosa* antibodies.

In this chapter the characteristics of a polyclonal monospecific anti-80 kDa *B. cepacia* antibody are investigated and its use in an immunofluorescence test for *B. cepacia* in sputum determined. A sputum immunofluorescence test for *B. cepacia* could be performed at the same time as the Gram stain when the sputum is plated for culture and could provide a result within several hours.

The 80 kDa protein was again chosen as the antigen for the same reasons outlined in chapter 5 (5.2.2) and because of the results obtained from the ELISA studies (5.2.3). The 80 kDa protein was prepared by the same method as used for the ELISA studies in chapter 5, as described in section 2.2. The production of the polyclonal antibodies is described in section 2.5 and the method for immunofluorescence described in section 2.6.

6.2 Results

6.2.1 *Characterisation of polyclonal monospecific anti-80kDa sera*

Immunoblotting was performed using the anti-80 kDa serum, diluted 1:50 TBS-Tween, from Rabbits A and B to three different strains of *B. cepacia* to determine the immune reaction of the polyclonal serum.

6.2.1.1 Whole outer membrane

a) *B. cepacia* OM strains 1,2 and 3 (see Table 2.1 for details of strains):

Pre-immunisation of rabbits A and B: No bands were detected on the immunoblot of strain 1,2 and 3.

Post-immunisation

Strain 1	Band at 36 kDa plus other faint bands
Strain 2	Bands at 80 kDa and 36 kDa
Strain 3	Bands at 80 kDa, 36 kDa, 27.5 kDa

b) *P. aeruginosa* OM strain from patient 1:

Several faint bands were seen in both the pre and post immunisation sera but no major bands were present. In particular there were no bands at the 80 kDa, 36 kDa and 27.5 kDa positions.

6.2.1.2 Lipopolysaccharide antigen

B. cepacia strain 2 (rough) proteinase K-treated OMs were probed with post-immunisation serum from rabbits A and B. No core lipid band was seen although this was clearly visible when the same immunoblot was probed with serum from whom the strain was isolated (patient 2).

6.2.2 *Immunofluorescence using the anti-80 kDa sera*

6.2.2.1 Immunofluorescence of bacteria grown in culture

Clinical strains of *B. cepacia* and *P. aeruginosa* were grown overnight on TSB and the cells fixed on the slide as described. *B. cepacia* cells fluoresced and with a small oval shape were easily identified. However, as expected the bipolar "safety pin" appearance seen on Gram staining (Fig 6.1) was not distinguishable on immunofluorescence (Fig 6.2). There was some fluorescence of the long rods of *P. aeruginosa* when the anti-80 kDa serum was used at a dilution of 1:50, however this was reduced when the serum was diluted 1:200, with no loss of intensity of the reaction to *B. cepacia*. Further reduction of this reaction occurred when the serum was preadsorbed with *P. aeruginosa* (see Chapter 2).

There was no immunofluorescence obtained when sera from the pre-immunised rabbits were used.

6.2.2.2 Immunofluorescence of sputum from CF patients

The immunofluorescence of sputum was much less successful because of the

amount of debris in the sputum. The debris tended to fluoresce, obscuring the cells. A variety of techniques was tried to break down the sputum, and differential centrifugation was attempted to separate bacteria from sputum. Sputasol (0.5 ml added to 0.5 ml of sputum) (Fig. 6.3) and mercaptoethanol (100 μl added to 1 ml of sputum) (Fig. 6.4) were used to break down the sputum, but without any reduction in the amount of debris present. Although it was possible to identify possible *B. cepacia* cells, they were only detected after a considerable time had been spent examining the slide and because pieces of sputum debris also fluoresced there was often uncertainty. Figures 6.3 and 6.4 show fluorescence of probable *B. cepacia* cells in the centre of the pictures.

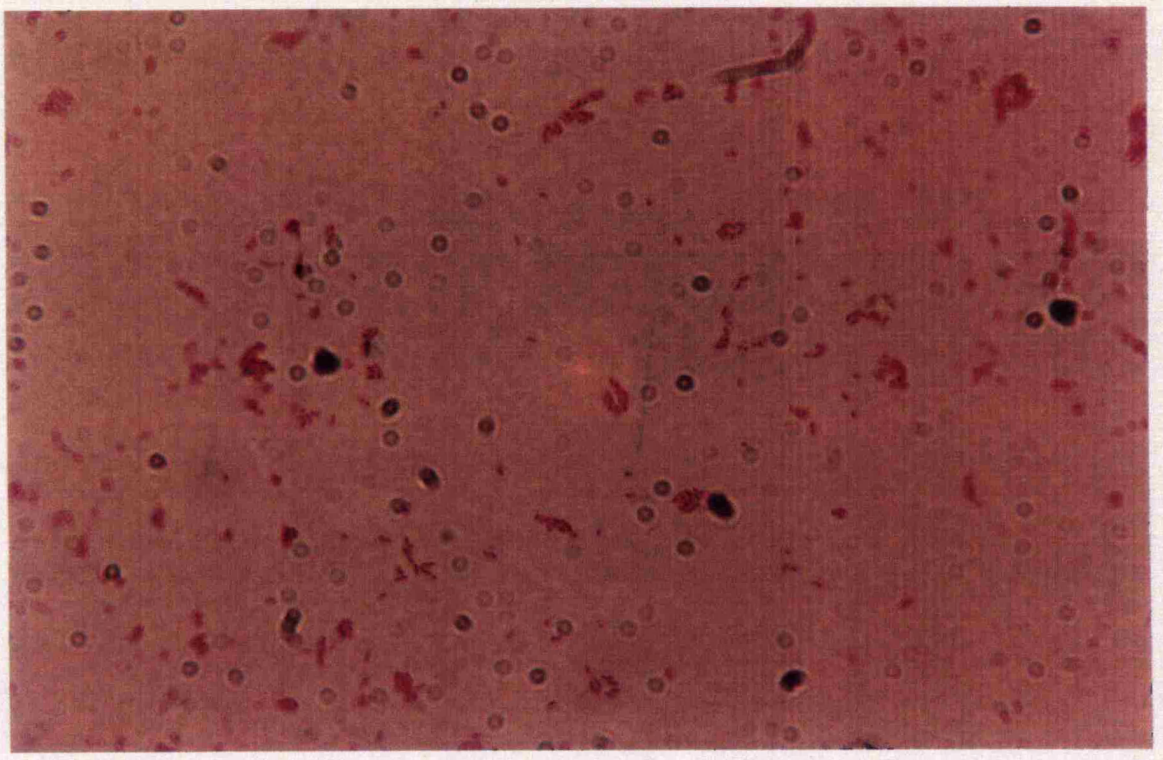

Figure 6.1. Gram stain appearance of *B. cepacia* culture (x 100).

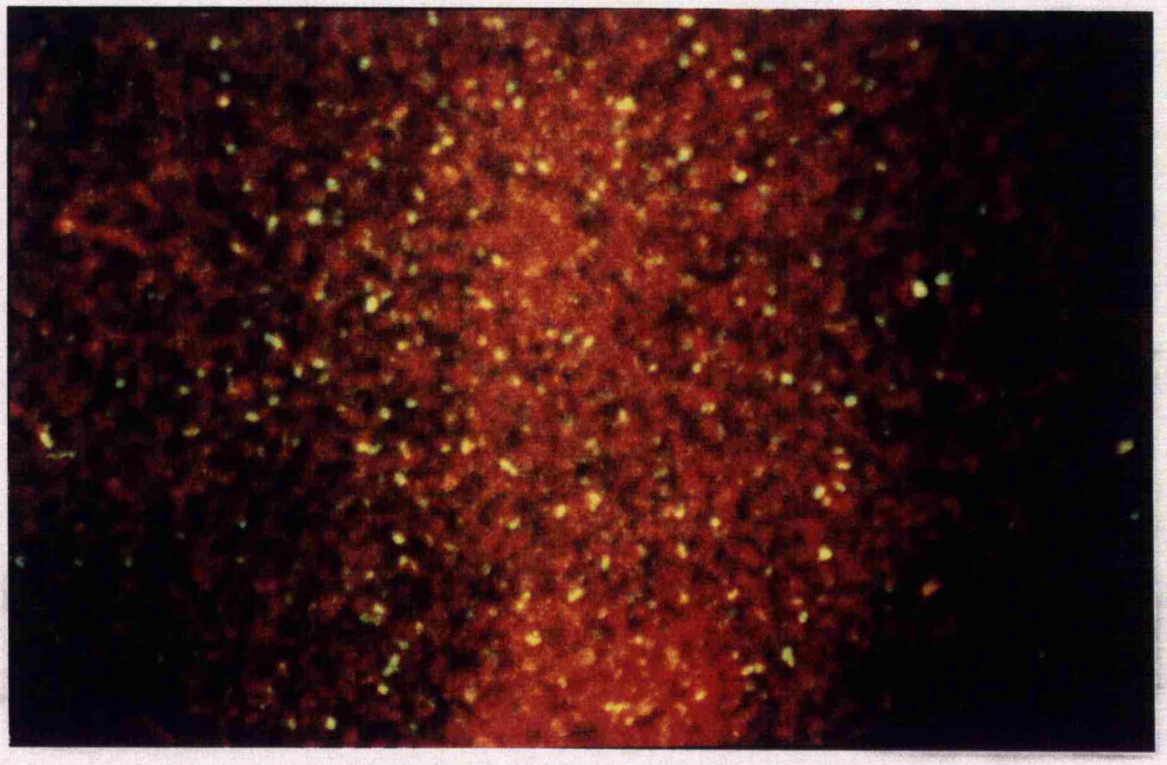

Figure 6.2. Immunofluorescence of *B. cepacia* cells using the anti-80 kDa rabbit antibody and anti-rabbit goat-FITC (UV light x 100).

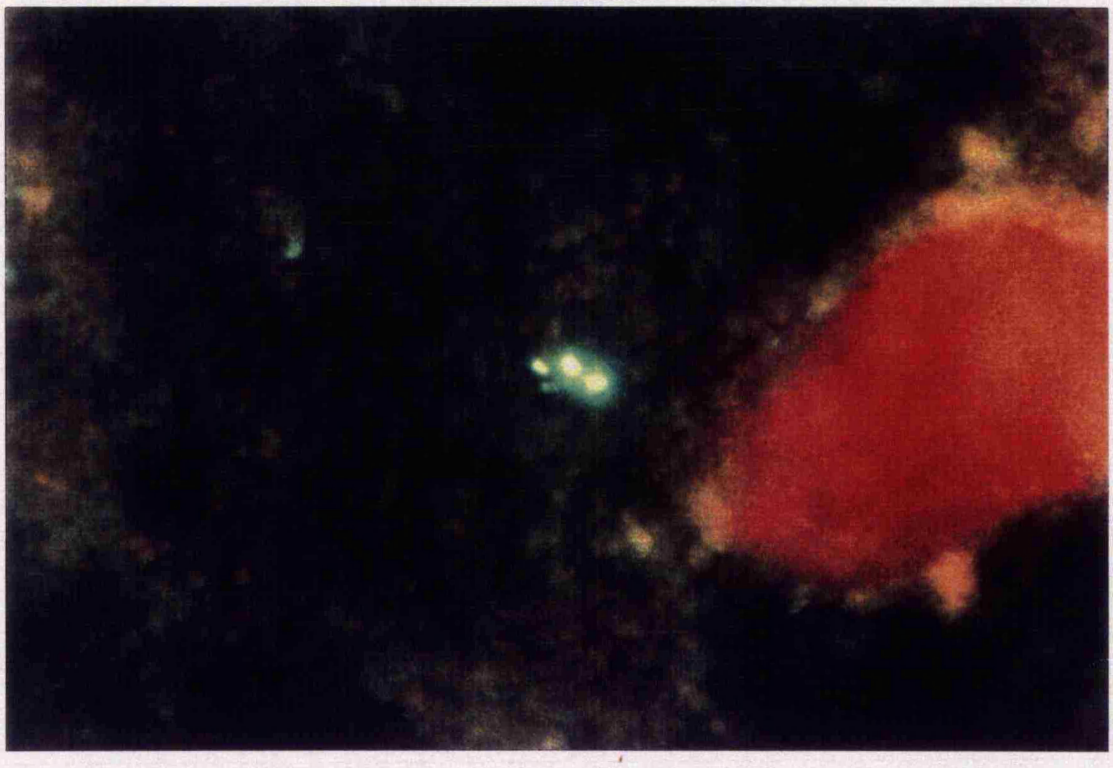

Figure 6.3. Immunofluorescence of sputum from a patient colonised with *B. cepacia* using the anti-80 kDa rabbit antibody and anti-rabbit goat-FITC after the sputum had been treated with Sputasol (UV light x 1000).

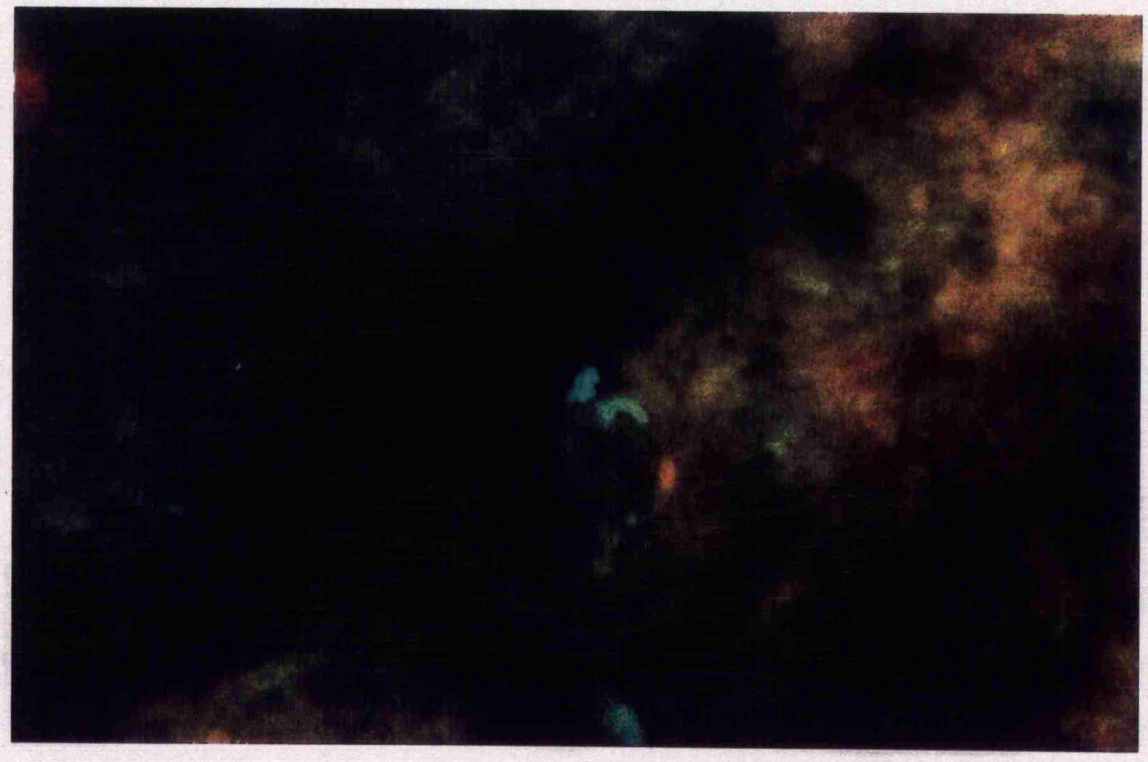

Figure 6.4. Immunofluorescence of sputum as in Figure 6.3 but treated with mercaptoethanol instead of Sputasol (UV light x 1000).

6.3 Discussion

6.3.1 *Polyclonal monospecific anti-80 kDa sera*

The serum from the rabbits prior to immunisation and after immunisation appeared to have some background reaction to *P. aeruginosa*. It is important to note that this reaction did not increase after immunisation and is therefore unrelated to the immunisation. This would be consistent with the prevalence of *P. aeruginosa* in the environment, particularly in water, soil and vegetables (Botzenhart and Doring, 1993). There was no detectable immune reaction to *B. cepacia* in the rabbits prior to immunisation. *B. cepacia* is also found in soil and water, but does not appeared to have evoked an immune reaction in the rabbits prior to immunisation.

In the post immunisation sera an immune reaction was detected using the polyclonal anti-80 kDa serum at the 80 kDa, and in some the 36 kDa and 27 kDa position on the immunoblots. As noted in chapter 5 (section 5.2.2), it is likely that the 80 kDa protein used in this study was composed of mainly 36 kDa protein as the 27 kDa protein could not be detected on SDS-PAGE.

When the immunoblot of *B. cepacia* OM from strain 1 was probed with post-immunisation serum the reaction was present only at the 36 kDa site suggesting that the 80 kDa protein had completely dissociated. The immunoblot of *B. cepacia* OM from strain 3 showed evidence of an immune reaction at the 27 kDa site, when probed with post-immunisation serum. Strain 3 is thought to be an environmental strain as it is known to be ribotype D, CHEF type IV (see table 2.1). Strains 1 and

2 which Ribotype A, CHEF type I (table 2.1), may have reduced amounts of the 27 kDa peptide as previously discussed (see section 5.3). Strain 3 may have increased amounts of the 27 kDa protein compared to strains 1 and 2, and thus an immune reaction occurred at this site. This finding also suggests that the 80 kDa protein used to immunise the rabbits did contain small but significant amounts of the 27 kDa protein.

6.3.2 *Immunofluorescence*

Immunofluorescence of ***B. cepacia*** was successfully demonstrated using the anti-80 kDa serum from the immunised rabbits (Fig 6.2). The immunofluorescence indicated that the 80 kDa protein is surface exposed and distributed evenly around the cell. The lack of the bipolar appearance present when the organism is Gram stained suggests that it is not due to the actual configuration of the cell.

The weak cross-reactivity of the immunofluorescence with ***P. aeruginosa*** is probably in part due to the non-specific binding of the FTIC-conjugated goat anti-rabbit immunoglobulin and also due to the weak non-specific immune reaction to ***P. aeruginosa*** that was demonstrated on the immunoblots. The former was addressed by increasing the dilution of the serum and the FITC-conjugated goat anti-rabbit immunoglobulin and the latter by preadsorbing the serum with ***P. aeruginosa.*** After these adjustments the reaction to ***P. aeruginosa*** was undetectable.

The major difficulty encountered in detecting ***B. cepacia*** in sputum by immunofluorescence could not be overcome in the time available. However with

better breakdown of the sputum, washing and differential centrifugation, more of the debris could be removed and the cells concentrated to allow detection of *B. cepacia.*

Immunofluorescence would allow detection of *B. cepacia* cells in sputum with the opportunity for a "same day" result, in comparison to culture that can take four to seven days because of its slow growth. It is likely that it would be specific for *B. cepacia* especially if combined with Gram stain. The Gram stain appearance of short oval gram-negative rods with bipolar appearance has been shown to have a specificity of 87.7% for *B. cepacia* (Sadeghi *et al,* 1994) but it is rarely used to detect *B. cepacia.*

Most clinical laboratories simply report the presence of either gram-positive cocci or gram negative rods without commenting on the morphology of the cells on routine sputum samples. Because the morphology of the *B. cepacia* is so distinct and different from the long slender rods of *P. aeruginosa* it should be possible to identify the gram negative ovoid cells of *B. cepacia* in sputum from CF patients. However, this "safety pin" morphology is not specific for *B. cepacia.* It has been described for other organisms such as *Pasteurella* species. This morphology has also been reported for *Burkholderia (Pseudomonas) pseudomallei* an organism closely related to *B. cepacia. Burkholderia gladioli* is another closely related organism that has been identified in CF sputum (Christenson *et al,* 1989). *B. gladioli* resembles *B. cepacia* on selective and differential medium but its morphology has not been studied.

Further study is needed to determine whether the described immunofluorescence

technique using the anti-80 kDa monospecific serum or other monospecific serum would be more specific than Gram stain in the detection of *B. cepacia.*

6.3.3 *Other applications for the monospecific anti-80 kDa serum*

The monospecific anti 80 kDa serum could also be used to detect *B. cepacia* in sputum using an ELISA technique. This could be performed either by the double antibody sandwich assay or the indirect immunosorbent assay. In the double antibody sandwich assay the monospecific anti-80 kDa antibody is placed in the wells of the microtitre plate and becomes absorbed onto the plate. The test sputum is denatured and is added to each well. Any unbound antigen-antibody is removed during washing and the antigen-antibody complex is detected by using an antibody conjugate specific for the antigen. The final complex is formed of an outer antibody-enzyme, middle antigen, and inner antibody. A substrate is added to cause a colour reaction, which can be measured by optical density scanning of the plate.

The indirect immunosorbent assay, as used in chapter 5, would be performed as follows. The denatured sputum is placed into the wells of the microplate and absorbed onto the plate. The monospecific anti-80 kDa serum is added and unbound antibody is washed away. The antibody-antigen complex is then reacted with an antibody enzyme complex (conjugate) and a chromogen added to cause a colour reaction which is measured by optical density.

These ELISA techniques would have the additional benefit of being quantitative and allowing some estimation of the amount of *B. cepacia* in the sputum. The

monospecific anti 80 kDa serum could be used to detect *B. cepacia* antigen in the urine. This is a well established technique for the detection in urine of *Streptococcus pneumoniae* in pneumonia and meningitis. Urine is used as it can be collected easily in children. The latex agglutination inhibition immunoassay relies on the competition for antibody between a latex-antigen conjugate and antigen in the urine. A urine sample is placed in the mixing well of a slide containing the 80 kDa protein coated onto latex particles and the monospecific anti-80 kDa serum. If the urine contains the 80 kDa antigen this will bind to the antibodies and no agglutination will occur. If the 80 kDa antigen is absent the latex-antigen complex will bind to the antibodies and agglutination will occur.

A urine latex agglutination inhibition assay would avoid the problem of thick mucopurulent sputum and would be advantageous in younger children who cannot produce sputum. Because the test would detect antigen rather than antibody the test would indicate present infection and not past exposure or past infection. It would not be affected by antibiotics taken by the patient.

6.3.4 *Monoclonal antibodies*

Many of the applications for the anti-80 kDa antibody outlined above may be made more specific if a monoclonal anti-80 kDa antibody was produced. However this may reduce the affinity and therefore sensitivty of the test. The method is now well established (Kohler and Milstein, 1975). Monoclonal antibodies have been raised to *P. aeruginosa* pili (Saiman *et al*, 1989) and to *B. cepacia* extracelluar protease (Kooi *et al*, 1994). Monoclonal anti-80 kDa antibodies could be used in the diagnosis of *B. cepacia* as part of a immunofluorescence test or as part of an ELISA test on serum or sputum.

6.4 Conclusion

Immunofluorescence using the monospecific anti-80 kDa serum was successful using *B. cepacia* grown in culture. Due to the extreme viscosity and mucopurulence of CF sputum the immunofluorescence was unsuccessful when applied to sputum. The specificity of the anti-80 kDa serum has not been tested against similar organisms such as *B. gladioli*. Specificity may be enhanced if a monoclonal antibody is used.

CHAPTER 7

CONCLUDING REMARKS

In 1993 a group of experts met together, under the chairmanship of Prof G Doring to publish a consensus document on *P. aeruginosa* and *B. cepacia* (Doring and Schaffar, 1993). A consensus statement was that studies using appropriate technology for the detection of *B. cepacia*, including non-culturable forms, are to be encouraged. The clinical management of *B. cepacia* infection depends on firstly the correct identification and secondly on its early detection; the fulfilment of these two objectives will allow effective segregation of patients and the prevention of transmission of *B. cepacia.*

Despite the introduction of a *B. cepacia* selective medium and commercially available biochemical identification kits, correct identification of *B. cepacia* is still difficult. For many laboratories in the UK, especially those dealing with small numbers of cystic fibrosis sputum specimens, these techniques may not be in regular use. Furthermore, Cimolai *et al* (1994) have suggested that some selective media, by their action of inhibiting the growth of other organisms may also inhibit growth of *B. cepacia,* thus resulting in failure to grow *B. cepacia* in some colonised patients.

Burdge *et al* (1994) have recommended the following steps for proper characterisation of *B. cepacia:* (1) confirmation that the organism is a nonfermentative, Gram negative bacillus; (2) use of a commercially available identification system such as API 20 NE system (Gilligan *et al*, 1985); (3) assessment of oxidase (read within 1 minute of testing), DNase (32°C for 72 hours), and Oxidation-Fermentation tests of sucrose and lysine decarboxylase (assess for 7 days at 32°C). However, most commercial test systems do not differentiate *B. gladioli* from *B. cepacia* and complete biochemical

testing is necessary, including oxidation fermentation tests for glucose, maltose, lactose, mannitol, and xylose (Simpson *et al,* 1994). Even if an organism is identified as *B. cepacia,* further confirmation is recommended by ribotyping or pulsed field gel electrophoresis.

There is increasing evidence that currently available culture methods are not able to detect *B. cepacia* at an early stage of infection. Infection may be occurring may months prior to positive sputum cultures, as indicated by both the serological results and by the pattern of transmission of the organism from one patient to another as determined by genotyping method (Lipuma *et al,* 1994). Lipuma *et al* reported two patients in whom *B. cepacia* was not isolated for 12 and 24 months respectively after their contact with the CF patient with the same strain. Although they could not exclude the possibility that this was due to inadequate culture methods they thought this unlikely and suggested that pulmonary colonisation with *B. cepacia* may not be detected for as long as 2 years using the currently recommended culture methods.

The presence of *B. cepacia* in the sputum raises clinical management issues. How should colonization status be defined? Should the simple identification of *B. cepacia* from any site, regardless of quantity define a patient as being colonised? Clearly a single isolation of *B. cepacia* does not always mean that colonisation will ensue and *B. cepacia* can be present in the sputum on several occasions and then disappear. On how many occasions or for how long should *B. cepacia* be isolated before the patient is said to be colonised? I would suggest a definition of three separate isolations within a six month period.

In this thesis an ELISA-based serological test has been developed that will differentiate those sputum-culture-positive for *B. cepacia* from those sputum-culture-negative CF patients. Longitudinal studies need to be performed to determine at what stage seroconversion occurs, particularly to determine whether seroconversion occurs before or after positive sputum culture. Further studies need to be performed to determine whether any cross-reacting immune reaction occurs in patients colonised with other organisms such as *B. gladioli* and *Xanthomonas maltophilia* species.

Further investigation is necessary to determine whether an ELISA-based test may be useful for screening CF patients for *B. cepacia* infection and comparing it with routine culture (with or without a *B. cepacia* selective medium) to determine which has a higher sensitivity. Serial measurements showing an increasing serum reaction to the 80 kDa may give an early indication of infection, whilst an intermediate reaction that does not increase may demonstrate exposure without colonization.

Nelson *et al* (1993) measured the sputum IgA immune reaction to *B. cepacia* LPS antigens but did not find an immune reaction as great as the serum IgG reaction to the same antigen. They suggested that the antibody levels may have been underestimated because of the possibility of immune complex formation and fragmentation of antibody by elastase derived from neutrophils or *P. aeruginosa.* In this thesis an alternative method of examining sputum for evidence of *B. cepacia* infection has been attempted using immunofluorescence. However problems were encountered due to the viscosity and mucopurulence of the sputum. If these problems could be overcome and the immunofluorescence was confirmed to be specific for *B.*

cepacia this test would be extremely useful as it would give a very quick indication of the presence of *B. cepacia.* Once *B. cepacia* has been cultured immunofluorescence could also be used to confirm its identity.

Many of the aims and objectives set out at the beginning of this thesis have been achieved. An iron-limited medium has been developed and the antigens expressed compared with the antigens expressed in an iron supplemented medium. The immune reaction to *B. cepacia* infection has been studied and cross-reactivity with *P. aeruginosa* has been investigated. A *B. cepacia*-specific antigen, the 80 kDa protein,has been isolated and an ELISA-based serological assay of the IgG reaction to *B. cepacia* has been developed. The same 80 kDa *B. cepacia*-specific antigen has been used to produce rabbit polyclonal antibody, from which a *B. cepacia*-specific immunofluorescence test has been developed.

Much more research is needed into the development of tests that will provide early and accurate diagnosis of *B. cepacia.* Further studies are needed to look in greater depth at the immune reaction to *B. cepacia* and to determine whether some antibodies might be protective against *B. cepacia* infection. Further studies are needed to look at *B. cepacia* virulence factors. Studies to determine the feasibility of using the polymerase chain reaction (PCR) to detect *B. cepacia* nucleic acid in sputum are needed as this could be an extremely sensitive and specific test but less likely to be suitable for routine use in hospital laboratories.

It is clear that *B. cepacia* can be a significant cause of morbidity in CF patients. While the prognosis and quality of life for CF patients continues to improve in general, acquisition of *B. cepacia* can have devastating consequences for the CF patient. Much further work is needed.

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Journal of Infection (1993) 27, 301–304

CASE REPORT

Chronic granulomatous disease presenting in childhood with *Pseudomonas cepacia* septicaemia

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Accepted for publication 5 July 1993

Summary

Two children who presented with fever, enlarged liver and spleen and ascites were found to have *Pseudomonas cepacia* septicaemia which proved fatal despite appropriate antibiotics and maximum supportive care. Chronic granulomatous disease of childhood (CGD) was subsequently diagnosed in both children. The possibility of CGD needs to be considered in any child with unexplained *P. cepacia* infection.

Introduction

Pseudomonas cepacia, a catalase producing Gram-negative bacterium is an uncommon pathogen in human disease. It has, however, been reported as a cause of infection in chronic granulomatous disease of childhood (CGD).[1–8] Previously reported cases of *P. cepacia* infection in CGD have predominantly involved the respiratory tract and we are only aware of one previous case of *P. cepacia* septicaemia occurring in CGD.[6]

Case reports

Case 1

A previously apparently healthy $3\frac{1}{2}$-year-old boy presented to a district general hospital with a 9 day history of diarrhoea and vomiting. On examination he was febrile (38·8 °C) and 5% dehydrated. The liver edge was palpable 5 cm below the costal margin. His chest X-ray showed a right sided pleural effusion and an ultrasound scan of the abdomen revealed an enlarged liver and spleen and considerable ascites. He was commenced on oral co-trimoxazole and after 6 days was transferred to this hospital for further investigation and management.

On arrival he was still febrile (39·8 °C), the spleen was now palpable 4 cm below the costal margin, the liver remained enlarged, and the abdomen was tense with ascites. Liver function tests were abnormal: bilirubin 50 μmol/l, alkaline phosphatase 422 IU/l, alanine transferase 386 IU/l, aspartate trans-

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0163-4453/93/060301+04 $08.00/0

ferase 1757 IU/l, and albumin 20 g/l. There was evidence of disseminated intravascular coagulation: prothrombin time 23 seconds (control 13 seconds) and partial thromboplastin time 46 seconds (control 33 seconds), fibrin degradation products raised at 0·75 mg/l.

After blood for culture had been taken he was commenced on IV cefotaxime, flucloxacillin and metronidazole. During the next days there was little improvement. His temperature remained high but all cultures were sterile. His ascites persisted despite fluid restriction and diuretics and a diagnostic and therapeutic peritoneal tap was therefore performed. The antibiotics were changed to IV ciprofloxacin and amoxycillin, while the metronidazole was continued. After 5 days incubation *P. cepacia*, sensitive to cefotaxime, trimethoprim, cefuroxime, azlocillin and ceftazidime, but resistant to amoxicillin, aminoglycosides and partially sensitive to ciprofloxacin, was isolated from peritoneal fluid. Peripheral venous blood taken 14 days after transfer and injected aseptically into culture bottles, also grew *P. cepacia* after 3 days. Pleural fluid was sterile. A liver biopsy showed epitheloid granulomata without giant cells or caseation. A nitrobluetetrazolium test (NBT) demonstrated only 3% positive stimulated granulocytes compared with 90% for a healthy control, and chemiluminescence showed no response.

His clinical condition deteriorated and 10 days after transfer intermittent positive pressure ventilation was commenced. He continued to worsen and 6 days later, despite maximal ventilatory and inotropic support, he developed refractory hypotension and died. Immediate *post mortem* cultures of blood and liver tissue grew *P. cepacia*. Consent for necropsy was declined.

His mother, who was pregnant at the time of his death, subsequently gave birth to another son in whom the diagnosis of CGD has been confirmed.

Case 2

A 7-month-old boy developed right cervical lymphadenitis which was treated with oral amoxycillin and flucloxacillin. However, an abscess developed requiring incision and drainage, and *Staphylococcus aureus* was cultured from the pus. The abscess required further incision and drainage, before eventually healing after 6 weeks. At 10 months of age he developed another abscess in the left cervical region which was also drained. *Serratia* sp. was cultured from the pus, but the abscess healed uneventfully without antibiotic treatment. As he was thriving and in good general health, he was discharged from further review.

When he was 11 months old he presented again with an 8 day history of fever and abdominal pain. On examination he was irritable and drowsy. At that time blood pressure and peripheral perfusion were normal. His liver and spleen were enlarged 9 cm and 2 cm below the costal margin respectively and there was ascites. Chest X-ray showed right upper lobe consolidation. He was commenced on IV cefotaxime and metronidazole after peripheral venous blood, CSF and urine had been collected for culture.

Liver function tests were abnormal: bilirubin 44 μmol/l, alkaline phosphatase 626 IU/l, alanine transferase 568 IU/l, aspartate transferase 1560 IU/l, albumin 30 g/l. There was evidence of disseminated coagulation: prothrombin time 20 seconds (control 13 seconds), partial thromboplastin

time 53 seconds (control 33 seconds), fibrinogen 0·5 g/l and fibrin degradation products 1·50 mg/l.

His clinical condition deteriorated rapidly and despite ventilatory and inotropic support he developed refractory hypotension and died after 72 hours.

After 5 days the initial blood cultures grew *P. cepacia* which was subsequently isolated from immediate *post mortem* samples of liver, spleen, lung, peritoneal and pericardial fluid. The organism was sensitive to cefotaxime, trimethoprim, cefuroxime, ceftazidime, azlocillin and ciprofloxacin but resistant to amoxycillin and the aminoglycosides.

At necropsy the lungs were collapsed, oedematous and haemorrhagic with focal pneumonia and early abscess formation. Microscopic examination of liver, spleen and bone marrow showed multiple necrotizing epithelioid granulomata.

An NBT test was not performed as the diagnosis of CGD was not considered before death. However, this test was subsequently performed on his parents and his sister. His mother was found to have only 69% positive stimulated granulocytes compared with 98·8% in the control. Father and sister had normal NBT reducing capacity (99·0% and 99·4%, respectively). These results strongly suggest that the mother is a carrier of X-linked CGD. Further female members of the family are being investigated.

Discussion

CGD is a rare disorder characterised by recurrent life threatening infections with catalase-positive bacteria and fungi as a consequence of defective neutrophil function. The underlying biochemical defect is a failure of nicotinamide-adenine dinucleotide phosphate oxidase activation. This results in failure to generate free radical superoxide and other toxic cellular metabolites required by neutrophils to kill phagocytosed organisms. Persistence of unkilled organisms results in excessive inflammatory reactions leading to granuloma formation in many organs, including the liver. *P. cepacia* is a catalase-positive pleomorphic Gram-negative rod, ubiquitous in the environment and initially identified as the phytopathogen causing onion rot. *P. cepacia* can grow in water with minimal nutritional requirements and survive in a number of disinfectants.

P. cepacia infection accounts for less than 5% of all infections in CGD.[8] It rarely causes infection in healthy children[9,10] but nosocomial infections are increasingly recognised.[11,12] Nosocomial infections most commonly involve the blood stream or urinary tract, in contrast to community acquired infections which more often involve the lower respiratory tract. The lung is the most frequent site of infection in CGD.[1-8] Septicaemia has only been described in one previous case,[6] which also proved fatal. This child also had disseminated infection in multiple organs, as did the two patients described in this report.

P. cepacia infection is an increasing problem in cystic fibrosis, and can cause a septicaemic illness.[13] This infection may occur in patients with acquired neutrophil dysfunction, as in a case of fatal *P. cepacia* pneumonia in a girl with an acquired bacterial killing defect.[14] There have been no formal studies of

neutrophil function in cystic fibrosis patients with *P. cepacia* infection, but a generalised defect in neutrophil function is unlikely as patients with cystic fibrosis are not prone to septicaemia caused by other organisms.[15]

The diagnosis of CGD should be considered in any child presenting with *P. cepacia* septicaemia. Although the prognosis of patients presenting in this manner is poor, those who survive may benefit from the use of long term prophylactic antibiotics[16] and gamma-interferon.[17] In addition, other family members can be screened, the parents offered genetic counselling and prenatal diagnosis offered in future pregnancies.

(D. E. Lacy is funded by the Cystic Fibrosis Trust U.K.)

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FEMS Immunology and Medical Microbiology 10 (1995) 253–262

Published by Elsevier

FEMSIM 00484

Serum IgG response to *Burkholderia cepacia* outer membrane antigens in cystic fibrosis: Assessment of cross-reactivity with *Pseudomonas aeruginosa*

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(Received 13 September 1994; revision received 29 November 1994; accepted 30 November 1994)

Abstract: Burkholderia cepacia (*Pseudomonas cepacia*) is now recognised as an important pathogen in cystic fibrosis patients, and several reports have suggested that sputum-culture-proven colonisation occurs despite the presence of specific antibody. In an attempt to establish the use of antibody studies as diagnostic and prognostic indicators of *B. cepacia* infection, we have examined the IgG response to *B. cepacia* outer membrane proteins and lipopolysaccharide in patients also colonised with *P. aeruginosa*. The *B. cepacia* strains were grown in a modified iron-depleted chemically defined medium and outer membrane components examined by SDS-PAGE and immunoblotting. IgG antibodies were detected against *B. cepacia* outer membrane antigens, which were not diminished by extensive preadsorption with *P. aeruginosa*. The response to *B. cepacia* O-antigen could be readily removed by adsorption of serum either with *B. cepacia* whole cells or purified LPS, whereas we were unable to adsorb anti-outer membrane protein antibodies using *B. cepacia* whole cells. The inability to adsorb anti-outer membrane protein antibodies using *B. cepacia* whole cells maybe due to non-exposed surface epitopes. Several *B. cepacia* sputum-culture negative patients colonised with *P. aeruginosa* had antibodies directed against *B. cepacia* outer membrane protein. This study suggests that there is a specific anti-*B. cepacia* LPS IgG response, which is not due to antibodies cross-reactive with *P. aeruginosa*. Our studies indicate that much of the *B. cepacia* anti-outer membrane protein response is specific and not attributable to reactivity against co-migrating LPS.

Key words: Outer membrane antigen; Lipopolysaccharide antigen; Smooth lipopolysaccharide; Rough lipopolysaccharide; Immunoblotting

Introduction

Progressive lung disease resulting from chronic colonisation with *Pseudomonas aeruginosa* continues to be a major cause of mortality and morbidity in patients with cystic fibrosis (CF). However, *Burkholderia cepacia* (*Pseudomonas cepacia*) is now recognised as an important pathogen in patients already colonised with *P. aeruginosa* [1–3]. *Burkholderia cepacia*, a plant pathogen ubiquitous in soil and water, rarely causes infection in healthy subjects. Exceptions include immunocompromised hosts and nosocomial acquisi-

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SSDI 0928-8244(94)00093-X

tion from contaminated equipment. Environmental strains are usually susceptible to multiple antibiotics whereas strains found in patients with CF are frequently multiresistant [4]. Some CF patients remain stable after acquisition of *B. cepacia*, but many show a progressive deterioration. A small group of CF patients have a systemic infection, with recurrent fever. In some patients deterioration is rapid, leading to death within weeks of *B. cepacia* infection [5,6].

It is well documented that CF patients not previously exposed to *P. aeruginosa* lack a detectable immune response to the organism; however, on subsequent infection a rapid response is mounted against surface components [7–9]. The characteristic chronic persistence of *P. aeruginosa* in CF lungs is attributed to the failure of this immune response to opsonise the bacteria effectively. Moreover, attempts at opsonophagocytosis are frustrated by conversion from the lipopolysaccharide (LPS) smooth state to the LPS-rough state and the production of mucoid alginate by the bacteria. Indeed, Pier et al. [10] have demonstrated the presence of opsonophagocytic killing antibody to *P. aeruginosa* mucoid alginate in older non-colonised patients, suggesting that the specific lack of this antibody may result in the chronic colonisation observed in other CF patients.

In contrast to *P. aeruginosa* infection in CF, *B. cepacia* infection occurs frequently in patients already colonised with *P. aeruginosa* and in whom high levels of anti-*P. aeruginosa* serum antibodies exist. Studies by Aronoff and Stern [11] have shown that anti-*B. cepacia* antibodies can be detected in the serum of *P. aeruginosa*-infected patients not culture proven to be colonised with *B. cepacia*. Longitudinal studies indicate that antibodies against 27 and 36 kDa *B. cepacia* outer membrane proteins (OMPs) can be detected up to four years prior to colonisation [12]. Another longitudinal study demonstrated that *B. cepacia* LPS core antigen antibodies are higher in patients colonized with *B. cepacia* and the increase in antibody titre sometimes precedes sputum culture by several months [13]. Therefore, at least in these patients, this raises the question of whether *B. cepacia* colonisation occurred despite the presence of specific anti-OMP and anti-core LPS antibodies.

In order to assess the degree of cross-reactivity between *B. cepacia* and *P. aeruginosa*, we have studied the immune response to *B. cepacia* and *P. aeruginosa* outer membrane components in CF patients colonised with both organisms.

Patients and Methods

Patients

Nine patients from Birmingham Heartlands Hospital and five patients from Nottingham City General Hospital colonised with both *B. cepacia* and *P. aeruginosa* were studied. The five patients from Nottingham City General Hospital were compared with five age and sex matched patients colonised with *P. aeruginosa* alone.

Bacterial strains and growth conditions

Nine clinical isolates of *B. cepacia* and *P. aeruginosa* were collected from adult CF patients at The Heartlands Hospital, Birmingham, UK. Of the nine patients, six were studied in greater detail and three of these were colonized with the ribotype strain thought to be common to many centres in the UK. Strains were stored in nutrient broth supplemented with 15% glycerol at −70°C and cultured in a modified iron-depleted chemically defined medium (CDM-Fe) consisting of: 40 mM glucose; 0.62 mM KCl; 40 mM $(NH_4)_2SO_4$; 0.4 mM $MgSO_4$; 50 mM 3-(N-morpholino) propane sulphonic acid (pH 7.4) supplemented with 0.1% casamino acids (Difco). Bacteria were grown to early stationary phase (E_{470} 0.9) in an orbital shaking incubator at 37°C, harvested by centrifugation at $10\,000 \times g$ and washed once with saline. For LPS isolation, strains were grown in tryptone soy broth (TSB, Oxoid) to an E_{470} of 5.

Preparation of outer membranes

Outer membranes (OMs) were prepared by the method of Filip et al. [14]. The washed bacterial pellet was suspended in 20 ml distilled water and broken by passage through a french pressure cell (Aminco). Unbroken cells were removed by centrifugation at $5000 \times g$ for 5 min. Sarkosyl

(sodium N-lauroyl sarcosinate, Sigma) was added to the supernate to 2% w/v. After 1 h at room temperature the mixture was centrifuged at $40\,000 \times g$ for 40 min. The OM pellets were washed in distilled water and stored at −20°C.

Preparation of purified lipopolysaccaride from B. cepacia

LPS was prepared by the hot phenol extraction method [15]. Cells were harvested by centrifugation, suspended in 300 mM Tris · Cl (pH 8.0), broken by sonication and incubated with DNase (0.125 mg ml^{-1}, EC number 3.1.21.1) and RNase (0.125 mg ml^{-1}, EC number 3.1.27.5) at 37°C for 1 h. Proteinase K (Sigma, EC number 3.4.21.14) was added to 0.125 mg ml^{-1} and incubated at 37°C overnight. The mixture was heated to 80°C and extracted with an equal volume of phenol (80% w/v). After separation of the two phases by centrifugation, the aqueous phase was retained. The phenol phase was extracted with an equal volume of water. EDTA was added to 1 mM to the pooled aqueous phase, which was dialysed overnight against water. Magnesium sulphate was added to the dialysate to 10 mM. The LPS was recovered by centrifugation at $100\,000 \times g$ for 4 h. The pellet was freeze-dried and stored at −20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

OM preparations were electrophoresed according to the method of Lugtenberg et al. [16] on 12% w/v acrylamide gels. Each lane was loaded with approximately 2.5 μg protein. For visualisation of LPS, OMs were heated to 100°C in denaturing buffer containing SDS 2.5% w/v and 2-mercaptoethanol 2% w/v, cooled and then incubated with an equal volume of proteinase K (1 mg ml^{-1} in denaturing buffer) for 60 min at 60°C before electrophoresis. Separated OMPs were stained with Coomassie blue R-250 0.1% w/v in methanol:acetic acid:water 50:10:40. LPS gels were silver stained according to the method of Tsai and Frasch [17].

Immunoblotting

Proteins or LPS separated by SDS-PAGE were electroblotted onto nitrocellulose (NC) paper and the antigens were visualised by a modification of the method of Towbin et al. [18]. After transfer, the NC paper was incubated at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBS-Tween) for 1 h to saturate non-specific binding sites and then incubated overnight at 4°C with serum diluted 1:50 in TBS-Tween. The NC paper was washed thoroughly with TBS and incubated for 2 h at 37°C with protein A-peroxidase (Sigma) 0.5 μg ml^{-1} in TBS-Tween. After incubation the NC paper was thoroughly washed again. The antibody binding sites were visualised by reaction with 3.5 mM H_2O_2 and 0.5 mM 4-chloronaphthol in 10 mM Tris.Cl (pH 7.4). Replicate blots were stained with amido black 1% w/v in methanol 10% v/v and acetic acid 7% v/v to show qualitative transfer of proteins.

Collection and preadsorption of sera

Blood was taken by venepuncture and allowed to clot at 37°C for 2 h. After centrifugation at $2000 \times g$ for 10 min, the serum was collected and stored at −20°C. Serum was preadsorbed with whole cells of *B. cepacia* and *P. aeruginosa* [19]. Bacteria were grown in CDM-Fe as described above and concentrated 10-fold by centrifugation. The cells from 1 ml of this suspension were collected by centrifugation and suspended in 0.5 ml of serum. The mixture was incubated at 37°C for 15 min, after which cells and immune complexes were separated by centrifugation. This process was repeated six times. Preadsorption with LPS was performed in a similar manner using 0.1 ml of LPS (10 mg ml^{-1} in water).

Results

Six independently isolated strains of *B. cepacia* were studied in detail. Five of these isolates were identified as the ribotype strain thought to be common to many CF centres in the UK (Table 1). The strains were grown in an iron-depleted chemically defined medium which had been developed for *P. aeruginosa*. Previous studies have shown that the outer membrane protein [20] and antigenic profiles [21] of strains grown in this medium are similar to those from cells recovered directly

from the CF lung. However, the clinical isolates of *B. cepacia* showed poor growth in this medium, but this could be improved by addition of casamino acids. When grown in this medium *B. cepacia* expressed six major OMPs (Fig. 1, lanes 2 and 3). *P. aeruginosa* expressed the characteristic outer membrane profile after growth in this medium [20] including the major porin, protein F at 39 kDa, proteins H/L at 21 and 20.5 kDa and two high molecular weight iron-regulated proteins at 80.5 and 72.5 kDa (Fig. 1 lane 4).

Fig. 2 shows a silver-stained SDS-PAGE gel of outer membranes from the four *B. cepacia* isolates after treatment with proteinase K. All strains possessed R-type LPS, but only two strains expressed smooth-type LPS (Fig. 2, lane 1 and 2), indicated by O-polysaccharide bands of increasing molecular weight.

Immunoblotting

The Birmingham Heartlands Hospital patients studied had a mean age of 23.3 years (range 10.2 to 29.5 years) and had been colonized with *B. cepacia* for a mean time of 13 months (range 4 to 28 months) and *P. aeruginosa* for a mean time of 36.5 months (range 18 to 89 months). Sera from the six patients selected for study in greater detail had IgG antibodies reactive against both *B. cepacia* and *P. aeruginosa* OMPs and representative immunoblots using the isolate and sera from patient 1 are shown in Figs. 3 and 4, respectively.

Table 1

Ribotype, CHEF (pulsed field gelelectrophoresis with the clamped homogenous electric field technique) type and LPS type from seven *B. cepacia* strains isolated from cystic fibrosis patients

Strain	Ribotype	CHEF type	LPS type
1	A	I	smooth
2	A	I	rough
3	D	IV	smooth
4	A	III	nd
5	A	III	rough
6	D	V	nd
7	nd	nd	rough
8	A	III	rough
9	A	nd	rough

nd: test not done.

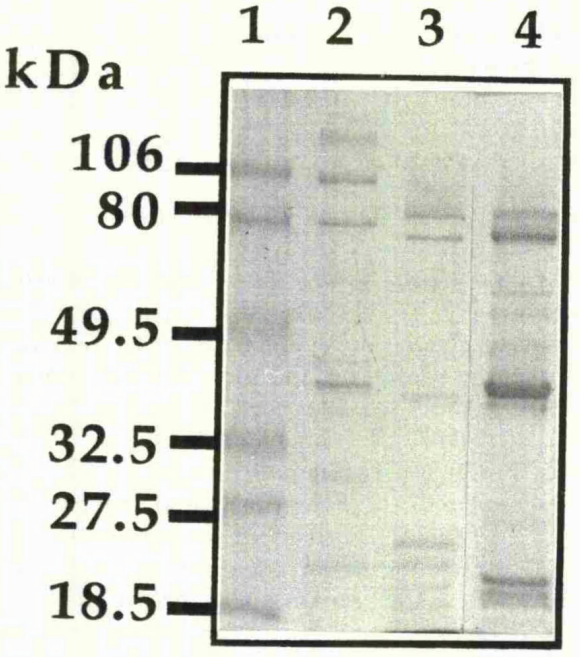

Fig. 1. SDS-PAGE analysis of outer membrane proteins of *B. cepacia* NCTC 10661 (lane 2) and representative clinical isolates of *B. cepacia* (lane 3) and *P. aeruginosa* (lane 4) after growth in an iron-depleted media. Molecular weight markers (lane 1).

Fig. 5 (lane 1) shows an LPS immunoblot of *B. cepacia* from patient 1 probed with serum from the same patient demonstrating a strong immune response to *B. cepacia* LPS, particularly against the O-chains of repeating polysaccharide units.

The IgG response to the *B. cepacia* OM from the five patients from Nottingham City General Hospital colonised with both *B. cepacia* and *P. aeruginosa* (mean age 24.5 years, range 22.3–29.6 years, Fig. 6, lane 1–5) was similar to the immune response of patients from Birmingham Heartlands Hospital. This response was compared with the immune response of five age- and sex-matched patients colonised with *P. aeruginosa* alone (mean age 22.7 years, range 19.6–25.9 years, Fig. 6, lane 6–10). Two of the non-*B. cepacia* colonised patient's serum did not react with the *B. cepacia* OM (Fig. 6, lane 6 and 10, approximately 10% reaction only), whereas three non-*B. cepacia* colonised patient's serum showed a response comparable with the response of a *B. cepacia* colonised patient, although the bands were less

distinct (Fig. 6, lanes 7–9). There was no response to *B. cepacia* OM by sera from both a non-colonised CF patient (not shown) and a non-CF patient (Fig. 6, lane 11).

Attempts were made to determine if the immune response against *B. cepacia* OM antigens was specific to that organism or attributable to cross-reactivity of anti-*P. aeruginosa* antibodies. Patient sera were adsorbed with *B. cepacia* or *P. aeruginosa* whole cells. In addition, sera were also adsorbed with *B. cepacia* LPS, to discriminate between a genuine anti-OMP response and one due to co-migrating LPS. Preliminary experiments indicated that six cycles of preadsorption were sufficient for optimum immunoadsorption. Serum preadsorbed with *P. aeruginosa* diminished the immune response to *P. aeruginosa* OMP by approximately 60–70% with some antigens still staining as strongly (Fig. 4, lane 4), whilst not affecting the immune response to *B. cepacia* OMPs (Fig. 3, lane 3). Serum preadsorbed with *B. cepacia* had no effect on the response to *P. aeruginosa* (Fig. 4, lane 3). The immune response to the major *B. cepacia* OMPs was not diminished significantly (Fig. 3, lane 4), but there was a reduction in the minor bands which we attributed to LPS O-antigen. This was confirmed by preadsorbing serum with purified LPS from *B. cepacia* (Fig. 3, lane 5).

The contribution of the anti-LPS response to the total serum immune response was confirmed by probing *B. cepacia* LPS blots with serum preadsorbed with *P. aeruginosa* and *B. cepacia*

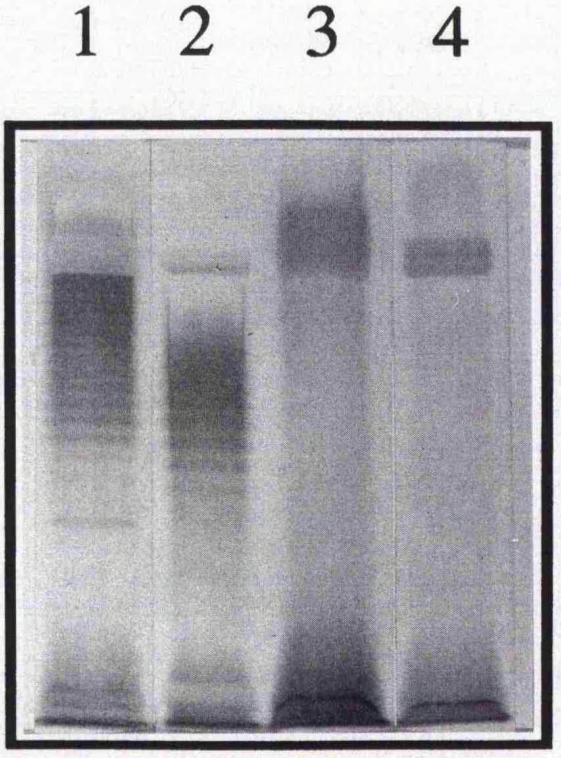

Fig. 2. Silver-stained SDS-PAGE showing LPS profile from four clinical *B. cepacia* isolates. Isolate from patient 1 (Lane 1), patient 3 (Lane 2), patient 2 (Lane 3), and patient 7 (Lane 4).

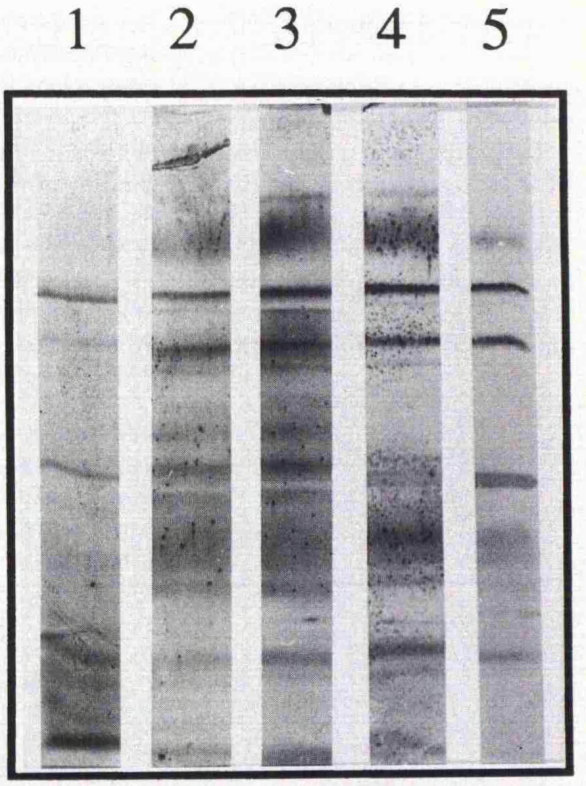

Fig. 3. Immunoblot of *B. cepacia* outer membranes from patient 1. Lane 1, OMP's visualised with amido-black; Lane 2, serum from patient 1; Lanes 3 and 4, serum from patient 1 preadsorbed with *P. aeruginosa* and *B. cepacia* whole cells, respectively; Lane 5, serum from patient 1 preadsorbed with *B. cepacia* LPS.

whole cells. Preadsorbing with *P. aeruginosa* (Fig. 5, lane 3) had no affect on the immune response, whereas it was abolished when *B. cepacia* whole cells were used (Fig. 5, lane 2).

Serum from a patient colonised with a R-type LPS *B. cepacia* strain (patient 5) showed no O-antigen response to LPS from the colonising strain, but did demonstrate a strong response to *B. cepacia* smooth LPS from patient 1, who was colonised with smooth type LPS. This was not diminished by preadsorption with his own colonising strains of *B. cepacia* and *P. aeruginosa*. Serum IgG responses to R-type LPS and smooth type LPS are summarised in Table 2.

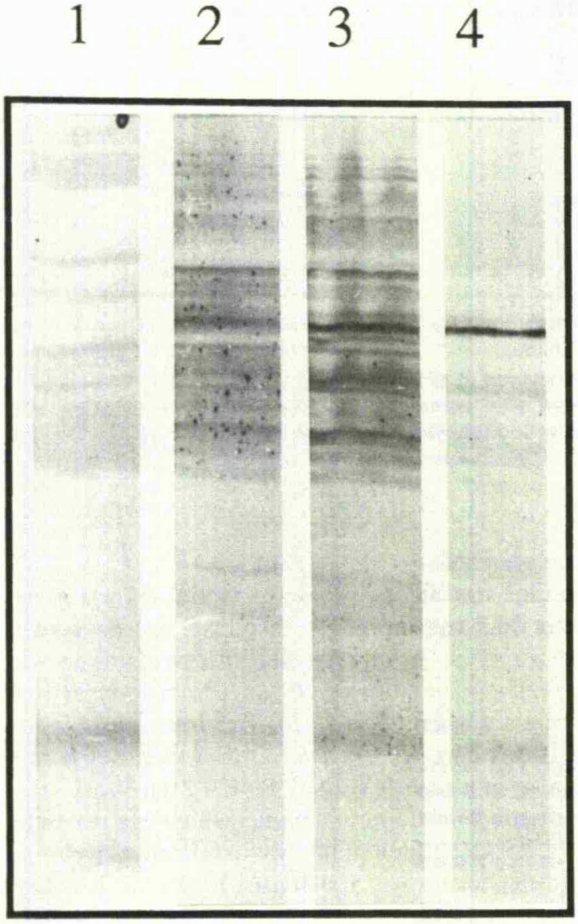

Fig. 4. Immunoblot of *P. aeruginosa* outer membranes from patient 1. Lane 1, OMP's visualised with amido-black; Lane 2, serum from patient 1; Lanes 3 and 4, serum from patient 1 preadsorbed with *B. cepacia* and *P. aeruginosa* whole cells, respectively.

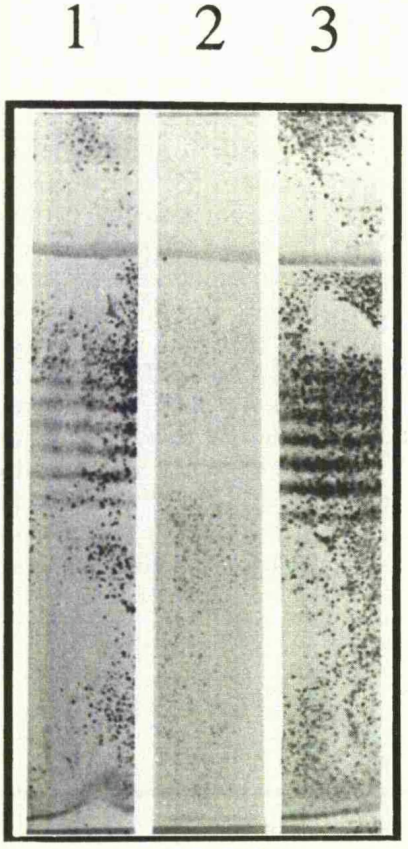

Fig. 5. Immunoblot of *B. cepacia* LPS from patient 1. Lane 1, serum from patient 1; Lanes 2 and 3, serum from patient 1 preadsorbed with *B. cepacia* and *P. aeruginosa* whole cells, respectively.

Table 2

Serum IgG response to two *B. cepacia* strains with smooth type LPS by five patients with different LPS types colonised with *B. cepacia*

Patient's serum	LPS type	Strain 1 (smooth LPS)	Strain 3 (smooth LPS)
1	smooth	positive	positive
2	rough	positive	negative
3	smooth	positive	positive
5	rough	positive	negative
7	rough	positive	positive

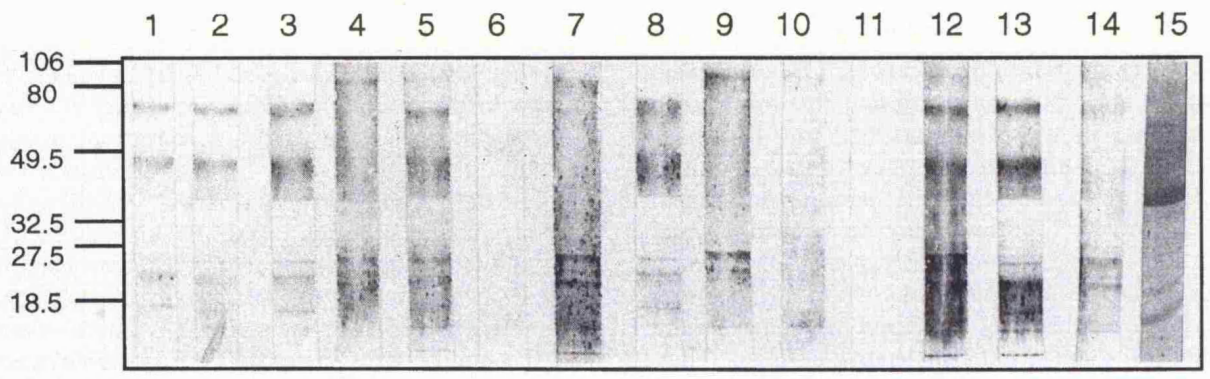

Fig. 6. Immunoblot of *B. cepacia* outer membranes from patient 9. Lanes 1–5, patients from Nottingham City General Hospital colonised with *B. cepacia* and *P. aeruginosa*; Lanes 6–10, patients from Nottingham City General colonised with *P. aeruginosa* only; Lane 11, non-CF patient; Lane 12, serum from patient 9; Lanes 13 and 14, serum from patient 9 preadsorbed with *P. aeruginosa* and *B. cepacia* respectively; Lane 15, outer membrane proteins stained with amido-black.

Discussion

Immunoglobulin G antibodies to outer membrane antigens of *Burkholderia cepacia* were demonstrated in patients colonised with *B. cepacia* and *P. aeruginosa*. Extensive pre-adsorption with *P. aeruginosa* did not diminish the response to *B. cepacia*, suggesting that the response was not due to cross-reactive antigens against *P. aeruginosa*. In contrast, the response to *B. cepacia* O-antigen LPS could be readily removed by adsorption of serum either with *B. cepacia* whole cells or purified LPS. Using ELISA studies, Nelson et al. [13] detected IgG antibodies directed against *B. cepacia* whole cell antigens in serum from both *B. cepacia*-colonised and non-colonised CF patients. Focusing on the response to *B. cepacia* core LPS, these workers found that a significant component of the response was specific and could not be reduced by adsorption with *P. aeruginosa* whole cells.

In the present study, three patients colonised with a *B. cepacia* R-type LPS strain had serum IgG antibodies against smooth type LPS, although in two of the three patients the response was against only one of two smooth strains tested (Table 2). Adsorption of serum with the patient's colonising rough *B. cepacia* strain did not diminish the response. Although preliminary, this may suggest that *B. cepacia* undergoes a transition from smooth to R-type LPS in the CF lung. In addition transient growth of smooth strains may have occurred without being observed by culture; examination of a strain's colonial appearance may not distinguish smooth and rough types.

The clinical importance of LPS type amongst colonising strains is unclear, although many strains isolated from CF patients produce rough LPS [22], including the epidemic strain CF5610, which now accounts for a significant proportion of *B. cepacia* colonisation in several centres in the UK [23]. *B. cepacia* infection is sometimes accompanied by fever and bacteraemia [24]. This is in contrast to *P. aeruginosa* bacteraemia which is rare in patients with CF, possibly due to the predominance of *P. aeruginosa* R-type LPS in the CF patient, which is more sensitive to the lytic action of serum [25]. Patients with a *B. cepacia* smooth type LPS strain maybe more susceptible to *B. cepacia* bacteraemia. One of the two patients with smooth type LPS developed a fatal *B. cepacia* septicaemia in the postoperative period after a heart lung transplant; however, fever and bacteraemia is not limited to patients with the smooth type LPS alone.

Serum adsorption experiments with *P. aeruginosa* whole cells did not affect the response directed against *B. cepacia* outer membrane proteins but reduced considerably the response to *P. aeruginosa*, with about 30–40% remaining. Again, these data may suggest that the immune response to *B. cepacia* is not attributable to cross-reactivity

with *P. aeruginosa*. In this study we have looked at the IgG response to *B. cepacia* OM antigens of five patients sputum-culture-negative for *B. cepacia*; three of these patients had anti-*B. cepacia* antibodies. Other workers have detected anti-*B. cepacia* antibodies in some patients up to two years prior to sputum-culture-proven colonisation [11,12]. Although some *B. cepacia* OM components may be cross-antigenic with *P. aeruginosa*, our immunoblotting experiments suggest that there are some specific *B. cepacia* antigens. These experiments are qualitative rather than quantitative, and currently we are developing an ELISA test based on a purified 80 kDa OMP component which we hope will distinguish *B. cepacia* colonised patients from those patients already colonised with *P. aeruginosa*.

It is not clear why we were unable to adsorb anti-*B. cepacia* OMP antibodies from serum using whole cells. However, the complete adsorption of anti-LPS antibodies indicates that the response is directed against protein epitopes and not co-migrating LPS [24]. Our failure to adsorb anti-OMP antibodies using either smooth- or rough-type LPS *B. cepacia* whole cells suggests that it is not due to O-antigen LPS preventing interaction between antibodies and proteins at the cell surface, previously postulated as a possible mechanism of bacterial defence in the presence of anti-*B. cepacia* antibodies [11,27]. However, it cannot be ruled out that the epitopes are not surface exposed in intact cells, and this would also account for the observation that there was remaining antibody response to *P. aeruginosa* after adsorbtion with *P. aeruginosa* whole cells. If this is the case then it could be that these hidden epitopes could give cross-reactive immune responses.

Despite previously being members of the same genus, *P. aeruginosa* and *B. cepacia* are phyllogenetically very different. *P. aeruginosa* belongs to the *Pseudomonas* rRNA group 1 and *B. cepacia* belongs to the rRNA group 2. This division has been further supported by studies based on the 16S rRNA gene sequences [28]. Therefore *B. cepacia*, along with 6 other pseudomonads, has been transferred to the new genus *Burkholderia* [29,30]. The marked phyllogenetic difference between the two organisms is in keeping with our findings of the OM antigens having at least partially differing epitopes. Even within the *B. cepacia* species there is marked variation in the OMP's, as demonstrated by the difference between the NCTC *B. cepacia* strain and the clinical strain (Fig. 1, lanes 1 and 2). This may reflect the diverse phenotypes of *B. cepacia*, evident even if the same strain is grown from the same patient, and from the same site [31]. The diversity of the phenotype appears to be differential gene expression, although there may also be marked heterogenity of the genotype within the species.

As first noted by Aronoff [12], *B. cepacia* colonisation of the CF lung occurs despite the presence of anti-*B. cepacia* antibody. This study supports the specificity of some of these antibodies in patients already colonised with *P. aeruginosa*. Immunoblotting of a patients serum alone and without consideration of other immune responses may give a false impression of the patient's ability to control *B. cepacia* infection. These antibodies may represent previous exposure to *B. cepacia* without colonisation, and may have a protective role in preventing infection. However, at present it is still highly speculative to assign a function to these antibodies, but if interpreted cautiously, they may have diagnostic and prognostic value.

Acknowledgements

D.E.L. is funded by the Cystic Fibrosis Trust. We thank Dr. T. Pitt for ribotyping of *B. cepacia* isolates and thank Dr. D Peckham for supplying serum samples from Nottingham City General Hospital.

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