

CYSTIC FIBROSIS

A STUDY OF PSEUDOMONAS AERUGINOSA FROM ADULT ~~CF~~ PATIENTS

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Abstract

Infection of the respiratory tract of cystic fibrosis (CF) patients with Pseudomonas aeruginosa presents a major problem for the clinical management of these patients. A previous study had shown that P.aeruginosa in CF apparently altered their surface antigens during infection and this change was clinically significant. As a result I carried out a) an immunological study of the surface antigens of P.aeruginosa, b) a longitudinal bacteriological survey of infection in CF patients and, c) an investigation of the immune response of patients to the varieties of P.aeruginosa recovered from their sputum.

Serologically typical and aberrant strains were detected in specimens and O-antigenic specificity was determined by lipopolysaccharide (LPS). Laboratory constructed serological variants and 80% of CF isolates were deficient in LPS components. These strains were often sensitive to normal human serum and hypersensitive to B-lactam antibiotics.

Eight hundred and forty four (80%) sputum samples yielded P.aeruginosa, 30%, Staphylococcus aureus and 67% Haemophilus influenzae. Staphylococci were significantly more often isolated from patients who remained free of P.aeruginosa and acquisition of pseudomonas was associated with a decline in clinical status. The incidence of mucoid P.aeruginosa did not correlate with length of colonisation. Patients often harboured more than one phenotype of P.aeruginosa but the most severely ill patients were colonised continuously with LPS-defective strains.

O-defective forms of P.aeruginosa were as antigenic as typical strains in experimental animals and antibody to both varieties of strains were found in patients sera. The magnitude of response was influenced by the length of colonisation but antibody to mucoid exopolysaccharide was rare. Local IgA antibody and other immunoglobulins towards pseudomonas were detected in the sol phase of sputum samples.

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CONTENTS

	<u>Page</u>
INTRODUCTION	12
Cystic Fibrosis	13
The Disease	13
History	13
Incidence	14
Genetics	15
Pathology	16
Diagnosis and Management	19
Pulmonary infection	21
The bacteriology of cystic fibrosis	21
Table 1	23
Figure 1	25
Mycoplasma and related organisms	29
<u>Legionella pneumophila</u>	29
Mycobacteria	30
Viral infection	30
<u>Pseudomonas aeruginosa</u>	34
Taxonomy	34
Characteristics of <u>P.aeruginosa</u>	35
Epidemiology	35
Morphology	36
Isolation	36
Identification	36
Table 2	37
Biochemical characteristics	38
Epidemiological typing of <u>P.aeruginosa</u>	39
Bacteriocins	39
Pyocin typing	40
Bacteriophages	41
Phage typing	42
Outer membrane antigens of <u>P.aeruginosa</u>	42
Figure 2	43
O-serological typing	46
Polyagglutination	47
Protein antigens	48
Fimbriae	49
Flagellar antigens	49
Outer membrane proteins	50
Common antigens	51

Resistance of <u>P.aeruginosa</u>	51
Antibiotic resistance	52
Resistance to normal human serum	55
The pathogenicity of <u>P.aeruginosa</u>	57
<u>P.aeruginosa</u> in cystic fibrosis	59
Mucoid exopolysaccharide	60
Table 3	61
Adherence properties	64
Antibiotic resistance	65
Lipopolysaccharide (LPS)	65
Virulence factors of <u>P.aeruginosa</u>	67
Proteases	67
Table 4	68
Exotoxin A	69
Exoenzyme S	70
Inhibition of leucocyte function	70
Phospholipase C	70
Pyocyanin	71
Aim of the investigation	72
MATERIALS AND METHODS	74
Strains and bacteriophages of <u>P.aeruginosa</u>	75
O-serotype strains	75
Experimental strains	75
Clinical strains	75
Bacteriophages	75
Media, Diluents and buffers	76
Media	76
Diluents	77
Buffers	78
Electrophoresis Buffers	78
Buffers for ELISA	78
Buffers for 'Western Blotting'	79
Stains	80
Characterisation of surface macromolecules	80
Acetone dried cells	80
Phenol-water extraction of LPS	81
Ethanol extraction of LPS	82
Trichloroacetic acid extraction of LPS	83
Preparation of outer membrane protein	83
Preparation of purified MEP	84
Chemical analysis of LPS	85
Total carbohydrate	85
Protein content	85
Determination of KDO	85

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)	86
Molecular weight standards	87
LPS standard	87
Proteinase K extraction of LPS	88
Extracts of whole cells for SDS-PAGE	88
Staining of lipopolysaccharide in SDS-PAGE	88
Staining for protein	89
Seroreactions of surface macromolecules	89
Preparation of O antisera	89
Titration of serum	90
Preparation of hyperimmune serum to MEP	90
Double diffusion in gel	91
Indirect haemagglutination test	91
ELISA	92
Competitive ELISA	93
Whole cell preparations for ELISA	93
LPS preparations for ELISA	93
Western Blotting	93
Staining of nitrocellulose sheets	94
Immunoblotting	94
'Dot-blot'	94
Longitudinal clinical and bacteriological survey	95
Patients	95
Collection of sputum	96
Serum samples	96
Bacteriological examination of sputum	96
Preparation of sol phase	97
Bacterial identification	98
Classification of <u>P.aeruginosa</u>	99
Colonial morphology	99
Serum sensitivity	99
Serotyping of <u>P.aeruginosa</u>	100
Phage typing	101
Determination of antibiotic sensitivity	101
Disc sensitivity test	101
Determination of minimum inhibitory concentration	102
RESULTS SECTION 1	103
Characterisation of antigenic variants of <u>P.aeruginosa</u>	104
Selection of O-antigenic variants	104
Polyagglutinating variant (PA)	
Non-typable variant (NT)	104
Table 5	106
Figure 3	107
Table 6	108
Table 7	109

Biochemical characterisation	110
Antibiotic sensitivity	110
Serological characterisation of experimental strains	110
Table 8	111
Table 9	113
Immunochemical characterisation of serological reactions	114
Extraction of LPS	114
Chemical characterisation of LPS	114
Table 10	115
Analytical chemistry of LPS	116
Figure 4	117
Table 11	119
Table 12	120
Table 13	121
Electrophoretic characterisation of LPS	122
LPS profiles of serotype strains	123
Figure 5	124
LPS of parent and variant strains	125
SDS-PAGE of outer membrane profiles	125
Figure 6	126
Figure 7	127
Precipitation in agar gel	128
Indirect haemagglutination	128
Figure 8	129
ELISA	130
Table 14	131
Table 15	132
Competitive inhibition ELISA	133
Table 16	134
Table 17	136
Table 18	137
Immunoblotting	138
Figure 9	139
Figure 10	141
Detection of isolates with variant antigens from CF Sputum	142
Serum resistance	143

Table 19	144
Frequency of O-serotypes among isolates	145
Table 20	146
Lipopolysaccharide of CF variant strains	147
Table 21	148
Analysis of CF isolates by SDS-PAGE	149
Figure 11	151
Figure 11 contd.	152
Immunoblotting of defective LPS	154
Figure 12	155
DISCUSSION SECTION I	158
RESULTS SECTION II	172
Longitudinal bacteriological survey	173
Patients	173
Table 22	174
Table 23	175
Table 24	176
Bacteriology of sputum samples	177
Table 25	178
Table 26	180
Table 27	181
Association of clinical data with the isolation of <u>P.aeruginosa</u>	182
Table 28	183
Table 29	185
Table 30	186
Table 31	187
The presence of mucoid <u>P.aeruginosa</u>	188
Table 32	189
Table 33	190
Table 34	191
Classification of <u>P.aeruginosa</u>	192
Figure 13	193
Table 35	194
Heterogeneity of <u>P.aeruginosa</u> from CF sputum	195

Colonial form	195
Table 36	196
Pigment production	197
Table 37	198
Figure 14	199
O-serology	200
Serum sensitivity	200
Hypersensitivity to antibiotics	200
Table 38	201
Table 39	202
Table 40	203
Table 41	205
Table 42	206
Association of pigment production in colony form and O-serotype reaction	207
Association of serum resistance and O-serotype reactions	207
Table 43	208
Table 44	209
Table 45	210
Association of phage type and O-serotype	211
Table 46	212
Association of phenotypes of <u>P.aeruginosa</u> with clinical condition of CF patients	213
Figure 15	214
Table 47	215
Figure 16	216
Table 48	219
Table 49	220
Table 50	221
Figure 17	223
Figure 18	224
Association of atypical phenotypes of <u>P.aeruginosa</u> and clinical status in individual patients	225
Table 51	226
Table 52	227
Table 53	229
Table 54	230
Table 55	231
Table 56	232
Table 57	234
Table 58	236
DISCUSSION II	237

RESULTS SECTION III	250
Introduction	251
Comparison of immunogenicity	251
Table 59	252
Table 60	253
Table 61	255
Table 62	256
Table 63	257
Measurement of patients' antibody	258
Table 64	259
Antibody response of patients	260
Table 65	261
Figure 19	263
Table 66	265
Figure 20	267
Table 67	269
Figure 21	270
Detection of local antibody production	271
Table 68	272
Comparison of antibody activity in sputum and serum	273
Table 69	274
Detection of antibodies to MEP in CF serum	275
Table 70	276
Production of hyperimmune serum to MEP	277
DISCUSSION III	278
GENERAL DISCUSSION	291
REFERENCES	296

INTRODUCTION

Cystic fibrosis

The Disease

Cystic fibrosis (CF) is the most common genetic disorder amongst Caucasians and constitutes a major health problem in Europe and North America. It is characterised by an abnormality of the exocrine glands, the clinical effects of which are manifest in the sweat glands, gastro-intestinal tract and the lungs. Although once considered a lethal disease in children, advances in diagnosis and treatment have significantly increased the life expectancy of patients and as a consequence the disease often presents as a chronic condition which persists from childhood into adult life.

History

Although cases of congenital pancreatic disease and bronchiectasis had been recorded earlier (Fanconi, Uehlinger and Knauer 1936) the first comprehensive clinical and pathological description of CF was made by Andersen in 1938. She proposed the term 'cystic fibrosis of the pancreas' on the basis of autopsy findings and the clinical effects of pancreatic deficiency. At that time changes in the lungs were considered to be secondary and possibly attributable to a concurrent vitamin A deficiency. At about the same time several articles appeared (Harper 1938; Blackfan and May 1938) which described congenital steatorrhoea due to a pancreatic defect which, coupled with pulmonary disease seemed to confirm the description of Andersen (1938).

The apparent involvement of all mucous secreting glands in the pathogenesis of CF prompted Farber (1945) to propose the term

'mucoviscidosis'. He suggested that 'cystic fibrosis' did not adequately define the major pathological features seen post mortem in patients with the disease.

Di Sant'Agnese and colleagues (1953) demonstrated an abnormal sweat electrolyte composition in CF which was not secondary to pancreatic deficiency, chronic pulmonary disease or renal or adrenal dysfunction. They concluded therefore that the basic defect in CF was of the sweat glands and referred to the syndrome as 'fibrocystic' disease.

Today CF is recognised as a generalised disorder of the exocrine glands but is generally referred to as 'cystic fibrosis of the pancreas' (Rosenlund 1972).

Incidence

The incidence of CF varies considerably both geographically and among different ethnic groups. There is a high frequency of the disease in Caucasian populations, ranging from 1 in 1500 to 1 in 15000 live births but frequencies of 1 in 646 and 1 in 377 have been described in Namibia, S.W. Africa and Brittany, France (WHO report 1985). The generally accepted incidence among Caucasians in Europe and North America is 1 in 1600 to 1 in 2000 live births.

The CF gene is rare among other ethnic groups but in North American Black populations it is estimated to occur in about 1 in 17000. CF is virtually unknown in Mongoloid races but recent reports suggest that it is increasing in the Japanese (WHO report 1985). Polynesians appear to be free of the disease and Ashkenazi Jews show a similar incidence to Europeans, as do the Palestinian Arab population of Israel. It is therefore probable that other Arab

peoples of the Eastern Mediterranean countries have a similarly high frequency of the gene.

It may be that as world-wide health care improves then the incidence of CF in the developing countries will increase. The high infant mortality prevalent in these countries from common respiratory infections and gastrointestinal disorders may mask the clinical picture and recent reports from Pakistan seem to confirm this trend (WHO report 1985).

Genetics

Many studies of the genetics of CF have been carried out in the last twenty years. The basic defect in CF is still unknown but is thought to involve a single gene locus, although there is evidence that more than one gene mutation may cause clinical signs of the disease (Williamson et al. 1984).

CF is inherited as an autosomal recessive gene by simple Mendelian genetics. In Caucasians the carrier frequency is between 1 in 20 to 1 in 25 of the population and both sexes are affected equally, therefore theoretically, 1 in 500 families may produce a child with CF. Where both parents are heterozygotes the incidence of a child being homozygous and thus having CF is 1 in 4, but such families tend to have a greater number of children with CF than would appear probable from simple genetic calculations (Rosenlund 1972).

Heterozygotes are asymptomatic and are usually not detectable due to the lack of reliable and reproducible laboratory tests for the gene product (Brock 1984). The high gene frequency in Caucasians has led to the suggestion that a heterozygote advantage analogous to sickle cell anaemia and malaria may exist but the nature of this advantage

remains obscure (Thompson 1980).

Recent research has identified two markers that are genetically linked to CF. By the production of DNA probes to these markers and consanguinity studies the CF locus has been identified on chromosome 7cen-q22 (Knowlton et al. 1985; Wainwright et al. 1985 and White et al. 1985). The work of these groups indicate that the DNA probe for this gene may provide accurate heterozygote detection and allow the antenatal diagnosis of CF in some cases. Nevertheless, further markers need to be identified before the exact location of the CF gene or genes can be established.

Pathology

The basic pathological lesion in CF is the accumulation of excess secretions which lead to dilation of the affected secretory gland. Pathological changes occur in the lung, upper respiratory tract, liver, gall bladder, pancreas and other mucus producing glands. These changes are secondary to the obstruction of the organ passages by excess mucus secretions and have been well documented (Di Sant'Agnese and Talamo 1967; Farber 1944). A more recent comprehensive review of the cellular pathology is given by Mangos and Boyd (1984).

Chronic pulmonary disease dominates the clinical picture in CF and its severity often determines morbidity and mortality (Hodson 1984). The lungs in CF are structurally normal at birth but during the first year of life 60-70% of patients experience some bronchial symptoms (Mearns 1974). There is hypersecretion of mucus which because of its viscid nature forms plugs in the lumen of the bronchi and smaller air passages although ciliary action appears normal (Hodson 1984).

The initial pulmonary abnormalities are similar to those seen in acute bronchitis or bronchiolitis (Mearns 1974). The patients suffer from recurrent infections and chronic lung inflammation which eventually cause bronchiolar scarring, fibrosis and bronchiectasis. When areas of local scarring and stenosis occur they may lead to distal hyperinflation and atelectasis.

There is metaplasia of goblet cells and the resultant hypersecretion of mucus causes areas of bronchiectasis which become more widespread with age. Bronchiolar obstruction results in air trapping, anoxia, poor perfusion and carbon dioxide retention. In older patients the presence of pneumothorax and haemorrhage may complicate the already severe pulmonary disease (Hodson 1984).

The pathological processes are generally reflected by gross abnormalities in pulmonary function. Many patients experience shortness of breath, especially on exertion and have a persistent cough which produces large amounts of sometimes purulent sputum (Levison and Tabachnik 1983). The degree of lung damage present is assessed by lung function tests, the principles of which are discussed by Levison and Tabachnik (1983). The clinical information provided by peak expiratory flow rate (PEFR) litres/minute, forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC), both measured in litres, are generally sufficient for the routine assessment of the clinical condition of patients (Hodson 1984).

As many as 85% of CF patients suffer from pancreatic insufficiency and maldigestion. The primary defect is thought to be an abnormality in electrolyte and bicarbonate secretion which leads to

precipitation of calcium salts and protein which occludes the ductules of the pancreas. Inflammation is followed by fibrosis with a decrease in the secretion of digestive enzymes. Malabsorption, characterised by steatorrhoea is a direct result of failure to digest fat and protein. If the malabsorption is left untreated gross malnutrition ensues (Hodson 1984).

Blocking of the ileum by meconium (meconium ileus) is present in 10-15% of neonates and it is considered diagnostic of CF (Mearns 1974). It is not clear whether the obstruction is due to the abnormal composition of the meconium alone (Di Sant'Agnese and Talamo 1967) or if the gut undergoes morphological changes before birth (Dodge 1983) which contribute to the failure to pass meconium. Neonates with CF are generally below the national mean for birth weight irrespective of the presence of meconium ileus and this indicates that factors which restrict growth may operate before birth (Dodge 1983).

Meconium ileus equivalent is an uncommon complication of older children and adults and more recently it has become known as distal intestinal obstruction syndrome. The cause is unknown and it does not occur in any other pancreatic deficiency disease; it appears to be related to the abnormal electrolyte content of the CF gut (Zentler-Munro 1983).

Other complications related to the gastro-intestinal tract that may occur in CF include intussusception, rectal prolapse, acute pancreatitis, gallstones and bile duct obstruction, cirrhosis, splenic enlargement, disaccharide intolerance and/or diabetes mellitus.

Diagnosis and Management

The diagnosis of CF is based on the clinical history and physical examination of the patient and a raised level of sodium or chloride in the sweat. The 'sweat test' developed by Gibson and Cooke (1959) is a quantitative pilocarpine iontophoresis technique and for an accurate result 100mg of sweat needs to be collected. In children a level above 60-70 mmol/l is considered as abnormal but in adults a level of 70 mmol/l or more is required. At a specialist centre for adult CF patients the range indicative of CF is 80-120 mmol/l. Pancreatic function tests may be performed if the results of sweat tests are equivocal.

As the number of patients that reach adult life has increased, the management of the disease has altered. Patients generally leave the care of the paediatrician at 16 years old and attend either specialised CF units or chest clinics. In recent years the trend has been to minimise hospitalisation and encourage patients to lead as normal a life as possible and take responsibility for their own disease under the regular supervision of their consultant (Hodson 1984).

The main aims of treatment are firstly to prevent or control pulmonary infection and secondly to ensure optimal nutrition. Precautions to avoid infection through contact with others is recommended in the first two years of life, although this may restrain normal social activity (Batten and Matthew 1984). Although some doctors prescribe prophylactic antistaphylococcal agents in early childhood (Lawson and Porter 1976) staphylococcal infections still occur. The normal immunisation programme for infants is

followed in CF, but there is a particular need for vaccination against pertussis (whooping cough) and measles as lung disease is a common complication of both infections. Annual vaccination against influenza is often recommended for CF patients (Batten and Matthew 1983). The management of pulmonary disease varies between hospitals but physiotherapy and prophylactic or therapeutic antibiotics as described by Dinwiddie (1986) with reference to the Hospital for Sick Children, Great Ormond Street, are the mainstay of treatment programmes. For physiotherapy to be beneficial it must be performed regularly and research has concentrated on the development of methods which will combine good compliance with effectiveness such as the Forced Expiration Technique (FET). As an adjunct to conventional physiotherapy the Positive Expiratory Pressure (PEP) Mask (Webber et al. 1985; Steen et al. 1985) and physical activity (Geddes 1984) have proved effective.

Mucolytic agents and bronchodilators are used in the management of CF but their effectiveness in either improving lung function or preventing infection is not proven (Batten and Matthew 1983). In patients with deteriorating lung function the use of corticosteroids may be indicated if conventional therapy has failed (Hodson 1984). However an American study suggests that prednisone may prevent an increase in lung damage (Auerbach et al. 1985).

Invasive techniques are avoided wherever possible and surgical intervention is indicated only in severe cases of pneumothorax, haemoptysis, lobar bronchiectasis, meconium ileus and intussusception.

Untreated malabsorption results in protein-energy malnutrition and

nutritional status has been shown to correlate with lung function (Szeinberg, McLaughlin and Levison 1985) and the incidence of infection (O'Loughlin et al. 1984). Control of malabsorption is achieved by the use of pancreatic enzyme supplements. The calorie content of the diet should be as high as possible and medium chain triglycerides can be substituted for normal dietary fats as they are better absorbed. Regular vitamin supplementation, especially of fat soluble vitamins, is often prescribed. A thorough review of supplemental nutrition in CF is given by Silk (1984).

Pulmonary Infection

Pulmonary infection has been recognised as a major complication of CF since the earliest reports of the disease. The increased survival and improved quality of life now experienced by many CF patients can be attributed to a major extent to better management of pulmonary infection. However pulmonary disease remains the most common cause of morbidity and mortality in CF (Batten 1983).

The Bacteriology of Cystic Fibrosis

Historically, the bacterium Staphylococcus aureus is most often associated with early respiratory infection in CF (Farber 1944; Di Sant'Agnese and Andersen 1946). It has been suggested that S.aureus initiates the lung damage seen in CF which may then predispose the patient to colonisation by opportunistic pathogens such as Haemophilus influenzae and Pseudomonas aeruginosa (Di Sant'Agnese and Talamo 1967; May, Herrick and Thompson 1972; Rosenlund 1972). This widely held view is compounded by the practice of many clinicians to include the use of prophylactic antistaphylococcal

agents in the treatment of all cases of CF (Lawson 1969). However, more recent studies of CF bacteriology (Table 1) imply that a more complex situation exists. It may be that respiratory infections which develop during early infancy result from pulmonary colonisation by various microorganisms such as Haemophilus, Staphylococci, Pseudomonas and coliform bacilli which become transient pathogens when exposed to aberrant mucous-secreting cells (Doggett and Harrison 1969).

Table 1 demonstrates an increase in the isolation rate of P.aeruginosa from CF in the last 40 years. Data from the studies of Mearns, Hunt and Rushworth (1972) and Corey et al. (1984) suggest that the isolation rate of S.aureus has declined while, since the mid 1960s, P.aeruginosa has increased to become the most frequent pathogen in CF today. There exists a close correlation between the age and clinical condition of the patients and the species of bacteria isolated from their sputum (Mearns et al. 1972; Kulczycki, Murphy and Bellanti 1978; Isles et al. 1984) Although P.aeruginosa infection can occur at any age, the incidence of the species increases as the patient population becomes older (Corey et al. 1984).

P.aeruginosa is also more frequently isolated from patients with acute exacerbations of infection (Højby 1982) and those who are terminally ill (Kulczycki et al. 1978). In as many as 70% of these patients the mucoid form of the organism predominates and the appearance of this colonial form has been shown to be correlated with a poor prognosis (Doggett and Harrison 1969; Kulczycki et al. 1978). Antimicrobial therapy may also influence the isolation rate of

TABLE 1
Prevalence of Bacterial Species Isolated from CF Sputum

Year	Patients Number (age range)	Per cent Prevalence:				Other Species Recorded	Author
		<u>S.aureus</u>	<u>P.aeruginosa</u>	<u>H.influenzae</u>	<u>S.pneumoniae</u>		
1946	*13 (4-40 mths)	84	8	8	15	<u>Streptococcus</u> sp.	DiSant'Agnese & Andersen (1946)
1952	49 (9mths-16yrs)	82	12	4	2	<u>Proteus</u> sp. <u>Candida</u> sp.	Schwachman <u>et al.</u> (1952)
1958	83 (2mths-16yrs)	78	65	5	5	coliforms	Huang <u>et al.</u> 1961
1963	34 (2days-21yrs)	85	65	21	3	coliforms <u>Proteus</u> sp. <u>Streptococcus</u> sp. Coagulase negative staphylococci	Iacocca <u>et al.</u> 1963
1971	102 (1yr-15yrs)	12	28	20 ⁺	ND	ND	Mearns <u>et al.</u> (1972)
1976	134 (1yr-34yrs)	88	78	43	38	<u>E.coli</u> <u>Klebsiella</u> sp. <u>Proteus</u> sp.	Kulczycki <u>et al.</u> (1978)
1981	500 mean 14.8yrs	18	70	15	ND	<u>P.cepacia</u> <u>E.coli</u> <u>Klebsiella</u> sp.	Corey <u>et al.</u> (1984)

* Isolated from lung tissue, post mortem.

+ No organism isolated but precipitins to the organism found in 20% of sera.

ND No data provided.

organisms. Iacocca, Sibinga and Barbero (1963) and Kulczycki et al. (1978) found that the incidence of persistent infection with P.aeruginosa was highest in those patients who had received continuous antibiotic therapy. Moreover studies in Edinburgh (Govan 1983) have shown no significant increase in the incidence of infections due to either S.aureus or P.aeruginosa when prophylactic antibiotics were withheld.

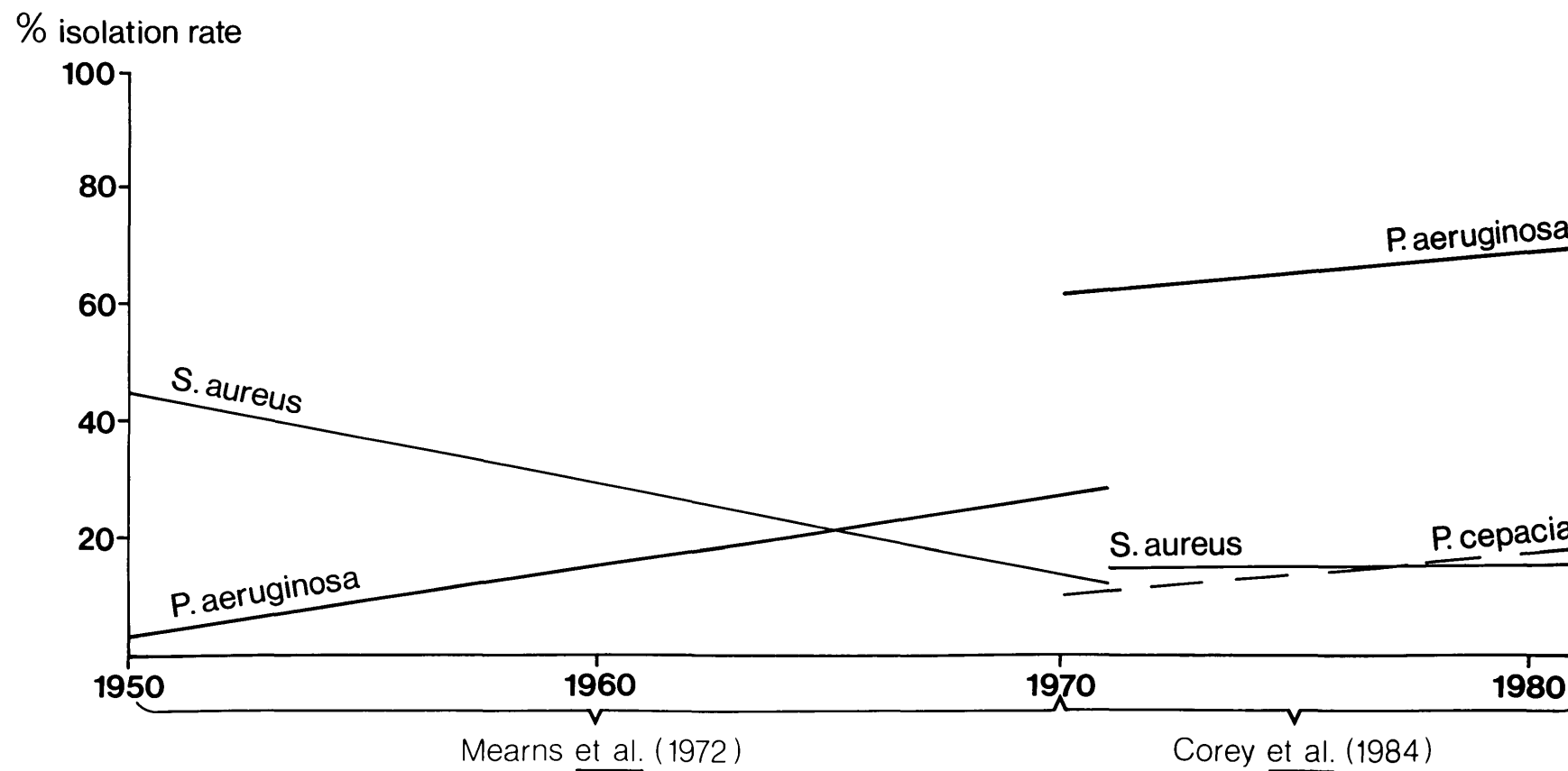
The isolation rate of H.influenzae has remained relatively constant over the 40 year period shown in Table 1. It was once considered to be a transitional stage between infection with S.aureus and the emergence of P.aeruginosa (Burns and May 1968) but more recent data does not support this (Kulczycki et al. 1978). H.influenzae is of major pathogenic significance in chronic bronchitis (Iacocca et al. 1963) but no definite conclusions can be drawn about its significance in CF (Mearns et al. 1972).

Longitudinal data from the studies of Mearns et al. (1972) and Corey et al. (1984) are summarised in Figure 1 and show the bacteriological findings in CF from 1951 to 1981. There was a progressive decline in the isolation rate of S.aureus from 45% in 1956 to 12% in 1971 and this was paralleled by a rise in the frequency of P.aeruginosa from 3% to 28% (Mearns et al. 1972). The data from the Canadian study (Corey et al. 1984) showed that by 1981, 70% of patients were colonised by P.aeruginosa while the figure for S.aureus remained constant at about 18%.

The number of patients from whom no pathogens were isolated had remained generally constant during the period of the study. Children under 5 years of age were most often free of S.aureus and

Figure 1

The emergence of P. aeruginosa as a major pathogen in CF (1951 – 1981) *



* Data from two longitudinal studies

P.aeruginosa, but in older patients colonisation with pseudomonas was common. When the sputum bacteriology was correlated with the clinical condition of patients, P.aeruginosa was most frequent in those patients with poorest clinical score determined by weight, X-ray picture and lung function tests.

The presence of precipitating antibodies to bacterial products in the serum of CF patients has been used both as indirect evidence of the presence of a bacterial species and to monitor the degree of respiratory infections. A review of the diagnostic and prognostic significance of serum precipitins in CF is given by Høiby (1977).

May et al. (1972) found in younger patients that serum precipitins to P.aeruginosa were more common than those to any other 'respiratory' organisms. The numbers of patients with precipitins to S.aureus and H.influenzae increased linearly with age and some older patients also had antibodies to Klebsiella pneumoniae and Streptococcus pneumoniae. In the oldest age group (>21 yrs) which contained the smallest number of patients, the proportion of serum samples with precipitins to P.aeruginosa was markedly decreased. These patients may have had less severe pulmonary disease than patients in other age groups as in a study by (Mearns et al. 1972) the presence of precipitins to P.aeruginosa was correlated with the severity of the disease. The data from this study (May et al. 1972) are at variance with a hypothesis that S.aureus is the initial pathogen (Burns and May 1968) as, even in the youngest age group (0-5 years old), precipitins towards P.aeruginosa were more common than those to S.aureus. May et al. (1972) suggested that the use of effective antistaphylococcal agents eradicated the organism before precipitins could be formed but this

seems unlikely as serum precipitins towards bacteria were frequently observed prior to positive culture (Høiby 1977).

The trends seen in the isolation rates of the two species, S.aureus and P.aeruginosa, (Table 1 and Figure 1) may be a result of the increased use of antibiotics. At the time of the earliest reports (Farber 1944; Di Sant'Agnese and Andersen 1946) chemotherapy was not extensively used and the large numbers of potentially pathogenic Gram-negative bacteria seen in hospitals today were not evident (Wolff and Bennett 1974). Nasal carriage of S.aureus was, as today, widespread (Williams 1963) and therefore patients susceptible to infection were more likely to acquire S.aureus.

The importance of H.influenzae in lung infections was not universally recognised until the 1950s (Turk and May 1967). It can be difficult to culture from clinical specimens and its growth is often inhibited or masked by other organisms, especially P.aeruginosa (Riley and Hoffman 1986). H.influenzae may have been prevalent in samples from early studies but not recognised. Burns and May (1968) found precipitins to H.influenzae in 80% of serum samples from CF patients aged 11-28 years and Penketh (1983) was able to recover the species in 80% of sputum samples from CF patients aged >16 years when a specific isolation medium was used.

Improved culture techniques may account for the reports of increasing rates of isolation of other bacterial species from CF sputum, in particular Pseudomonas cepacia. Corey et al. (1984) showed an increase in the rate of isolation of P.cepacia from CF since 1970 in all age groups studied. In 1981, 21% of patients were colonised with P.cepacia compared with 10% in 1970 (See Figure 1). Gilligan

and Schidlow (1984) found a similar increase of this species in older patients (>16 yr) attending a CF centre in Pennsylvania between 1976 and 1982. They suggested that the organism may have been overlooked in the past due to the difficulties inherent in its isolation.

The clinical significance of P.cepacia has yet to be established but its resistance to many commonly used antimicrobial agents is of concern (Isles et al. 1984; Gilligan and Schidlow 1984). The organism has been associated with long-term chronic asymptomatic carriage (either alone or in combination with P.aeruginosa) and with recurrent exacerbations of infection and gradual deterioration of health. It has also been implicated in a lethal pneumonia which affects patients, especially females, with previously minimal lung disease (Isles et al. 1984).

Other Gram-negative nonfermentative bacilli associated with CF include P.maltophilia, P.fluorescens, P.putida, and Achromobacter xylosoxidans (Klinger 1984). Species of enterobacteriaceae are often isolated from CF. In a survey of 72 patients, Seidmon et al. (1975) found that 49 harboured a variety of enterobacteriaceae, but these organisms seemed to be of little pathological importance. The survey by Baltimore et al. (1982) was also unable to determine the clinical significance of the presence of these organisms.

The emergence of a mucoid form of species is not restricted to P.aeruginosa as there have been reports of mucoid E.coli in CF (Kilbourn, Grach and Campbell 1968). Macone et al. (1981) isolated mucoid strains of E.coli in 11% of patients with CF followed for a year. The mucoid material was biochemically and antigenically distinct from that produced by P.aeruginosa but appeared to be of no

clinical significance. The authors suggested that selective pressures in the environment of the CF lung promote the emergence of mucoid forms of bacteria.

Anaerobic bacilli such as Bacteroides and Fusobacterium sp; anaerobic cocci and Corynebacterium sp. have been implicated as pathogens in pleuropulmonary infection (Hunter et al. 1985). When paired samples of saliva and sputum for 14 patients with CF were incubated under anaerobic conditions there was heavy growth of Bacteroides. However, no clinically significant anaerobic pleuropulmonary infection was demonstrated in this group of patients (Hunter et al. 1985).

Mycoplasma and Related Organisms

The evidence for infection with mycoplasma and chlamydia has invariably been based on serological tests. The number of seropositive patients is generally low and a Scandinavian study showed only four of 332 acute exacerbations of respiratory infection could be attributed to mycoplasma or chlamydia (Petersen et al. 1980). Nevertheless a survey at the Brompton Hospital found serological evidence of infection with Mycoplasma pneumoniae and Coxiella burnetti in 29% of 46 adult patients (Efthimiou et al. 1981).

Legionella pneumophila

High titres of antibody to Legionella pneumophila are common in CF sera but their clinical significance has not been established. It has been suggested that the high titres found in CF may be due to a cross reactive antibody directed towards P.aeruginosa and L.pneumophila, induced by a hyperactive immune system (Katz and

Holsclaw 1982, Efthimiou et al. 1981).

Mycobacteria

Mycobacterial infections are rare in CF. In a series of over 700 patients only two cases of active pulmonary tuberculosis were diagnosed (Wood, Boat and Doershuk 1976). Diagnosis of these infections in CF is complicated by the clinical picture produced by the longstanding respiratory symptoms, frequent infective exacerbations and pre-existing often extensive radiographic abnormalities. The introduction of routine examination of sputum samples for acid fast bacilli has increased the numbers of mycobacteria isolated from patients at the Brompton Hospital. Over a period of 16 years, 286 CF patients had attended the hospital. In the first 10 years when patients were not routinely screened, no mycobacterial isolations were recorded but the change in policy revealed positive cultures in 7 of 223 patients in the last 6 years of the study (Smith et al. 1984).

Mycobacterium tuberculosis was isolated from three of these patients, the others were represented by single cases each of M.fortuitum, M.chelonae, and two of an unclassified species. The patients with M.tuberculosis responded well to conventional treatment but in the others deterioration of the chest radiographs was evident and two of the patients subsequently died. Poor prognosis associated with M.chelonae infection was also indicated by the work of Boxerbaum (1980), who suggested that the mycobacterium acts as an opportunistic pathogen the significance of which should not be overlooked.

Viral Infection

Viruses are the most common cause of respiratory infection in health

and disease. Viral infection has been linked to pulmonary exacerbations in chronic respiratory diseases such as asthma, obstructive pulmonary disease and bronchitis (Green et al. 1977). Respiratory viral infections predispose to acute bacterial infection and the mechanism by which this occurs has been attributed to the suppression of pulmonary antibactericidal defences by the production of oedema in the alveoli, necrosis and desquamation of the tracheobronchial epithelium and impairment of mechanical transport out of the lung. Jakab and Green (1974; 1976) demonstrated these mechanisms in an animal model with Sendai viral infection. They also demonstrated a defect in intracellular killing of S.aureus by alveolar macrophages of mice previously infected with the Sendai virus and found multiplication of the bacteria in both consolidated and nonconsolidated areas of lung tissue.

A study of patients with naturally occurring acute respiratory tract infection and those who received an attenuated live influenza A vaccine showed an increased adherence of S.aureus to the cells of the respiratory mucosa. There was also a slight increase in the adherence of H.influenzae and S.pneumoniae but that of P.aeruginosa and K.pneumoniae was significantly decreased (Fainstein, Musher and Cate 1980).

It is widely accepted, as a result of the suggested synergy between viruses and bacteria in respiratory infections, that viral infection in CF could be of considerable importance in pulmonary disease. Wang et al. (1984) carried out a two-year prospective survey of the effect of viral infection on pulmonary disease in 49 CF children (mean age 13.7 years) and used their non-CF siblings (mean age 14

years) as controls. They measured antibody in serum, taken every 3 months, against a range of viral antigens and any exacerbations were recorded as evidence of infection. They found 105 cases of viral infection in the CF patients but failed to isolate any viruses from the patients. Interestingly 40% of these infections were asymptomatic. Serological tests indicated that infection was most often due to parainfluenza virus types 1, 2 and 3, adenoviruses and respiratory syncytial virus (RSV). Although parainfluenza infection was normally associated with lower respiratory tract disease there was no significant difference between the clinical syndrome and the type of viral infection.

Wang et al. (1984) also found no correlation between the annual incidence of viral infection and colonisation with P.aeruginosa, S.aureus or H.influenzae. There was, however, a slight correlation with colonisation by P.cepacia. Overall there was no difference in the number of viral infections experienced by the CF and non-CF groups although the CF children had a higher frequency of respiratory exacerbations. There seemed to be no association between initial pulmonary status and frequency of viral infection but there was evidence to suggest that frequent viral infection contributed to irreversible pulmonary deterioration.

Wright et al. (1976) studied 153 CF children in a double blind crossover trial to assess the effectiveness of amantadine-hydrochloride used prophylactically against an epidemic strain of influenza A virus. They used serological assays for a range of viral antigens when a patient had an exacerbation of infection. They recorded 48 episodes of respiratory exacerbation in

the two months of the trial, 33% of which were associated with viral infection, the most frequent being with RSV. They were unable to assess the effectiveness of amantadine in this population as infection with the virus did not reach epidemic proportions during the study.

A study of 116 CF patients for 8 months in Denmark, resulted in 332 cases of acute exacerbation of which 6% were attributable to non-bacterial infection. The most commonly encountered virus was RSV and the authors suggested a possible synergism between colonisation with P.aeruginosa and RSV (Petersen et al. 1980).

More recent work in this country by Stroobant et al. (1985) seemed to indicate an association with chronic P.aeruginosa infection and non-bacterial exacerbation of respiratory disease. In a group of CF children colonised by P.aeruginosa there were 42 cases of upper respiratory tract illness, 19 of which were attributable to non-bacterial pathogens. This was compared to a group of CF children free of P.aeruginosa in whom 25 exacerbations were reported, only three of which were of non-bacterial origin.

All these studies reported a decline in pulmonary function as a result of viral infection. A possible mechanism for this was postulated by Strunk et al. (1977) who reported a depression of the levels of complement fractions C4 and C3 in all the CF patients studied with acute viral infection. They concluded that this may have been due to the formation of localised immune complexes possibly triggered by viral antigens which may initiate pulmonary damage.

Pseudomonas aeruginosa

Taxonomy

The first description of the microorganism Pseudomonas aeruginosa was made in 1872 by Schröeter who named the species Bacterium aeruginosa (Palleroni 1984). In 1882 Gessard described the bacterium responsible for the 'blue-green' pus frequently observed in septic surgical wounds as Bacillus pyocyaneus. In 1894 Migula gave the genus the name Pseudomonas (Gk. false unit) and proposed the species epithet pyocyanea (Gk. blue pus). The epithet aeruginosa (Lat. full of copper green rust) has temporal priority over pyocyanea (Schroeter 1872; Migula 1894) and is the preferred species name today. (For early references see Bulloch 1929).

Members of the genus Pseudomonas are widely distributed in nature. Some species are pathogenic for animals or plants but P.aeruginosa, P.fluorescens and P.cepacia in particular cause disease in both. Morphologically, cells of the genus are straight or slightly curved non sporing, Gram-negative rods, 1.5-5.0 µm long and 0.5 µm wide. Most species are motile by means of a single polar flagellum but some may possess two, three or even four flagella. The genus is nutritionally unexacting and although strictly aerobic, can on occasion utilise nitrate to allow aerobic metabolism in an anaerobic environment. They do not ferment carbohydrates or photosynthesise. Nearly all members of the genus produce a cytochrome oxidase and all produce catalase.

Three species, P.aeruginosa, P.fluorescens and P.putida are taxonomically closely related and form a group known as the 'fluorescent

pseudomonads' by virtue of their production of the yellow water-soluble pigment, fluorescein. P.fluorescens and P.putida are psychrophilic and grow at temperatures as low as 5°C while P.aeruginosa, a mesophile, can grow at 42°-44°C. P.aeruginosa produces a characteristic blue-green pigment, pyocyanin, a phenazine derivative, and its presence is diagnostic for the species.

Other species of pseudomonads that are pathogenic for man include; P.pseudomallei (Whitmore's bacillus) and P.mallei the causative agents of melioidosis and glanders respectively. The latter species is most often associated with cattle. P.cepacia is primarily a plant pathogen causing onion rot but in recent years it has become implicated increasingly in human infections.

Many other species have been isolated from clinical material and the hospital environment but with the exception of P.maltophilia and P.stutzeri their pathogenic significance is questionable. Detailed reviews of the taxonomy and ecology of the genus are given by Stanier, Palleroni and Doudoroff (1966) and Palleroni (1984).

Characteristics of P.aeruginosa

Epidemiology

P.aeruginosa is widely distributed in nature and is often found in aquatic environments, both fresh water and marine. It is not strictly 'free-living', as it is uncommon in water sources not contaminated by man or animals. It is invariably found in slime-enclosed microcolonies which adhere to the surface of soil, water or plants and also to the epithelial lining of animal tissues. P.aeruginosa colonises the human intestine as a saprophyte, the rate

of faecal carriage in the normal population varies between 3% and 15%, but rates as high as 54% have been reported in hospitalised patients (Rhame 1980).

Morphology

P.aeruginosa is a Gram-negative, aerobic, motile, non-sporing rod. It is 1.5-3 μ m in length and 0.5 μ m wide with a single polar flagellum.

Isolation

P.aeruginosa is easily isolated on simple media but selective media have been developed for its isolation from clinical specimens. Selective agents include; cetyltrimethylammonium bromide (Cetrimide), 2,4,4'-trichlor-2'-hydroxydiphenylether (Irgasan,^(R)), Dettol^(R), antibiotics and antiseptics (Park and Billing 1965 ; Thom et al. 1971 ; Wong et al. 1984). There is evidence that the growth of some clinical strains, especially those from CF may be suppressed by the use of selective media (Fonseca, MacDougall and Pitt 1986).

Identification

Identification of the species is based on colonial appearance on agar culture, production of the pigment pyocyanin and the characteristic 'fruity' odour of trimethylamine.

There is heterogeneity in the morphology of the colonial types of P.aeruginosa which are shown in Table 2 (Wahba and Darrell 1965; Phillips 1969). However, 80% of strains from clinical sources grow as flat, diffuse, irregularly edged colonies (type 1). Changes in colonial form are often observed on subculture from liquid to solid medium and may be related to the degree of aeration of the culture (Homma 1971).

Many strains exhibit a metallic sheen on agar culture, with

TABLE 2

Colonial types of Pseudomonas aeruginosa

(Wahba and Darrell 1965)

Type	Size (mm)	Description	Shape	Elevation	Comment
1	2-3	Feathery edge slimy and moist	irregular	flat	most common (81%) occasionally coarse granular appearance may have irridescant patches with metallic sheen - autolysis
2	1-2	small slimy smooth colonies 'coliform'-like	round	raised	
3	1-2	rough or rugose, dry and crenated	irregularly round	raised	may also have irridescant patches
4	2-3	muroid	irregular	raised	may have loss of shape due to collapse of muroid; inclusion of glycerol in medium increases muroid production
5	1-2	gelatinous, similar to 3 but not as dry	round	raised	very rare
6	0.5	similar to 2	round	raised	after overnight incubation
	1-2				after 48 h incubation

Descriptions apply to colonies on nutrient agar at 24h 37°C, except for Type 6 which requires 48h incubation for visible growth.

iridescent patches of lysis. This is considered to be a form of autolysis the nature of which is not known (Cowan 1974).

Typically P.aeruginosa forms two extracellular pigments, fluorescein (yellow) and pyocyanin (blue) which combine to produce the characteristic green appearance surrounding colonies on agar. The chemical composition of the medium influences the production of the pigments. King, Ward and Raney (1954) formulated two media, A which enhances the production of pyocyanin and B, fluorescein.

Other pigments may be produced, but there is controversy as to whether they are true pigments or oxidation products of pyocyanin. The study of Ogunnariwo and Hamilton-Miller (1975) showed that two further pigments are produced; the dark brown, tyrosine induced pyomelanin and the red, pyorubrin, another phenazine derivative.

Biochemical Characteristics

The optimum temperature for growth of P.aeruginosa is between 30°-37°C. However, growth occurs over a wide temperature range, but not below 4°C (Gilardi 1980). Growth at 42°C may be used to distinguish P.aeruginosa from other fluorescent pseudomonads (Cowan 1974).

Although a strict aerobe, P.aeruginosa may grow anaerobically in nitrate-containing media (Gilardi 1980). This process leads to the production of nitrogen as the organism utilises nitrate as an alternative terminal electron acceptor (Stanier et al. 1966).

P.aeruginosa utilises a number of carbohydrates oxidatively with the production of acid but not gas; these include hexoses and polyalcohols but not disaccharides (Gilardi 1980). Diagnostic biochemical features of P.aeruginosa include the reduction of

gluconate with the production of slime (Haynes 1951), production of ammonia from arginine, reduction of selenite (Lapage and Bascombe 1968), deamination of acetamide (Buhlmann, Vischer and Bruhin 1961) and growth over three successive subcultures at 42°C (Wahba and Darrell 1965).

Epidemiological Typing of *P.aeruginosa*

Although primary identification of *P.aeruginosa* is rarely a problem to hospital microbiologists they may require to subdivide the species to distinguish between strains from the same epidemiological incident. The introduction of typing schemes based on differences in O-antigen, phage resistance patterns and bacteriocin production enables the similarity of strains to be assessed and helps to determine the source of infection. The methods available for the epidemiological typing of *P.aeruginosa* will detect strains that differ from the outbreak strain but otherwise can only indicate that similar strains of the same type are indistinguishable, and not that they are identical.

Bacteriocins

Bacteriocins are antibiotic substances produced by bacteria that are active against members of the same or closely related species. Bacteriocinogeny is encoded by chromosomal or extrachromosomal genes and possession of the gene by a cell affords immunity from the action of that bacteriocin. A small proportion of cells (approximately 1 in 10^4) per population elaborate bacteriocin and lysis of these cells occurs on release of bacteriocin.

Bacteriocin production is widespread in nature and several types may

be formed by the same bacterial strain. Although some are similar in structure to bacteriophages, but without a nucleic acid containing capsid, bacteriocins do not multiply within the sensitive cell. Bradley (1967) distinguished two basic kinds of bacteriocins which differed essentially in their molecular weights. The particle with the lower molecular weight may represent a portion of the high molecular weight particle and both are lethal to sensitive cells. The bacteriocins produced by P.aeruginosa are known as pyocins (Jacob 1954). The pyocins are of three types, R and F which are particulate in nature and the smaller molecular weight S-type pyocins which are diffusible. The R- and F-pyocins have a bacteriophage type structure and attach to the cell membrane of a sensitive strain leading to disruption of the cell. The smaller S-pyocin is amorphous and has no discernible structure by electron microscopy; it is thought to inhibit protein synthesis in the susceptible cell. The pyocin from one strain is only active on closely related strains and the producer strain is immune from its action (Govan 1974^a;1974^b).

Pyocin typing. The production and sensitivity of different strains to pyocins (particularly R-pyocins) has led to the development of typing systems to distinguish between strains of P.aeruginosa from outbreaks of infection. This method of typing, particularly that of Gillies and Govan (1966), is widely used in hospital laboratories (Pitt 1980). Isolates are tested for their bacteriocin activity against eight standard indicator strains and the patterns of inhibition are designated type numbers. At least 105 types have been described (Govan 1978) and new types are often reported

(Carvajal and Flores 1985).

Strains may also be typed by their susceptibility to pyocin containing filtrates (Falkiner and Keane 1977) and the patterns of sensitivity of isolates to bacteriocin activity are compared. The discriminatory power of this method is high but lytic patterns are not reproducible possibly due to mixtures of pyocins and bacteriophages in the lysate (Rampling, Whitby and Wildy 1975).

Although reproducible the discriminatory power of pyocin typing by the method of Gillies and Govan (1966) is only fair as four types; 1, 3, 5 and 10 account for about 60% of the strains of P.aeruginosa typed throughout the world (Pitt 1980). A revised technique has recently been described by Fyfe, Harris and Govan (1984). In this method the activity of R, F and S pyocins can be distinguished on the basis of inhibition zone size thus allowing greater discrimination. The method is more rapid than the earlier procedure (24h vs. 72h) and it is suitable for the typing of mucoid strains from CF.

Bacteriophages

Bacteriophages are viruses that infect bacteria. Phages exist in the mature form outside the host cell but are metabolically inert. Phages attach to the bacterial cell via specific receptor sites and these can be located on the cell surface (somatic), flagella, fimbriae (pili) or slime and capsular polysaccharides. Following adsorption to the cell surface the nucleic acid content of the phage is injected into the cell and two distinct states are recognised. In the lytic or virulent state, phage encoded genes mediate the production of numerous new phage particles which are released on lysis of the host cell. In the temperate state the phage matures

and becomes integrated into the bacterial chromosome or plasmid (extrachromosomal) and phage nucleic material is replicated with bacterial DNA. Successive daughter bacterial cells carry the phage genes and this lysogenic phage confers immunity on the cell from attack by similar phages.

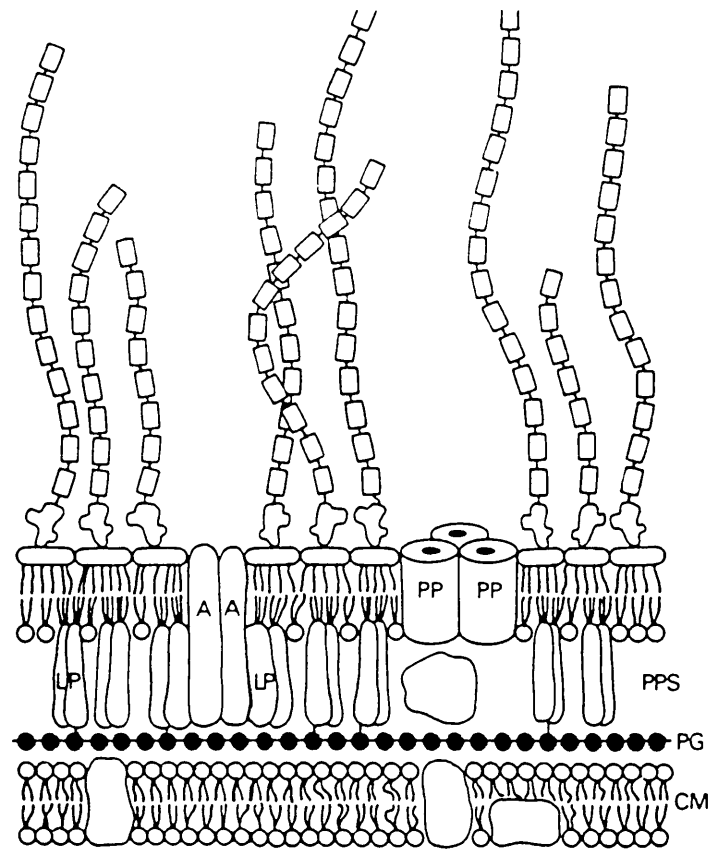
Phage typing. Numerous phages active on P.aeruginosa have been described and receptor sites range from lipopolysaccharide (Jarrell and Kropinski 1976; Temple, Ayling and Wilkinson 1986^a), slime polysaccharide (Bartell et al. 1971) and fimbriae (pili) (Bradley and Pitt 1974). Several of these phages have been used for the epidemiological typing of clinical isolates; the set in use at the Central Public Health Laboratory (CPHL) consists of 21 phages, most of which were isolated from sewage (Asheshov 1974) and adhere to a variety of cell receptors (Mutharia, Nicas and Hancock 1982; Temple, Ayling and Wilkinson 1986^b).

In routine phage typing, cultures are tested for their susceptibility to a phage set at a standard concentration of phage. Although typability is high (80-85%), phage typing is not completely reproducible and therefore can not be used alone for epidemiological typing (Pitt 1980). When used in conjunction with O-serotyping it provides finer discrimination between apparently related isolates (Pitt 1981).

Outer membrane antigens of P.aeruginosa

The outer membrane (OM), of Gram-negative bacteria is the uppermost layer of the cell wall (see Figure 2) and comprises two membranes separated by a periplasmic space containing peptidoglycan. The OM is responsible for the rigidity of the cell and for many phenotypic

Figure 2 Molecular organisation of the outer membrane of Enterobacteriaceae



Taken from Lugtenberg & Van Alphen (1983). Pore protein (PP), Omp A protein (A), lipoprotein (LP), periplasmic space (PPS), peptidoglycan layer (PG) and cytoplasmic membrane (CM).

properties of the living bacterium including ; resistance to toxic agents, antigenic state and provision of receptor sites for phages and pyocins. The major constituents of the OM are protein, phospholipid and lipopolysaccharide. Some proteins form pores or porins through which substances can be exchanged across the cell wall and others have specific functions such as the binding of iron or adhesion to mucosal surfaces (Benz and Hancock 1981).

Lipids in the OM of Gram-negative bacteria account for 10-25%(w/w) of the cell wall (Reitschel et al. 1982), phosphatidylethanolamine is the predominant compound but phosphatidylglycerol and diphosphatidylglycerol are also present in P.aeruginosa (Hancock, Humphreys and Meadow 1969). The major fatty acids present are palmitic (n-hexanoic), palmitoleic (cis 9-hexadecanoic) and vaccenic acids.

Six major outer membrane proteins (OMP) were identified in P.aeruginosa by Mizuno and Kageyama (1978) by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and were designated as D, E, F, G, H and I. Further work by Hancock and Carey (1979) subdivided proteins D and H to give D1, D2, H1 and H2. More recently a new protein, labelled P, a major porin has been described (Hancock, Poole and Benz 1982).

The cell wall of P.aeruginosa contains (by weight) 17-30% lipopolysaccharide (LPS) (Wilkinson and Galbraith 1975). In addition to being the immunodominant antigens LPS also mediates many biological activities including pyrogenic activity, stimulation of macrophage and cellular responses and induction of interferon (Reitschel et al. 1982).

LPS of the family Enterobacteriaceae consists of three regions. 1) The O-specific antigen, which is a long chain polysaccharide consisting of repeating units of monosaccharides, 2) the oligosaccharide core composed of monosaccharides including KDO, heptose, glucose, galactose and N-acetylglucosamine and 3) lipid A, a unique lipid backbone consisting of diglucosamine in a B1-6 linkage with fatty acids, and with phosphate, 4-amino-arabinose substituents or both on the reducing and non-reducing sugars (see Figure 2). The lipid A deep in the OM is structurally conserved and is the major toxic element of LPS. It is a rather unusual phosphoglycolipid having as its backbone the disaccharide B-glucosaminyl-1,6-glucosamine. Lipid A is bound to the core oligosaccharide through 3-deoxy-D-manno-octulosonic acid (KDO) linked to the inner core component L-glycero-D-manno-heptose (see Figure 2).

Wilkinson and Galbraith (1975) showed that the LPS of P.aeruginosa had an unusually high phosphorus content (4-5%) which correlated with the finding that only 5-11% of core oligosaccharides carry O-specific side chains (Drewry et al. 1975). The short core sequence of P.aeruginosa is predominantly comprised of aminosugars, and the monosaccharides glucose and rhamnose. Galactosamine is the aminosugar most commonly present and the oligosaccharide L-glycero-D-mannoheptose also occurs frequently. L-alanine has been identified in the core region of some strains.

The O-specific region of the LPS has a more variable composition. Side chains from most strains of P.aeruginosa contain either fucosamine, quinovosamine or both (Chester, Gray and Wilkinson 1972) and some serotype strains contain small amounts of D-bacillosamine

and 2-amino-2-deoxy-L-galacturonic acid (Wilkinson 1977; Koval and Meadow 1977).

O-serotype specificity is determined by the chemical components of the lipopolysaccharide and nine chemotypes have been described within the major serogroups of P.aeruginosa (Chester, Meadow and Pitt 1973). As yet O-specific chemical sequences have not been identified unequivocally but recent work by Stanislavskii et al. (1985) has identified the structure of the epitope of P.aeruginosa serotype 03 which is shared by different strains of this O-group.

O-serological typing. The group specific, LPS-based O antigens can be detected by agglutination with hyperimmune rabbit antiserum and this forms the basis of the variety of O-serological schemes for P.aeruginosa that have been described in different countries (Habs 1957; Verder and Evans 1961; Meitert 1964; Lányi 1966-67; Fisher, Devlin and Gnabasik 1969; Homma et al. 1970). Many of the types in each scheme correspond to those in others, but as no two schemes are directly comparable an international panel, headed by Professor Liu of the University of Kentucky, Louisville, USA, was organised to establish an internationally recognised serotyping scheme. The International Antigenic Typing Scheme (IATS) scheme comprises seventeen serotype strains of P.aeruginosa each of which represent distinguishable heat-stable somatic antigens (Liu et al. 1983).

The O-serological typing set used at the CPHL corresponds to the IATS but two of the O-groups are subdivided further by minor antigens (Véron 1961), to give a total of 21 serotypes. O-serological typing is reproducible and 90-95% of clinical strains can be allocated to a single O-type. However the scheme, if used alone, lacks

discrimination as about 40% of strains in the UK are of type 06 or 011 (Pitt 1980).

Polyagglutination. Most of the 0-serogroups of P.aeruginosa are antigenically distinct, but some strains, although stable in saline, are agglutinated by two, three or more antisera prepared against unrelated serogroups. This feature has been termed polyagglutination (PA) and several workers have made reference to it (Christie 1948; Lányi 1966-67; Homma et al. 1970).

Pitt and Erdman (1978) found that polyagglutinable strains of P.aeruginosa were most commonly agglutinated by antisera to 0 groups; 3, 6, 10 and 13 and less often with antisera to 0 groups; 4, 5, 9 and 11. The cross-reacting antibodies were not removed by absorption with the appropriate serotype strains or protein A-containing staphylococci. Lányi (1966-67) suggested that a heat-stable antigenic determinant was responsible for polyagglutination and it was common to all PA strains. This was confirmed by Pitt and Erdman (1978) who were able to distinguish between PA and 0-type specific antibody in rabbit antisera. PA antigen elicited only an IgM response but 0 antibody was found to be both IgM and IgG.

Studies of strains of P.aeruginosa isolated from the sputum of CF patients suggest that polyagglutinability may be due to the loss of 0-specific side chains and the subsequent exposure of a 'common' antigen which may correspond to core oligosaccharide (Hancock et al. 1983; Meadow, Rowe and Wells 1984; Ojeniyi, Baek and Højiby 1985). The frequency of polyagglutinable strains from all clinical sources is low (3.8%, Pitt and Erdman 1978), but the incidence increases

dramatically with strains from patients with chronic respiratory infections especially in CF where Penketh et al. (1983^a) found 55% of cultures from 49 patients were PA.

Loss of O-serotype determinants by members of the Enterobacteriaceae is usually manifest by a variation in the colonial morphology from a smooth to a rough or granular appearance (S → R variation). Through this spontaneous mutation the bacteria lose the ability to synthesise the O-specific polysaccharide moiety of their LPS and thus their serological O-specificity. The resulting R mutants have R-LPS which consists of only lipid A and the core oligosaccharide. In addition, R mutants exhibit increased cell surface hydrophobicity and often spontaneously agglutinate in saline without the addition of antibody. Variation in the O-specificity of P.aeruginosa as shown by polyagglutination is not reflected in the colonial form. Immunologically rough strains i.e. those lacking O-side chains are not colonially rough and vice-versa. Furthermore, PA strains have not been reported to spontaneously agglutinate in saline.

Some of the variation in the frequencies of PA reported in non-CF strains may be due to technical factors such as poorly absorbed antisera or the use of high cell concentration vaccines to prepare antisera (Mikkelsen 1968).

Protein Antigens

Sera prepared in animals against unheated suspensions of P.aeruginosa contain, in addition to anti O antibody, other antibodies to various inadequately defined heat-labile bacterial cell constituents. The major protein (heat-labile) antigens of P.aeruginosa are flagella, fimbriae or pili and outer membrane proteins of the cell.

Although several groups have studied the heat-labile antigens of P.aeruginosa (Gaby 1946; Christie 1948; Lányi 1970), the structures responsible for antigen specificity have yet to be completely characterised. The preparation of mutant strains of P.aeruginosa that lacked flagella or fimbriae, or both, made possible the study of the contribution of each of these structures to the reactions of cells with antisera prepared against whole unheated bacterial cells (Pitt and Bradley 1975).

Fimbriae. Fimbriae are long, thin appendages of the bacterial cell that occur in many species of enterobacteria and are highly antigenic (Duguid and Campbell 1969). Some fimbriae are retractile and can be withdrawn into the bacterial cell on stimulus. The fimbriae of P.aeruginosa differ from those of the enterobacteria in that they do not cause haemagglutination of red blood cells, which is a cardinal test for enterobacterial fimbriae. They are antigenic and the vaccination of rabbits with strains that have non-retractile fimbriae give rise to sera with high agglutinating antibody titres (Bradley and Pitt 1975). These workers demonstrated four different fimbrial antigens in six strains of P.aeruginosa. The fimbrial antigenic types were unrelated to the O or flagellar antigens and were potentially useful as epidemiological markers. Unfortunately, the expression of fimbriae appeared to be unstable in culture and fimbriation was rapidly lost on the acquisition of a plasmid.

Flagellar Antigens. Flagella are long, uniformly thin, unbranched filaments thicker than fimbriae (120-300 Å). Flagella have a characteristic sinusoidal wave and the basic protein unit is known as

flagellin. Whiteside and Rhodes-Roberts (1985) purified flagella from three species of Pseudomonas; P.aeruginosa, P.putida and P.fluorescens. Three flagellins were obtained but an exact determination of their molecular weight could not be made although the range was found to be between 38-40 Kdaltons. The molecular weight of 40 Kdaltons is that commonly reported for hook proteins (Homma et al. 1984), but this study was able to confirm a definite filament location for the purified flagellins by direct immunofluorescent serology (Whiteside and Rhodes-Roberts 1985).

Six H-type strains were used as the basis of an H-typing scheme (Pitt 1979). Routine typing was performed by agglutination of unheated formalin-fixed bacterial suspensions with antisera raised towards the six H-type strains. Although there was apparent O group association of the H-antigens there was sufficient variety in the patterns to give further subdivision of O groups 1, 6 and 11. A thorough study of the heat-labile antigens of P.aeruginosa is provided by Pitt (1979).

Outer Membrane Proteins. The results of Mutharia et al. (1982) suggested that the outer membrane proteins are antigenically highly conserved throughout the 17 IATS O-serotypes of P.aeruginosa. It was suggested that this feature may be potentially useful for the development of protective antigens and associated vaccines. Patients with chronic infections due to P.aeruginosa have high levels of antibody to OMP (Lam et al. 1983) but the efficacy of the antibody response is doubtful.

It would seem that proteins F, H2 and I are antigenically related in all strains and the work of Meadow et al. (1984) indicated that F and

H porins were accessible and strongly antigenic in animals. No typing schemes based on OMP antigens have been described for P.aeruginosa as there is insufficient heterogeneity between strains for such a scheme to be of practical value.

Common antigens. The occurrence of serological cross-reactions between different species of Gram-negative bacteria has stimulated interest in the potential of a single antigen to induce protection in humans against a range of bacteria. Sompolinsky et al. (1980^a) described an antigen which was expressed in P.aeruginosa and was common to a wide range of bacteria. Biochemical studies showed the antigen to be a protein composed of polypeptide units of a molecular weight of c. 62 Kdaltons. The antigen was heat and acid labile and degraded by trypsin (Sompolinsky et al. 1980^b). More recently Mutharia and Hancock (1985) made a monoclonal antibody directed towards the outer membrane lipoprotein H2, present in 50 of 52 P.aeruginosa strains tested. This antibody reacted with the fluorescent group of pseudomonads and Azotobacter vinelandii but not with several other species of Gram-negative bacteria.

Resistance of P.aeruginosa

P.aeruginosa can survive at ambient temperatures for long periods in moist conditions and for months in water but is killed on exposure to a temperature of 55°C for one hour. It is resistant to quaternary ammonium compounds such as cetyltrimethylammonium bromide (cetrimide) and this compound has been used extensively in liquid and agar media as a selective agent for the species.

P.aeruginosa is generally more resistant than the enterobacteriaceae

to many antiseptics (Stickler and Thomas 1982) and resistance is mediated primarily by the permeability of the cell envelope. The addition of ethylenediamine tetraacetate (EDTA) enhances the activity of quaternary ammonium compounds and chlorhexidine against P.aeruginosa (Brown and Richards 1965).

The species is often resistant to concentrations of other agents markedly above their cidal concentrations for most bacteria and include; benzalkonium chloride, Irgasan(^R) and other bis-phenol compounds. Most strains of P.aeruginosa are fully susceptible to chlorhexidine at the concentration recommended for hospital use (Pitt, Gaston and Hoffman 1983).

Antibiotic resistance

In terms of treatment efficacy P.aeruginosa must be considered to be resistant to most of the commonly used antibacterial compounds such as ampicillin, cefoxitin and cotrimoxazole. Resistance of P.aeruginosa to antimicrobial agents is complex and varied. Some strains have modified target enzymes, penicillin-binding proteins (PBP) which negate the effects of B-lactams (Mirelman, Nuchamowitz and Rubinstein 1981). Compounds such as ampicillin and the antistaphylococcal penicillins are not effective against Pseudomonas as they are unable to traverse the outer membrane and have a low affinity for the PBP of Pseudomonas. Conversely, the antipseudomonal penicillins such as carbenicillin and the ureidopenicillins are active because of their ability to reach a suitable concentration within the periplasmic space and bind to PBP. P.aeruginosa produces a wide range of B-lactamases and, except for the Sabath and Abraham enzyme (Sabath, Jago and Abraham 1965) which

is chromosomally encoded, they are all plasmid mediated and hydrolyse a variety of B-lactams. Resistance of P.aeruginosa to B-lactams is essentially a combination of B-lactamase activity and the inability of the compound to reach a receptor site (Sykes and Bush 1982). Antibiotics such as azlocillin and piperacillin are more active against P.aeruginosa than carbenicillin because of their ability to pass readily through the cell wall and their high affinity for PBP. Many of the third generation cephalosporins such as cefotaxime, ceftriaxone and cefuroxime have been shown to have good activity against P.aeruginosa but in therapeutic terms they are not a good choice of antibiotic (Neu 1982). Alternatively, the newer agents; cefoperazone, cefsulodin and ceftazidime are highly active against the great majority of strains and are resistant to most of the B-lactamases produced by Pseudomonas (Neu et al. 1979).

The monobactams such as aztreonam and moxalactam are generally resistant to hydrolysis by B-lactamases but the latter antibiotic is not considered a first choice in treatment. An excellent B-lactam compound is the thienamycin class of drug, imipenem, (Neu and Labthavikul 1982) and clinical trials are in progress.

The aminoglycosides especially gentamicin and tobramycin are generally active against most strains of P.aeruginosa and resistance is uncommon in clinical isolates. Aminoglycosides enter the cell through porin channels and are moved across the cell membrane by proton motive forces (Bryan and Van den Elzen 1977). Once in the cytoplasm the antibiotic binds to the 30S ribosomes and causes faster uptake of the drug by inducing a protein which facilitates its transport. Enzymes mediated by plasmids may modify the

aminoglycosides causing them to bind less well to ribosomes and thus inhibit further uptake of the drug. Most strains of P.aeruginosa are resistant to streptomycin, kanamycin and neomycin by virtue of 6-phosphotransferase and 3-phosphotransferase enzymes (Shannon and Phillips 1982). Resistance to gentamicin and tobramycin is not common and generally more than 90% of clinical isolates are sensitive to these antibiotics. Amikacin resistance is rare and when enzyme mediated, is due to a 6'acetyl-transferase (AAC-6') which does not hydrolyse gentamicin and therefore strains are usually gentamicin sensitive.

Chloramphenicol and trimethoprim both penetrate the cell wall of P.aeruginosa poorly and in the case of chloramphenicol, strains produce a transacetylase which impairs the uptake of the antibiotic by the cell. Resistance to sulphonamides is due to altered dihydropteroate synthetase enzyme.

The quinolones, nalidixic acid and cinoxacin are inactive against P.aeruginosa because of the efficient permeability barrier and/or the production of a DNA gyrase which inactivates these drugs. The recently introduced fluorinated carboxy quinolones such as enoxacin, ofloxacin, perfloxacin and ciprofloxacin are active against Pseudomonas and ciprofloxacin in particular has been very effective. The advantage of ciprofloxacin is that it can be administered orally as well as intravenously and this may be of value in the treatment of chronic infections in diseases such as cystic fibrosis.

High level resistance to quinolones can be produced in vitro and the emergence of resistant strains during treatment has been reported as a major problem (Scully et al. 1986). Resistance of Pseudomonas to

the new quinolones does not appear to be due to reduced permeability since enhancement of permeability with chelating agents such as EDTA does not increase sensitivity. A chromosomal change in the DNA gyrase is the most probable cause of resistance.

Many antibiotics show synergistic activity against P.aeruginosa, notably the aminoglycosides and ureidopenicillins. Synergy between the 'antipseudomonal' cephalosporins and the monobactam aztreonam also occurs (Fu and Neu 1978).

Resistance to normal human serum

The bactericidal activity of normal human sera has long been recognised as a factor in nonspecific resistance to infection. Many species of Gram-negative bacteria show a loss of viability and some are lysed when exposed to suitable concentrations of fresh normal human serum. Serum killing is mediated by the insertion into the bacterial envelope of the aggregated terminal components of the complement cascade, also called the membrane attack complex. Activation of complement can occur by either the classical or alternative pathways. The classical pathway often requires recognition of a target antigen on the bacterial surface by antibody whereas alternative pathway killing is not dependent on antibody (Taylor 1983).

The classical and alternative pathways are dependent on the presence of Mg^{2+} ions, but the classical pathway also requires Ca^{2+} and is therefore specifically inactivated by the chelating agent ethyleneglycol tetraacetic acid (EGTA). Fine et al. (1972) showed that chelation of serum with EGTA did not affect the ability of serum to kill E.coli while treatment with ethylenediamine tetraacetic acid

(EDTA), which chelates both Mg^{2+} and Ca^{2+} destroyed serum bactericidal activity.

The classical pathway can be activated by IgM and some classes of IgG which interact with the C1 component of the complement cascade. Michael and Rosen (1963), demonstrated that IgM antibodies in normal human serum were a hundred times more active at fixing complement with Gram-negative bacteria than IgG on a weight for weight basis. Studies on E.coli and Salmonella suggest that normal and hyperimmune sera contain complement activating antibodies specific for the LPS (Schwab and Reeves 1966) and bactericidal activity is O-type specific (Muschel, Chamberlin and Osawa 1958). Other non-specific factors may also enhance the killing of bacteria by serum e.g. lysozyme which is necessary for the lysis of the bacterial cell (Inoue et al. 1959). Glynn and Ward (1970) showed that normal and immune sera lost bactericidal activity against gonococci after absorption with erythrocytes coated with LPS. Furthermore LPS from Gram-negative strains inhibited the bactericidal activity of both normal and immune sera against the homologous strain (Michael, Whitby and Landy 1962). Antibody for classical pathway activation may be directed towards antigenic determinants in the core region of the LPS (Rowley 1968). Experiments with E.coli 0111:B4 and its rough mutant J5 suggested that antibody independent classical pathway activity may be more important in the killing of rough rather than smooth strains (Muschel and Larsen 1970). The lipid A moiety of LPS activates C1 in the absence of antibody but this region lies deep in the outer membrane and is not exposed on the surface of smooth strains. The loss of side chains in a rough LPS mutant would probably expose the lipid A

and lead to activation of the classical pathway of the complement cascade.

There has been little published on the mechanism of serum resistance or sensitivity in P.aeruginosa. Young (1972) reported that most strains from natural or clinical sources were resistant to serum. However, serum sensitive cultures are frequently isolated from patients with cystic fibrosis (Penketh et al. 1983^a; Pitt et al. 1986) and these strains were invariably defective in their LPS (Hancock et al. 1983; Pitt et al. 1986).

The Pathogenicity of P.aeruginosa

Nosocomial infections due to Gram-negative bacteria have increased in the last forty years (Rhame 1980; Shanson 1982) and P.aeruginosa is the most frequently encountered human pathogen of the genus Pseudomonas. It can produce serious infections especially in compromised individuals (Gilardi 1980) and it has emerged as an important opportunistic pathogen in burns and cancer patients.

Most clinical isolates reflect colonisation rather than infection and rates are highest in the immunocompromised, those receiving antibiotics and those who have been hospitalised for long periods. The proportion of Gram-negative bacteraemia due to P.aeruginosa has remained constant since the 1950s (Rhame 1980), but Schimpff et al. (1973) suggested that it is responsible for up to 25% of cases of hospital acquired septicaemia, being particularly prevalent in centres treating leukaemics. In District General Hospitals in the U.K. it is, by contrast, a rare cause of septicaemia (1-2%) (Gould and Wise 1985).

Infections in healthy individuals, with the exception of new-born infants, are most often superficial. There is a strong association between dermatological infection with P.aeruginosa and water, or moist environments for example; 'soggy dermatitis' of the interdigital spaces, otitis externa from swimming pools (Hoadley and Knight 1975) and more recently folliculitis from whirlpools and Jacuzzis (Sausker et al. 1978; Jones and Bartlett 1985).

If the organism can obtain direct entry into tissues e.g. surgical wounds, there is localised formation of pus but extension into the bloodstream is rare except in patients with extensive burns (Pruitt 1980). P.aeruginosa can be isolated from most burn wounds (60%) and it contributes to the rejection of skin grafts and in rare instances to life threatening septicaemia (Rabin et al. 1961). The case fatality is between 40-80%, but if specific anti-pseudomonal therapy is given early enough survival rates increase (McKendrick and Geddes 1981).

Pseudomonas eye infections in previously healthy patients can result in loss of the infected eye. In one outbreak due to contaminated eye drops used at operation, panophthalmitis caused partial or complete blindness in 6 patients (Ayliffe et al. 1966).

In the urinary tract, infection with P.aeruginosa may occur post operatively as a result of a structural abnormality or the continuous presence of an indwelling catheter.

Endocarditis due to P.aeruginosa is not common but may be encountered in heroin addicts or patients who have had heart surgery. The organism may also cause infection in bones and joints as a complication of puncture wounds. Extensive reviews of P.aeruginosa

infections in burns and wounds are provided by Pruitt (1980), Geddes (1980) and Gould and Wise (1985).

Newborn infants are susceptible to infection with P.aeruginosa. The organism may enter via the umbilical wound or the gastrointestinal tract or mucous membranes and infection may range from localised skin lesions to meningitis or generalised septicaemia. At particular risk are those neonates in Special Care Baby Units (SCBU) if equipment such as humidified incubators or resuscitation equipment has been incorrectly cleaned following contamination with Pseudomonas which proliferates in the moist environment of the SCBU (Hoffman and Finberg 1955; Asay and Koch 1960; Barson 1971).

Serious primary respiratory infection with P.aeruginosa is rare in healthy humans but if a patient receives inhalation therapy that incorporates reservoir nebulisation, aerosol inhalation of the organism can occur. Increased awareness of this hazard has reduced the incidence of infection following inhalation therapy (Reynolds and Fick 1980). It is more common for P.aeruginosa to be implicated in a secondary pneumonia in a compromised host e.g. in acute bone marrow failure, leukaemia or after prolonged chemotherapy (Reynolds and Fick 1980).

P.aeruginosa in Cystic Fibrosis

P.aeruginosa has emerged as the principle pulmonary pathogen in older CF patients. Once acquired, eradication is seldom achieved and morbidity and mortality are high (Hodson 1983).

The strains of P.aeruginosa isolated from patients with CF differ from those in other clinical sources. There is an increased isolation

rate of the mucoid colonial phenotype (Doggett 1969), the strains are sensitive to the bactericidal action of normal human serum (Penketh et al. 1983^a), lack O specific antigen (Hancock et al. 1983), are often hypersensitive to antibiotics (Govan, Fyfe and Baker 1983) and there is evidence that these strains are avirulent with altered physiological properties (Luzar and Montie 1985), (Table 3).

Mucoid exopolysaccharide (MEP)

The mucoid appearance on agar of many of the strains of P.aeruginosa isolated from CF is caused by the production of a mucoid exopolysaccharide (MEP). This substance closely resembles alginic acid, a seaweed polysaccharide. It is primarily D-mannuronic and L-guluronic acid joined by a B(1-4) linkage but unlike the seaweed alginate, MEP is highly acetylated (Linker and Jones 1966).

The predominance of mucoid phenotype in CF, 60-90% of chronically colonised patients compared with 1% from other clinical sources (Doggett 1969), has generated much interest in MEP. However, the significance of the mucoid variant in CF is still not clear (Pier, Matthews and Eardley 1983).

Mucoid strains of P.aeruginosa can be isolated in vitro from wild type, non mucoid strains by selecting for carbenicillin resistance (Govan and Fyfe 1978) or resistance to a virulent phage (Martin 1973; Miller and Renta Rubero 1984). The mutations responsible for the alteration in phenotype have been located on the bacterial chromosome (Fyfe and Govan 1980), which suggests that all wild type strains carry the necessary information for the expression of the mucoid phenotype.

TABLE 3

Altered characteristics of Pseudomonas aeruginosa isolated from
patients with cystic fibrosis (CF)

Strain	
Clinical (Non CF)	CF
93% Specific O-serotype ⁽¹⁾	15% specific O-serotype ⁽³⁾
4% polyagglutinable ⁽¹⁾	55% polyagglutinable ⁽⁴⁾
80-90% resistant to normal human ⁽¹⁾ serum	69% sensitive to normal human ⁽³⁾ serum
1% mucoid appearance ⁽²⁾	60-90% mucoid colonial ⁽²⁾ morphology
not hypersensitive to antibiotics	20% hypersensitive to ⁽⁵⁾ antibiotics
production of virulence factors	appear to be avirulent ⁽⁶⁾

1. Pitt (1981)

2. Doggett (1969)

3. Pitt et al. (1986)

4. Penketh et al. (1983^a)

5. Govan, Fyfe and Baker (1983)

6. Luzar and Montie (1985)

Selection for the mucoid phenotype occurs in CF but the mechanism of the change and its pathogenic significance remain unclear. The CF lung may become a selective environment due to continuous antibiotic therapy, especially antistaphylococcal (Govan and Fyfe 1978) or because of iron depletion (Boyce and Miller 1982; Brown, Anwar and Lambert 1984), or variation in calcium and magnesium concentrations (Dunne and Buckmire 1985) or by the selective pressure of the patient's immune response (Høiby 1982). The situation is complicated by the heterogeneity of mucoid colonies which even within a single specimen may differ on primary isolation in appearance, nutritional requirements and resistance to antibiotics (Irvin et al. 1981).

It has been suggested that MEP acts as a physical barrier to the clearance of P.aeruginosa by pulmonary macrophages. Mucoid variants have been shown to be more resistant to phagocytosis than isogenic nonmucoid strains (Schwarzmann and Boring 1971; Ruhen, Holt and Papadimitriou 1980). Oliver and Weir (1983) demonstrated inhibition of binding to macrophages when a nonmucoid strain was incubated with alginate extracted from its mucoid variant. Furthermore, they found that the binding of Staphylococcus albus was also greatly inhibited, which implied that if such a mechanism did exist in vivo it would not be specific for P.aeruginosa.

The model for phagocytosis employed by Schwarzmann and Boring (1971), showed that phagocytosis by leucocytes was inhibited by the presence of MEP and the degree of inhibition was directly proportional to the concentration of MEP in the assay. Inhibition was more complete for mucoid P.aeruginosa than for the non mucoid variant or other

microorganisms tested in the system. The addition of normal serum to the assay reduced the protective role of the MEP for all but the mucoid strain of P.aeruginosa but protection was destroyed by the addition of antiserum raised towards the mucoid variant.

It is probable that MEP exerts a barrier effect which acts to mask the surface charges necessary for phagocytosis to occur (Ruhen et al. 1980). However as specific antibody appears to neutralise this effect in vitro (Ames, DesJardins and Pier 1985) this suggests that MEP elicits an antibody response. Initially, it was thought that MEP was non-immunogenic but Pier et al. (1983) and Speert, Lawton and Mutharia (1984) have demonstrated high antibody titres to MEP in CF patients, especially those harbouring mucoid strains of P.aeruginosa. There is no evidence that antibody to MEP minimises the progressive pulmonary damage associated with P.aeruginosa colonisation but may indeed perpetuate it (Pier and Elcock 1984).

The role of MEP production in vivo has been studied in animal models. Cash et al. (1979) established a non-fatal, chronic P.aeruginosa infection in the rat by encasing strains in agar beads. However, they were unable to demonstrate predomination of the mucoid phenotype over nonmucoid strains. Similarly, when mucoid or nonmucoid P.aeruginosa were introduced into guinea pig lung there was no apparent difference in the rates of intrapulmonary killing, and the animals which received mucoid strains were no worse affected (Blackwood and Pennington 1981). These authors concluded that the pathogenesis of chronic lung disease in CF patients involved a more complex series of host-parasite considerations than the production of mucoid exopolysaccharide alone.

Adherence properties. It has been proposed that MEP is stimulated by the cation concentration of CF sputum and that calcium promotes gelation of alginate (Costerton, Brown and Sturgess 1979). The glycocalyx, or loose capsule, is composed of highly organised linear strands of polysaccharide protruding from the cell surface and may mediate attachment to respiratory epithelium. Baker and Marcus (1982) observed a network of fine fibres in the extracellular matrix surrounding mucoid strains of P.aeruginosa, these attached the bacteria to each other and to the epithelium. They found similar fibres in post mortem lung specimens from CF patients who had been colonised with P.aeruginosa and also in chronically infected rats. They proposed that colonisation of the lower respiratory tract in CF may result from a combination of impaired mucociliary clearance in the lung and the mucoid nature of the bacteria. In a later study (Marcus and Baker 1985) they were able to show that adherence of mucoid strains of P.aeruginosa to epithelial cells was better than that of nonmucoid strains. They suggested that this may explain the prevalence and persistence of this phenotype in CF.

The buccal epithelial cells from CF patients appear to allow greater adherence of P.aeruginosa than those from normal controls. Woods et al. (1980) showed that adherence of P.aeruginosa correlated inversely with the amount of cell surface fibronectin and directly with the presence of salivary proteases. Fibronectin is a high molecular weight (440 Kdalton) glycoprotein present in plasma and produced locally in the lung. It is secreted by fibroblasts and alveolar macrophages and functions as an opsonin for many bacteria. When normal buccal cells were treated with trypsin, which decreased the

surface fibronectin, there was a concurrent increase in bacterial binding (Woods et al. 1981).

Antibiotic resistance. There are conflicting reports in the literature as to the susceptibility of mucoid strains to antibiotics. Thomassen et al. (1979) found mucoid strains of P.aeruginosa from CF to be more susceptible to tobramycin and carbenicillin than nonmucoid variants which is in direct contrast with the findings of Govan and Fyfe (1978). In vivo the production of MEP may retard the diffusion of antibiotics by acting as a simple physical barrier or by inhibiting the diffusion of positively charged antibiotics such as gentamicin (Slack and Nichols 1981).

Lipopolysaccharide (LPS). Penketh et al. (1983^a) studied strains of P.aeruginosa isolated from 49 chronically infected adult CF patients. Only 32% of these strains exhibited serotype specificity and 52% were sensitive to normal human serum. The bacteriological findings were correlated with clinical data for each patient and it emerged that those patients most recently colonised generally harboured strains with specific O-serotype antigen that were resistant to normal human serum. Strains from patients with chronic infection were invariably atypical in these properties and strains that exhibited characteristics transitional between the two extremes were commonly isolated from those patients with the most severe clinical symptoms. Investigation of variant strains from CF by polyacrylamide gel electrophoresis and silver staining (Hancock et al. 1983) revealed that their LPS lacked high molecular weight O-specific side chains. The relationship between O-specificity and the side chains has been

demonstrated in laboratory strains of P.aeruginosa. Meadow et al. (1984) showed that three polyagglutinating variants of a strain differed chemically from the parent strain in LPS composition. The amount of side chain retained by each of the three mutants varied, although all lost the O-specific determinant.

A major function of the LPS O side-chains is to protect the bacterial surface from the lytic effects of complement, which is activated by lipid A embedded deeper in the outer membrane (Mäkelä et al. 1980). The increased sensitivity to normal human serum of Salmonella species with variant LPS has been well documented (Muschel and Larsen 1970; Shaio and Rowland 1985) and a similar situation may occur in strains of Neisseria gonorrhoeae (Shafer et al. 1984).

The relationship between serum susceptibility and loss of O-specificity in P.aeruginosa does not seem to be absolute. Some strains which have lost O-specificity are still resistant to human serum (Hancock et al. 1983; Meadow et al. 1984; Pitt et al. 1986) and conversely serum sensitive strains may exhibit a specific serotype reaction. O-antigenic variants may alter the degree of complement binding by inducing the production of blocking factors or the core polysaccharide may cause steric inhibition of the binding to lipid A (Shafer et al. 1984).

The influence of environmental conditions, in vitro, on the expression of outer membrane components has been well documented. Williams and Brown (1986) showed that iron concentration effects the synthesis of some outer membrane proteins in H.influenzae. This group had suggested earlier that a similar mechanism may occur, in vivo, for P.aeruginosa in CF (Anwar et al. 1984). Gross changes in

P.aeruginosa LPS composition have been induced, in vitro, by altering the magnesium ion concentration of the medium, these were also accompanied by functional changes in the binding to the bacterial cell of charged molecules such as gentamicin and lysozyme (Day, Marceau-Day and Ingram 1978; Day 1980).

The environment in the CF lung may well influence the expression of bacterial components and continuous antibiotic therapy may contribute to the selection of strains of P.aeruginosa with atypical LPS and outer membranes.

Virulence Factors of P.aeruginosa

P.aeruginosa produces a wide range of extracellular products, many of which can be considered virulence factors which may help to establish colonisation in the CF lung (Table 4).

Proteases. P.aeruginosa produces at least three proteases and two of these, alkaline protease and elastase, have been implicated in the pathogenesis of CF. These proteases produce haemorrhagic skin lesions and can form necrotic lesions in the lung thereby altering its structure and function (Döring et al. 1983). Although Woods et al. (1982) showed these factors to be important in the animal model of lung damage, it is not clear if proteases contribute to lung damage in CF. All the evidence for their presence is indirect, i.e. from the detection of specific antibodies in sera and bronchial secretions (Jagger et al. 1982; Döring et al. 1983).

Elevated antibody titres to proteases in CF have been associated with poor prognosis (Granstrom et al. 1984) suggesting either an inability to neutralise enzyme activity or increased levels of proteolytic activity as colonisation progresses. P.aeruginosa elastase has been

TABLE 4

Virulence factors of Pseudomonas aeruginosa

Factor	Function
Mucoid exopolysaccharide	Adherence to epithelia, penetration barrier for phagocytes, antibiotics etc.
Fimbriae	Adherence to buccal epithelia
Proteases	Local necrosis, haemorrhagic lesion splitting of IgG, degradation of fibronectin
Phospholipase C	Destruction of pulmonary surfactant
Exotoxin A	Systemic toxicity
Exoenzyme S	Dissemination of infection
Pyocyanin	Inhibition of ciliary function Accumulation of iron
Lipopolysaccharide/ endotoxin (Lipid A)	Complications of bacteremia, e.g. shock, gastrointestinal bleeding, disseminated intravascular coagulation, acute renal failure, 'acute lung distress'.

shown, in vitro, to cleave human IgG (Fick et al. 1985); this was observed in both nonmucoid and mucoid variants of P.aeruginosa. Moreover, elastase inactivates complement components C1-C9 and also the human plasma 1 proteinase inhibitor. Examination of CF sputum for direct evidence of the presence of proteases did not reveal either alkaline protease or elastase in 21 patients, although all had high antibody titres to these proteases (Döring et al. 1985).

Exotoxin A. This toxin is a proenzyme with a molecular weight of 60-71.5 K daltons and it is the most toxic extracellular product of P.aeruginosa. Its mode of action is to transfer adenosine diphosphate ribose (ADPR) from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2). Transfer of ADPR to EF-2 irreversibly inactivates the latter and stops peptide elongation and hence protein synthesis in the mammalian cell. The toxin is known to be active, in vivo, and many pathological changes occur including diffuse liver cell necrosis, leucopaenia, pulmonary haemorrhage and renal necrosis.

The role of exotoxin A in CF is equivocal. Animal models have shown that it has a role in primary lung damage (Woods et al. 1982), but free exotoxin A could not be isolated from the sputum of CF patients (Döring et al. 1985). Antibodies towards exotoxin A are produced in CF, which although do not prevent the persistence of the organism in the lung may neutralise the effects of the toxin (Jagger et al. 1982).

Exotoxin A is toxic for human macrophages as phagocytosis by these cells is impaired at concentrations as low as 10 µg/ml. In mice, both T-cell dependent and independent responses are suppressed

(Speert 1985), and if suppression occurs in CF it may facilitate persistent colonisation. Antibody specific for exotoxin A has been demonstrated in immune complexes from CF (Berdischewsky et al. 1980) but antibody towards LPS was more frequently found and to a consistently higher titre.

Exoenzyme S. This has a similar action to exotoxin A, but it has been less well characterised (Nicas et al. 1985). Although it does not appear to be a contributory factor in initial colonisation of rat lung or burned mice, it probably causes tissue damage in the lung and dissemination of the infection.

Inhibition of leucocyte function. Leucocidin is a cell bound protein of molecular weight 27 Kdaltons produced by P.aeruginosa which is toxic for neutrophils and lymphocytes but not erythrocytes or platelets. Another leucocyte inhibitor of 65 Kdaltons is known to exist which is heat-labile and it may inhibit bacterial uptake and intracellular killing by leucocytes. The role of these inhibitors in the pathogenesis of CF lung damage has not been investigated (Sorenson 1984; Speert 1985).

Phospholipase C. This enzyme is one of two haemolytic toxins produced by P.aeruginosa (Liu 1979), the other is a heat-stable glycolipid. In the study of Berka, Gray and Vasil (1981) phospholipase C expression was highest in nonmucoid isolates from CF and mucoid strains were poor producers of the enzyme. Both haemolysins may contribute to the virulence of P.aeruginosa but their role in CF has not been confirmed.

Pyocyanin. The phenazine pigment pyocyanin, is toxic both for mammalian and some bacterial cells. Pyocyanin oxidises NAD and releases oxygen free radicals, in the absence of enzyme action, through the electron transport system. Recently it has been shown (Wilson et al. 1986) that both pyocyanin and its colourless derivative 1,hydroxyphenazine have an effect on human ciliary function. Pyocyanin from CF isolates and other clinical isolates of P.aeruginosa caused slowing of the ciliary beat and dyskinesia. The effect was reversible and the cilia remained viable. The significance of this finding in CF remains to be established but it provides a possible mechanism by which the organism can overcome primary host defences. The pigment is produced in vivo as evidenced by the presence of green colouration of sputum in CF from which the pigment can be readily extracted.

Pyocyanin has been shown to have an antibacterial activity which may suppress normal flora and thus facilitate colonisation of the lung with P.aeruginosa (Cox 1986). Pyocyanin may also participate in the iron metabolism of P.aeruginosa by releasing iron from transferrin hence allowing growth of the organism in an iron restricted environment (Cox 1986).

Aim of the investigation

The purpose of the study was to answer three basic questions;

- 1) What was the molecular basis of the atypical serological reactions of strains of P.aeruginosa from CF sputum specimens and could reagents be prepared to allow their rapid detection?

It was intended to isolate O-antigenic variants from strains of P.aeruginosa with O-specific antigens and to characterise the major epitopes which contribute to the reaction of these strains in conventional agglutination tests. A limited chemical analysis of lipopolysaccharide and protein antigens was made and correlated where possible with their serological activity in a variety of serological test systems. Antisera were prepared against the variant antigens and used routinely along with O-typing sera to detect O-defective and O-specific strains in CF sputa.

- 2) Did the phenotypes of P.aeruginosa isolated from CF patients correlate with clinical status and what was the pattern of colonisation in adult CF patients over a long period?

A longitudinal survey was made of approximately 200 CF patients and the incidence of various bacterial species determined. The frequency of phenotypes of P.aeruginosa in individual patients was compared with available clinical data and correlated with the onset, duration and outcome of infection.

- 3) Was the antibody response of CF patients who were colonised

with serologically typical and/or atypical P.aeruginosa,
directed equally against these forms?

Experiments were carried out in animals to determine the immunogenicity of LPS defective and serologically typical strains of P.aeruginosa. The levels of antibody in patients sera and their specificity for surface epitopes of strains isolated from them were measured and correlated with the clinical status. Bronchial secretions were also examined for hyperimmune antibody to pseudomonas isolates.

MATERIALS AND METHODS

Strains and bacteriophages of *P.aeruginosa*

O-serotype strains

The type strains used in this study for the production of hyperimmune sera and to examine surface macromolecules were those used routinely at the Central Public Health Laboratory. The set corresponds numerically to the IATS and comprises 21 serotypes which are; 01, 02a, 02b, 03, 04, 05d, 06, 06a, 06c, 06d, 07, 08, 09, 010, 011, 012, 013, 014, 015, 016, 017.

Experimental strains

The four strains used to characterise the surface chemistry and serological reactions of *P.aeruginosa* were isolated at the C.P.H.L. They were strain 7, the propagating strain for phage 7 and its variant 7/1 selected by its resistance to phage 7 and strains 18R and 18S isolated from a single sputum sample from a CF patient.

The mucoid strain of *P.aeruginosa* 217M was used for the production of mucoid exopolysaccharide.

Clinical strains

All other strains of *P.aeruginosa* were isolated from the sputa of CF patients attending The Brompton Hospital.

Bacteriophages

The bacteriophages used for typing *P.aeruginosa* were; 7, 16, 21, 24, 31, 44, 68, 73, F7, F8, F10, 109, 119X, 352, 1214, M4, M6, Col 21, Col 11, Col 18, 118/1. In addition the two LPS specific phages E79 and PLS1 were a gift from Dr. A.M.B. Kropinski, Ontario, Canada.

Media, Diluents and Buffers

Media

All media, unless otherwise stated, were prepared by the Media Production Department, CPHL, Colindale or to the manufacturers' instructions in the laboratory. All were sterilised at 121°C for 15 minutes or by filtration. All chemicals unless otherwise stated were Analar^(R) grade and supplied by BDH, Poole, Dorset, U.K.

Tryptone soy broth (Oxoid Ltd, Basingstoke, Hampshire, U.K.)

was used throughout for liquid culture, but where stated Nutrient broth was Oxoid Nutrient broth No.2.

The agar for solid culture was King's A agar which enhances the production of pyocyanin (King, Ward and Raney 1954). Its composition (w/v) was Bactopeptone (Difco Laboratories, Detroit, Michigan, U.S.A.) 20g; Bactoagar (Difco) 15g; glycerol 10 ml; potassium sulphate (anhydrous) 10g; magnesium chloride 1.4g; distilled water to 1L, pH 7.2.

Selective media for the primary plating of sputum samples was Pseudomonas Isolation agar (Difco) and MacConkey agar (Oxoid MacConkey No.3 CM 115). The formulation of Pseudomonas Isolation agar (PIA) was (w/v) Bactopeptone 20g; magnesium chloride 1.4g; potassium sulphate (anhydrous) 10g; Irgasan 0.025g; Bactoagar 13.6g; glycerol 20 ml (v/v); distilled water to 1L, pH 7.0. King's B agar was used for the detection of fluorescein, the formulation was (w/v) Difco proteose peptone No.3 20g; glycerol 10ml; potassium phosphate 1.5g; magnesium sulphate heptahydrate 1.5g; Bacto-agar 11.5g; distilled water to 1L, pH 7.2.

Isolation medium for the recovery of P.cepacia from respiratory secretions of CF patients was as described by Gilligan et al. (1985) the formula was (w/v) ammonium sulphate 1g; magnesium sulphate heptahydrate 0.2g; ferrous ammonium sulphate hexahydrate 0.01g; phenol red 0.02g; Bactoagar 15g dissolved in 950ml of 0.04M potassium phosphate buffer pH 6.2. In order to inhibit the growth of other organisms the following were added to 1L of medium before autoclaving; 1% (w/v) crystal violet (0.1ml); proteose peptone no.3 (Difco) 1.0g; and bile salts (Difco) 1.5g. The medium was heat sterilised at 121°C for 15 min and cooled to 50°C, 50ml of 10% (w/v) pyruvic acid sodium salt was added. Before use sterile solutions of ticarcillin sodium salt (Sigma T-5639, Sigma Chemical Co., Poole, Dorset, U.K.) and polymyxin B sulphate 300,000 U (Sigma P-1004) were added to 1L of medium.

The medium to enhance the production of mucoid exopolysaccharide (MEP) was Mueller-Hinton agar (Difco) containing (w/v) deoxycholate 5g; sodium thiosulphate 5.4g; and glucose 20g to 1L with distilled water.

Phage typing was performed on a medium prepared from Oxoid Nutrient broth No. 2 CM 67 to which was added Oxoid Agar No.1 (1%, w/v) and sodium chloride (0.5%, w/v) and 400 ug/ml (w/v) of calcium chloride. Antibiotic disc sensitivity testing was carried out on Diagnostic Sensitivity Test (DST) agar (Oxoid D.S.T. agar CM 261).

Diluents

Saline was 0.85% (w/v) aqueous sodium chloride and phosphate buffered saline (PBS) was prepared with tablets of PBS reagent (Dulbecco-'A',

Oxoid) dissolved in distilled water to give a final concentration of 0.002M, pH 7.4.

Ringer Solution was prepared from tablets (Oxoid BR52). A tablet was dissolved in 500 ml distilled water to give a "quarter strength" working concentration. Hanks Solution was prepared aseptically from stock solutions of Hanks A and Hanks B powders (Oxoid BR 19d) and sterilised by heat before use.

Buffers

Electrophoresis Buffers

Stacking gel buffer was 0.25M Tris (Tris (hydroxy-methyl) methyl-amine) and 0.224M sodium phosphate adjusted to pH 7.8 with concentrated HCl.

Resolving gel buffer was 0.375M Tris-HCl pH 8.8, the reservoir buffer was prepared from a 10 times concentrated stock solution which contained Tris 30.3g; glycine 144g; sodium dodecyl sulphate (SDS) 10g; distilled water to 1L, pH 8.6. Solubilising buffer was 0.1M Tris-HCl buffer with 2% (w/v) SDS; 20% (w/v) sucrose; 1% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue; pH 6.8.

Buffers for ELISA

Antigens under investigation were prepared by suspension to a working dilution in coating buffer, (carbonate-bicarbonate buffer) the formulation (w/v) of which was ; magnesium chloride 1.964g; sodium carbonate 1.6g; sodium bicarbonate 2.9g; sodium azide 0.2g; distilled water to 1L, pH 9.6. The coating buffer for the MEP ELISA was Sorensen's buffer (pH 6.8) diluted to give a 0.04M sodium phosphate solution (Mercia-Brocades Ltd, West Byfleet, Surrey, U.K.) purified MEP and commercial seaweed alginate were diluted in this

buffer to give a working solution of 1.2 or 0.24 ug per well, (6 or 1.2 µg/ml).

Washing buffer and antibody diluent for the procedure (PBST) was PBS containing 0.05% (v/v) Tween 20, pH 7.4.

Substrate buffer was 0.1M citric acid 24.3 ml; 0.2M sodium dihydrogen-phosphate 25.7 ml; orthophenylenediamine (Sigma P-1526) 34mg; distilled water to 100 ml, pH 5.0. Hydrogen peroxide (40ul) was added to the substrate buffer immediately prior to use.

Buffers for 'Western Blotting'

The transfer buffer for the electrotransfer of cell wall components from polyacrylamide gel to nitrocellulose sheets was (w/v); Tris 3.03g; glycine 14.4g; methanol 200 ml; distilled water to 1L, pH 8.3. Tris-buffered saline (TBS) used for the modified ELISA technique was (w/v) Tris 1.21g in 1L of saline adjusted to pH 7.4 with HCl. Primary incubation buffer was Tris-buffered saline containing 3% (w/v) bovine serum albumin (BSA). Conjugate was incubated in secondary incubation buffer which contained; Tris-buffered saline, 3% (w/v) bovine serum albumin and 0.05% (v/v) Nonidet p-40 (Sigma N-6507). The reaction was developed with 'chloronaphthol', which was 4-chloro-1-naphthol (Sigma C-8890) 50mg; methanol 16.6ml; Tris-buffered saline 83ml; 0.33ml of hydrogen peroxide was added immediately before use.

Stains

Coomassie blue stain. Coomassie blue stain for the detection of protein was Coomassie brilliant blue 0.1% (w/v) in glacial acetic acid 10% (v/v) and methanol 25% (v/v), (Sigma B-0630), in distilled water. The destain solution was glacial acetic acid 10% (v/v) and

methanol 25% (v/v) in distilled water.

'Amido black' stain. For the detection of protein on nitrocellulose sheets the 'amido black' stain was 0.1% (w/v) naphthalene black, glacial acetic acid 10% (v/v) and methanol 45% (v/v) in distilled water. The nitrocellulose sheets were destained in distilled water.

Reagents for silver stain. The staining solution was; 0.1M sodium hydroxide 28ml, ammonia solution 2ml; to which was added dropwise, with continuous stirring 20% (w/v) silver nitrate 5ml; distilled water to 150ml. Formaldehyde developing solution contained citric acid 50mg, 37% (v/v) formaldehyde solution 0.5ml to 1L with distilled water.

Characterisation of surface macromolecules

Acetone-dried cells

Mass culture for the preparation of dried bacterial cells was made as follows. Six polypropylene trays of dimension 30cm x 25cm x 5cm (Payne's Scientific Apparatus, Clapham, London) were used for each of the strains. The trays were washed in 1% (v/v) 7x detergent, rinsed thoroughly and dried. They were covered with sheets of aluminium foil, secured with tape and sterilised at 121°C for 15 min. Each tray was filled with approximately 750ml of nutrient agar to a depth of about 3cm. The agar was dried at 37°C for 1h and 2ml of an overnight broth culture of the strain was spread evenly over the surface with a sterile cotton swab.

The bacterial growth was harvested in distilled water after incubation at 37°C for 18h. The cells were deposited by

centrifugation at 6000g for 30 min. The supernatant was discarded and replaced with cold (-20°C) acetone. The acetone suspension was mixed thoroughly and the cells were allowed to settle for about 1h. The acetone was removed and the procedure repeated twice more. The final deposit of cells was suspended in approximately 20ml of acetone and this was rapidly poured into open glass trays and the solvent was driven off in a fume cupboard. The dry cells were collected, weighed and stored in a dessicator over calcium chloride.

Phenol-water extraction of LPS

Acetone-dried cells were suspended to approximately 6% (w/v) in distilled water and incubated at 37°C overnight to ensure complete dispersion. The suspension was heated at 65°C and an equal volume of 90% (w/v) crystalline phenol solution at 65°C was added. The extract was thoroughly mixed throughout incubation in a waterbath at 65°C for 15 min. The container was rapidly cooled in running water and then centrifuged at 6000g at 4°C for 30 min. The extract was cooled at 4°C for 3-4h to allow separation of the phenol and aqueous layers to occur. The top, aqueous, layer was pipetted off and the same volume of water was added to the phenol layer to repeat the extraction. The aqueous layers were pooled and centrifuged at 10,000g, 4°C for 30 min. and then dialysed extensively against running water for 2-3 days to remove all traces of phenol.

The dialysate was collected, clarified by centrifugation and ribonuclease and deoxyribonuclease were added to a final concentration of 30 µg/ml. The extract was incubated at 37°C for 1-2h before centrifugation at 10,000g, 4°C for 1h to remove peptidoglycan. LPS in the supernatant was deposited by

centrifugation at 100,000g for 3h at 4°C and if a clear gelatinous pellet was obtained this was recovered in 1ml of distilled water and lyophilised. In the event of the pellet showing brownish contamination it was resuspended in 5ml of water and centrifuged at 25,000g for 1 h followed by 100,000g for 3h.

Ethanol extraction of LPS

The method was as described by Darveau and Hancock (1983) but with the following modifications.

Acetone-dried bacterial cells (1g) were suspended in 30ml of buffer containing 10mM Tris, 2mM magnesium chloride pH 8 at 37°C overnight. Deoxyribonuclease (DNase) 100 µg/ml (w/v) and ribonuclease (RNase) 25 µg/ml (w/v) were added and the suspension was sonicated at 4°C for 4 cycles of 3 min duration with 3 min cooling in an ultrasonic disintegrator at an amplitude of 14 µ with an extrapotential microprobe (MSE Scientific Instruments, Crawley, W.Sussex).

DNase 200 µg/ml (w/v) and RNase 50 µg/ml (w/v) were again added to the lysate and it was incubated at 37°C for 2 h. To this was added 10ml of 10mM Tris-HCl pH 8 containing 0.5M tetrasodium ethylenediamine tetraacetic acid (EDTA), 5ml of SDS 20% (w/v) in Tris-HCl pH 8 and 5ml of Tris-HCl pH 8 to give a final volume of 50ml. The solution was vortexed and centrifuged at 50,000g, 20°C for 30 min. to remove peptidoglycan.

Pronase 200 µg/ml (w/v) was added to the supernatant and this was incubated overnight at 37°C with shaking. Crude LPS was precipitated, in ice, by the addition of 100ml of 0.375M magnesium chloride in 95% (v/v) absolute ethanol. The precipitate was collected by centrifugation at 12,000g, 4°C for 15 min.

The pellet was dissolved in 50ml buffer which contained 2% (w/v) SDS, 0.1M tetrasodium EDTA in 10mM Tris-HCl pH 8. The suspension was sonicated for two cycles of 30s each at 4°C. The sonicate was incubated at 85°C for 30 min and after cooling 25 µg/ml pronase was added and incubation was continued overnight at 37°C with agitation. LPS was precipitated in 150ml of 95% (v/v) absolute ethanol and collected by centrifugation as described above. The precipitate was dissolved in 30ml of Tris-HCl and sonicated again for two cycles of 30s and centrifuged at 1000g for 5 min to remove Mg²⁺ EDTA complexes. The supernatant containing LPS was centrifuged at 200,000g, 15°C for 2h in the presence of 25mM magnesium chloride. The LPS pellet was dissolved in 1ml of distilled water and lyophilised.

Trichloroacetic acid extraction of LPS

Approximately 5g of acetone dried cells were suspended in 100ml of distilled water and the suspension was stirred at 37°C stirred for 2h to allow complete dispersion. An equal volume of 10% (v/v) trichloroacetic acid was added and following incubation for 18h at 4°C the mixture was centrifuged at 5000g, 4°C for 1h. The supernatant was dialysed against running water for 48h and the dialysate was concentrated with solid polyethylene glycol (carbowax) at room temperature overnight.

RNase and DNase were each added to a final concentration of 3 µg/ml (w/v) and after incubation at 37°C for 1-2h the extract was centrifuged at 16,000g, 4°C for 45 min and the supernatant was lyophilised.

Preparation of outer membrane protein

The overnight growth from 3 King's A agar plates (approximately 3g

wet weight) was suspended in 9ml of distilled water. The cells were broken by sonication, (at 4°C); 3 cycles of 60s each with 30s cooling. To the lysate was added 1ml of 20% (w/v) sodium N-lauryl sarcosine sodium salt (Sigma L-5125) and after 30 min at room temperature the unbroken cells were removed by centrifugation at 5000g, for 10 min. The outer membranes in the supernatant were pelleted by centrifugation at 45,000g, 4°C for 30 min and the pellet was washed once with water, resuspended in 0.5ml of distilled water and stored at -20°C.

Preparation of purified MEP

The method was adapted from that of Bryan, Kureishi and Rabin (1983). The mucoid NCTC strain 217M was grown on plates containing media to enhance the production of MEP at 37°C for 7 days. The growth was harvested in 5-10ml of PBS per plate. The growth from 5 to 10 plates was combined and digested with (w/v) RNase (150 µg/ml) and DNase (150 µg/ml) at 37°C for 2h with continuous agitation. Pronase 1.5 mg/ml (w/v) was added and incubation was continued at 37°C overnight with stirring every 20 min for the first 2h. The preparation was centrifuged at 10,000g for 15 min and the crude MEP in the supernatant was precipitated by the addition of 4 volumes of 80% (v/v) ethanol. The precipitate was removed from the solution by winding it round a glass rod and redissolved in distilled water to give a concentration of 1mg MEP per ml (w/v). Calcium chloride 0.1% (w/v) was added and the crude MEP was centrifuged at 150,000g for 2h before the supernatant was dialysed against running water for 8h. The supernatant was concentrated with carbowax to 5mg/ml (w/v) MEP. Sodium chloride was added to obtain a final concentration of 9g/L and

precipitation in 80% (v/v) ethanol was repeated. The precipitate was collected by centrifugation at 30,000g for 30 min and lyophilised.

The resulting MEP was analysed for contamination with protein and KDO and the final precipitation step was repeated until these were undetectable.

Chemical analysis of LPS

Detailed chemical analysis of the LPS from strains 7 and 7/1 was carried out by Dr Stephen Wilkinson, Chemistry Department, University of Hull. The procedures described below were those used by the author to assess the quality of LPS prepared in this laboratory.

Total carbohydrate. Carbohydrate was measured as total hexose by the anthrone reaction of Trevelyan and Harrison (1952) against standards prepared from 0-250 µg/0.5ml of D-glucose and read at 625nm.

Protein content. Protein content was determined by the biuret method of Lowry et al. (1951). A standard curve was prepared with BSA to give a protein range from 0-200 µg/ml and the reaction was read at 750nm.

Determination of KDO. The KDO content of samples was determined by the thiobarbitone acid procedure of Osborn (1963). A KDO standard was kindly provided by Dr S. Wilkinson and LPS samples were read at 40°C at a wavelength of 548nm.

The concentration of KDO was calculated as below :-

$$\text{Concentration of KDO in test} = \frac{\text{absorbance of test}}{\text{absorbance of standard} \times 1}$$

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

Electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) in a discontinuous buffer system with a 12.5% acrylamide resolving gel, the stacking gel was 5% acrylamide modified from the method of Palva and Mäkelä (1980).

The equipment for SDS-PAGE comprised two clean glass plates (160mm x 160mm, 5mm thick). In one of the plates an indentation 130mm x 20mm was made. Perspex spacer strips 1.5mm thick were placed between the two plates and these were secured with clips. The edges of the plates were sealed with molten agar.

The resolving gel contained acrylamide:bisacrylamide (30:0.08) 12.5ml, resolving gel buffer 3.75ml, 10% (w/v) SDS 0.3ml, 1.5% (w/v) ammonium persulphate 1.5ml, distilled water to 30ml, pH 8.8. The solutions were thoroughly mixed under vacuum in a fume cupboard for 15 min and TEMED (NNNN' tetramethylethylenediamine, Sigma T-8133) (15 µl) was added to the deaerated mixture.

Approximately 15ml of the solution was pipetted between the glass plates to form a gel of 120 x 80mm (1.5mm thick). A small volume (1-2ml) of distilled water was gently run on to the top of the gel and polymerisation occurred at room temperature within 30 min.

The stacking gel was as follows acrylamide: bisacrylamide (30:0.08) 3.3ml, stacking gel buffer 5.0ml, 10% (w/v) SDS 0.2ml pH 7.8. The solution was deaerated and mixed under vacuum for 15 min before the addition of 15 µl of TEMED.

The water was removed from the polymerised resolving gel and the

surface rinsed with stacking gel solution. Approximately 10ml of stacking gel was pipetted on to the resolving gel to give a depth of about 2cm and a perspex 'comb' (120mm x 25mm, 1.5mm thick) was placed in the stacking gel to form the sample wells. The gel was allowed to set at room temperature for 1h.

The comb, bottom spacer and clips were removed and the gel was clamped to a perspex electrophoresis tank, the chambers of which contained reservoir buffer.

Samples for electrophoresis, 10-15 μ l in solubilising buffer at a concentration of 5mg/ml, were applied to the wells in the stacking gel with a Hamilton syringe 701-NW9 (Hamilton Co., Reno, Nevada, USA). Electrophoresis was carried out at a constant current of 28mA per gel at 50-200V at room temperature for 4h or until the bromophenol marker dye had migrated within 1cm of the bottom of the gel.

Molecular weight standards. Standards for molecular weight calculation were contained in the Low Molecular Weight Calibration Kit (Pharmacia, Uppsala A, Sweden) and included phosphorylase b mol wt 94,000, albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, trypsin inhibitor 21,000, lactalbumin 14,000; as a lysophilised mixture. When reconstituted in sample buffer, 10 μ l of the mixture was applied per gel.

LPS standard. Commercially prepared LPS from Salmonella typhimurium (Sigma L-7261) was suspended in solubilising buffer to a concentration of 5mg/ml for use as a control in SDS-PAGE and 10-15 μ l were applied to each gel.

Proteinase K extraction of LPS

The procedure was modified from that of Hitchcock and Brown (1983). Wet bacterial cells c.6mg were harvested from a overnight culture on King's A agar and placed in an Eppendorf centrifuge tube (Eppendorf Gerätebau Netheler + Hinz GmbH, Hamburg, W.Germany) and thoroughly mixed in 50 µl of solubilising buffer. The samples were heated at 100°C for 10 min and after cooling, 10 µl of a stock solution of proteinase K (25mg/ml) was added. The tubes were incubated in a water bath at 60°C for 1h and centrifuged in an Eppendorf (5414S) centrifuge at maximum speed (15,600g) for 3 min to remove cell debris. The supernatants were transferred to fresh tubes and boiled for a further 10 min to destroy residual enzyme activity. The samples were applied directly to SDS-PAGE using volumes of 10-20 µl or stored at 4°C.

Extracts of whole cells for SDS-PAGE. Whole bacterial cells were deposited from 3ml of an overnight broth culture, resuspended in 50 µl of solubilising buffer and boiled for 15 min. The samples were used immediately or stored at 4°C.

Staining of lipopolysaccharide in SDS-PAGE. Lipopolysaccharide was detected by the silver staining procedure of Tsai and Frasch (1982) and was carried out in thoroughly cleaned glass dishes; 200mm diameter, 40mm deep. All solutions were freshly prepared and gloves were worn throughout for the handling of gels.

Gels were fixed overnight in 150ml of a 40% (v/v) ethanol, 5% (v/v) acetic acid solution at room temperature. Periodic acid 0.7% (w/v) was added to the solution and the gel was agitated gently for 5 min.

The oxidising solution was replaced with c.200ml of distilled water and the gel was washed in repeated changes of water with gentle shaking in a waterbath at 30°C for 2h. The water was poured off, 150ml of silver stain solution was added and the gel was vigorously agitated at 30°C for 10 min. The gel was transferred to a clean glass dish and washed repeatedly in changes of distilled water and with gentle shaking. Formaldehyde developing solution (200ml) was added, the reaction was stopped in distilled water and the gel was photographed immediately.

Staining for protein. Gels were placed overnight in 150ml of Coomassie blue stain at room temperature. The stain was removed and replaced with the same volume of destain solution. Destaining was performed at room temperature with constant agitation and pieces of sponge foam were added to the solution to accelerate the removal of the stain. The destain solution was changed at least twice and destaining continued until the background was clear. The gels were photographed or dried in a slab gel drier (Bio-Rad Laboratories, Watford, Hertfordshire, U.K.)

Seroreactions of surface macromolecules

Preparation of O antisera

O antiserum was raised by the injection of rabbits with heat killed cells. The vaccine strain was grown in 20ml of broth in a 100ml bottle at 37°C overnight and the cells were deposited by centrifugation at 6000g for 30 min, the deposit was taken up in 10ml of saline and boiled for 2h. The opacity of the suspension was adjusted by dilution to contain approximately 2×10^9 cells per ml

with reference to standard opacity tubes (Wellcome Diagnostics, Dartford, U.K.). Rabbits were injected intravenously every 3-4 days with; 0.25ml, 0.5ml, 1.0ml, 1.5ml and 2.0ml of vaccine. Rabbits were test bled from the marginal ear vein (at 18-20 days) and the titre was determined by slide agglutination. If the titre was satisfactory the animals were sacrificed and bled out by heart puncture.

Titration of serum

Titration of freshly prepared serum was prepared by forming a one in ten dilution of the serum in 0.5ml of saline and then performing subsequent doubling dilutions to 1 in 1000. A drop (20 μ l) of each dilution was placed on a clean glass microscope slide and a portion of growth from an overnight plate of the vaccine strain was emulsified with a toothpick, in each drop. The slide was rotated gently for 30 seconds and the highest dilution in which complete agglutination occurred was considered to be the reciprocal of the titre of the serum.

For routine use in agglutination tests, serum was diluted in saline to that concentration which was four times higher than the homologous titre. The serum was sterilised by passing it through a sterilising-grade membrane filter (Gelman Sciences Ltd, Bracknell, Northamptonshire, U.K.).

Preparation of hyperimmune serum to MEP

Six high antibody producing (Biozzi) mice were inoculated with 60 μ g of purified MEP in 0.1ml of saline as described by Bryan et al. (1983). After three weeks, three of the mice were sacrificed and bled by heart puncture, the remainder received one further injection

(60 µg MEP in 0.1ml of saline) before they were sacrificed three days later. The serum was separated from the clotted blood and tested for activity to MEP by slide agglutination and ELISA.

Double diffusion in gel

Antisera were used undiluted in the double diffusion test in agar gel (Ouchterlony 1949). A 0.9% (w/v) solution of agar No.1 was heated at 100°C in distilled water and the molten gel (2.5ml) was pipetted on to clean microscope slides (75mm x 25mm). Equi-distant wells (3mm in diameter, 4mm apart) were cut with a template and the agar plugs were removed.

Undiluted antiserum (5 µl) was placed in the central well and 5 µl of antigen solution was added to each of the surrounding wells. The slides were placed in a moist chamber at 4°C and examined daily for up to 3 days. Where necessary lines of precipitation were enhanced by staining with Coomassie blue.

Indirect haemagglutination test

Sera were inactivated at 56°C for 30 min and absorbed with thrice washed sheep red blood cells (SRBC) by the addition of 100 µl of serum to 0.9ml of 10% (v/v) SRBC suspension. Fifty µl of LPS (1mg/ml) was added to 0.3ml of saline and 0.15ml of 10% (v/v) SRBC. The mixture was incubated at 37°C for 1h with shaking in a water bath and the sensitised cells were washed three times in saline by low speed centrifugation and resuspended in 5ml of PBS containing 0.1% (w/v) bovine serum albumin (BSA). Control mixtures not containing antigen were included in each batch of tests.

Sera were diluted serially in PBS-albumin by doubling dilutions of 25 µl in 'U' well microtitre trays (Dynatech Laboratories Ltd,

Billingshurst, W.Sussex) and an equal volume of sensitised SRBC was added to each of the serum and control wells. The trays were shaken gently and incubated first at 37°C for 2h and then at 4°C overnight.

ELISA

Flat bottomed microtitre plates (M29A; Sterilin, Feltham, Middlesex) were coated at room temperature overnight with 100ul of sample, in coating buffer pH 9.6, per well. For the MEP ELISA, samples in 200 ul coating buffer pH 7.0 were applied per well. The plates were washed three times in PBST with a plate washer (Titertek-Flow Laboratories) and drained thoroughly before the addition of 100ul of primary antibody, diluted serially in PBST to each well. The plates were covered and incubated at 37°C for 2h. To ensure complete removal of unbound antibody the plates were washed 5 times in PBST, before the addition of 100 µl of peroxidase conjugated anti-immunoglobulin antibody (Dako Immunoglobulins, Copenhagen, Denmark), diluted 1 in 1000 in PBST, to each well. The plates were reincubated at 37°C for 2h and washed with three changes of buffer. The reaction was developed with 100 µl of O-phenylenediamine per well and after 15 min the reaction was stopped by the addition of 50 µl of 12.5% (v/v) sulphuric acid.

The absorbance was read at 450 nm on a plate scanner (Multitek-Flow Laboratories, Rickmansworth, Hertfordshire, U.K.) and the data were stored on a computer disc (BBC microcomputer). The antibody titre was calculated as the reciprocal of that dilution which gave 50% of the maximum absorbance after adjustment for background absorbance had been made.

Competitive ELISA. The procedure was as for the standard ELISA except that the primary antibody was used at a constant dilution of 1 in 500 with the competing antigen in varying concentrations. One hundred microlitres of each were added simultaneously to the wells and incubation proceeded as above. Control wells were included which contained no competitive antigen and the absorbance value of these wells was taken as 100% reaction. The per cent inhibition of the reaction by competing antigens was calculated by comparison of the controls with the absorbance values of the test wells.

Whole cell preparations for ELISA. Bacterial cells harvested from the overnight growth on King's A agar were adjusted by dilution to give an optical density of 0.06 at 600 nm in carbonate buffer pH 9.6. The samples were stored at -20°C before use.

LPS preparations for ELISA. Purified LPS was diluted in carbonate buffer pH 9.6 at a concentration of 10µg/ml.

'Western Blotting'

On completion of electrophoresis the gel was removed from the glass slides and placed on a sheet of nitrocellulose of a similar size as the gel submerged in transfer buffer. Care was taken to ensure that no air was trapped between the gel and the nitrocellulose. Squares of filter paper (Whatman Chromatography No.17, Whatman Ltd., Maidstone, Kent, U.K.), were placed either side of the gel and nitrocellulose and the 'sandwich' was completed by Scotchbrite pads (Bio-Rad) saturated in transfer buffer. The sandwich was secured in the gel-holder (Bio-Rad) and placed in the Trans-BlotTM cell

(Bio-Rad) with the nitrocellulose nearest the anode.

The cell was filled with cold (4°C) transfer buffer and electrophoresis was performed at a constant voltage of 28V (0.12-0.16 amps) overnight at 4°C.

Staining of nitrocellulose sheets. For the detection of protein, nitrocellulose sheets were removed from the blot cell and were immersed in amido black stain for 10 min. They were destained in repeated changes of distilled water. The sheets were dried between filter paper and photographed.

Immunoblotting. The nitrocellulose sheet was removed from the blotting equipment and immersed in primary incubation buffer using the minimum volume which completely covered the sheet. Non specific binding sites in the nitrocellulose were blocked with BSA with gentle shaking at room temperature for 20 min. The primary antibody was diluted in this buffer and the sheet was incubated at room temperature for 2h with continuous slow agitation.

The sheet was washed at 4°C in 5 changes of TBS for 30 min. Peroxidase labelled conjugate, anti-immunoglobulin antibody, (Dako-Immunoglobulins) was diluted 1 in 1000 in secondary antibody buffer and the sheet was incubated in this solution at room temperature for 2h with shaking. The sheet was washed as before and 'chloronaphthol' was added to develop the reaction, which was stopped with distilled water after 5-10 min. The sheet was dried between filter paper and photographed.

'Dot-Blots'. Five µl of an overnight broth culture was spotted on to nitrocellulose paper and dried in air at room temperature. The

nitrocellulose sheet was used immediately or stored at -20°C. The reaction for the detection of immunoreactive colonies was as described for immunoblotting.

Longitudinal clinical and bacteriological survey

Patients

From September 1983 to September 1985, sputum samples were collected from 210 CF patients attending the Brompton Hospital, London. The patients ranged in age from 12-61 years and the majority were attending the hospital for routine assessment as outpatients, but some were hospitalised either because of clinical deterioration or for alterations in their treatment.

All clinical data was collected at appointment by Dr M E Hodson and her staff, the logging and transmission of samples to Colindale was supervised by Sister F Duncan.

A number of clinical factors were recorded for each patient and these included their age at diagnosis, dates of first chest symptoms and continuous sputum production, the first occurrence of P.aeruginosa in the sputum and the incidence of S.aureus and/or H.influenzae. The patients' weight and any relevant antibiotic therapy over the course of the investigation was also recorded. This included the number of courses of intravenous, nebulised and orally administered drugs. The presence of diabetes and allergic hypersensitivity reactions was also noted. Lung function was recorded as forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁), on a Vitalograph Spirometer, reading from the ATPS (ambient temperature and pressure) scale. Where lung function was recorded as per cent

predicted this was calculated with the use of nomograms relating indices of lung function to stature and age for healthy adults of European descent (Cotes 1979).

Collection of sputum

Sputum samples were collected following physiotherapy or natural cough, by staff at the hospital and were frozen at -70°C and transported to Colindale in ice. Included with each specimen were details of the name, age and sex of the patient, lung function (FVC and FEV₁) values and current antibiotic therapy.

Serum samples

If a routine blood test was to be performed on the patient an aliquot (1.5ml) of the serum collected from clotted blood was stored at -70°C before transportation to Colindale. Serum samples remained frozen until use in ELISA or immunoblotting experiments.

Bacteriological examination of sputum

Samples of sputum were processed directly on receipt at the laboratory or were stored at -20°C and examined within 48h. Sputum was processed in the Category B laboratory in accordance with Safety Regulations (Code of Practice for the Prevention of Infection in Clinical Laboratories and Post Mortem Rooms). Samples were thawed at room temperature and liquefied by the addition of approximately 0.1g/ml of pancreatin (Rawlins 1953), and incubated at 37°C for 2h. A loopful (5mm diameter loop) of each digested sample was streaked on to one plate each of King's 'A' agar, PIA and MacConkey agar. Plates were incubated at 37°C for 48h, aerobically. Early in the survey chocolate agar was included for the isolation of Haemophilus sp. but due to the large number of samples it was decided to abandon

this practice and obtain data on the incidence of H.influenzae from the Brompton Hospital as routine microbiology was performed there on duplicates of the samples sent to Colindale.

The plates were examined visually for bacterial growth and the number of Pseudomonas colonies on PIA and King's A agar were quantified crudely as 3+, 2+ or 1+. In order to obtain an approximation of the number of colony forming units (cfu) per ml of sputum, pilot experiments were carried out in which overnight broth cultures of mucoid and nonmucoid strains of P.aeruginosa were centrifuged at 1000g for 20 min. The cells were resuspended in 3ml of Ringer solution and diluted logarithmically in 0.5ml of Ringer solution. One drop 0.02ml from each dilution was spotted on to King's A agar and a loopful (5mm diameter) of each was also spread over the surface of a King's A agar plate. When dry the plates were incubated at 37°C for 48h. The number of colony forming units/ml was calculated at each dilution (Miles, Misra and Irwin 1938) and the growth from the spread plates was compared visually with the 3+, 2+ and 1+ growth from CF sputum. The number of cfu/ml in the CF sample could then be estimated.

Preparation of sol phase

Before the addition of pancreatin c.2ml of sputum was pipetted into an Eppendorf tube. The tube was centrifuged at maximum speed (15,000g) for c.10 minutes. The resulting clear supernatant (sol) was removed and stored at -20°C. The remaining material was resuspended in 1ml of PBS and centrifuged again. These steps were repeated three times to ensure that all soluble components had been removed from the sputum. All aliquots were stored at -20°C before

use in dot blots and ELISA.

Bacterial identification

Colonies that produced the 'diagnostic' blue-green or yellow-green pigment on King's A agar or PIA were classified as P.aeruginosa and subcultured for storage on nutrient agar slopes at room temperature and in 10% (w/v) skimmed milk at -20°C. Colonies which grew on PIA but were not pigmented were presumptively identified as Pseudomonas sp. and stored as above for further identification. Lactose and non-lactose fermenting bacteria on MacConkey agar which were unlikely to be Pseudomonas sp. were also subcultured for further tests. Yellow pigmented and non-pigmented staphylococcal-like colonies were picked from King's A agar and subcultured on nutrient agar.

The strategy for the identification of Pseudomonas, Staphylococci and enterobacteria was as follows. Non-pigmented colonies that grew on PIA were tested by the scheme of King and Phillips (1978). This separated species into the fluorescent pseudomonads; P.aeruginosa, P.fluorescens and P.putida on the basis of their oxidative metabolism of glucose (Hugh and Leifson 1953), production of cytochrome oxidase and production of fluorescein on King's B agar.

Non-fluorescent oxidative species were tested in the API 20 NE system (API Basingstoke, Hampshire) and identified according to the manufacturers' instructions.

Colonies presumptively identified as staphylococci were plated on nutrient agar and after incubation at 37°C for 18h were examined for pigment and identification of Staphylococcus aureus was confirmed by Gram stain and tube coagulase test.

Coliform bacteria from MacConkey agar were identified by the API 20 E

system.

Gram-negative bacteria which gave atypical biochemical reactions were submitted to the Computer Trials Laboratory at Colindale for further identification.

Classification of *P.aeruginosa*

Colonial morphology. The colonial morphology of each isolate of *P.aeruginosa* was recorded according to the numerical scheme of Wahba and Darrell (1965), the colour of pigment produced and the presence of autolysis were noted.

Serum sensitivity. Normal human serum (NHS) was obtained by venepuncture of healthy volunteers. Serum was collected from clotted blood and samples from at least two individuals were pooled. The pooled serum was dispensed in 1-2ml aliquots and stored immediately at -70°C for use within 1 month.

The sensitivity of isolates to the bactericidal activity of NHS was determined by the method of Penketh et al. (1983^b). Fifty µl of a vortexed overnight (37°C, 18h) broth culture was added to 5ml of Hanks solution (HBSS) and mixed thoroughly. A 100 µl sample of this was mixed with an equal volume of NHS and incubated at 37°C for 1h, with shaking, in a water bath. Control tubes contained 100 µl of diluted bacterial suspension and 100 µl of HBSS.

Following incubation, 20 µl of both the test and control solutions were spotted on to King's A agar. A further 20 µl of each was added to 1ml of HBSS and 20 µl of this was applied to plates. When the spots had dried the plates were incubated at 37°C overnight.

The colonies in each spot were counted and the serum sensitivity of cultures was expressed by comparing the growth from the test and

control samples. Serum sensitive cultures were defined as those that yielded no more than 50 colonies from 20 ul of the undiluted test sample and gave no growth when diluted. Partially sensitive cultures were those that yielded 51-200 colonies from the undiluted samples but on dilution gave semi-confluent growth. Resistant cultures grew equally well in both test and control samples. Reduced resistance was recorded if the growth from the test sample was not quite confluent on dilution.

Serotyping of P.aeruginosa. Isolates of P.aeruginosa were serotyped by slide agglutination with the 17 standard antisera used in the Division of Hospital Infection which corresponded numerically to the IATS serotypes. Antisera were raised in rabbits by the method described by Pitt and Erdman (1978). O-typing sera were not prepared by the author as adequate laboratory stocks were available.

The slide agglutination test was performed by emulsifying a portion of bacterial growth taken directly from agar with a toothpick in a drop of antiserum, on a clean glass microscope slide. The slide was rotated for approximately 30 s and the strength of agglutination reactions was recorded as follows;

- 3+: heavy coarse or completely flocculent agglutination, clear supernatant.
- 2+: moderate agglutination with many cells remaining in suspension.
- 1+: fine agglutination with slightly turbid background fluid.

(Pitt 1979).

All cultures from CF patients were tested in saline to detect non emulsifiable and autoagglutinable isolates. For typing, cultures were tested first with four pools of antisera each of which contained

four to five antisera specific for each serotype. Cultures which gave agglutination in a single pool were serotyped with each of the individual sera that comprised the pool.

Phage Typing. The typing procedure was that described by Asheshov (1974). Isolates of P.aeruginosa in overnight broth culture were flooded onto plates of phage typing agar (PTA). The excess fluid was pipetted off and the plates were dried in air at room temperature for approximately 20 mins. Phage filtrates at Routine Test Dilution (RTD) - that dilution of phage which just failed to give confluent lysis of its propagating strain - were spotted onto the dried lawns and the plates were incubated at 32°C overnight. Phage lytic reactions were scored as follows:-

Strong (++; > 50 plaques - confluent lysis)

Moderate (+; 20-50 plaques)

Weak (+; < 20 plaques)

Determination of Antibiotic Sensitivity

Disc sensitivity test. An overnight broth culture was diluted 1 in 3000 in 10 ml of Ringer solution with the aid of a standard 3mm loop. DST plates were flooded and the excess fluid was removed with a pipette, the plates were dried in air at room temperature for approximately 15 min. The following antibiotics were then tested, by placing antibiotic-impregnated filter paper discs on the bacterial lawn; amikacin (30 µg), azlocillin (75 µg), carbenicillin (100 µg), chloramphenicol (50 µg), mezlocillin (75 µg), latamoxef (moxalactam) (30 µg), (Oxoid) and cefotaxime (30 µg), ceftazidime (30 µg) and cefsulodin (30 µg) (Mast Laboratories, Liverpool U.K.). The plates

were incubated overnight and the diameter (mm) of the zone of inhibition was measured. Isolates were classified as resistant, intermediate resistant and sensitive with reference to the zone diameters recommended by the manufacturers.

Determination of minimum inhibitory concentration. Commercially prepared microtitre plates (Seward Laboratory, London, U.K.) containing increasing concentrations of antibiotics (mg/ml) were used. An overnight broth culture of the strain under test was thoroughly mixed and diluted 1 in 100 in double strength broth, to give approximately 5×10^6 cells/ml, 25 μ l of this suspension was added to each of the wells which contained a similar volume of antibiotic. The plates were incubated overnight at 37°C and read with the aid of a mirror (Dynatech) in oblique light. The lowest concentration of antibiotic that inhibited the growth of the culture as judged by a lack of turbidity was taken as the minimum inhibitory concentration (MIC).

Pyocin typing:

The method of Gillies and Govan (1966) was used.

RESULTS

SECTION I

Characterisation of antigenic variants of *P.aeruginosa*

Earlier studies had shown that strains of *P.aeruginosa* isolated from CF gave atypical serological reactions with O-typing sera in that many expressed polyagglutinability or were non-typable. It was considered necessary to examine the surface macromolecules which confer antigenicity on the cell and determine if these altered during the course of infection and expressed new or variant antigens.

To facilitate the identification and characterisation of *P.aeruginosa* from CF, laboratory variants similar in antigenic characteristics to those isolated from CF were produced and basic chemical and serological studies were performed. The variants were selected by the action of phage on a parent strain which expressed serologically-typical surface antigens and sub-populations of this strain which lacked the attachment site for the phage were selected by their resistance to the isolating phage.

Selection of O-antigenic variants

Polyagglutinating Variant (PA). Phage 7 was used to select variants of its propagating strain. Resistant colonies that grew in the area of near confluent lysis were selected and purified by subculture on agar over 3 successive days. A representative culture, derived originally from a single phage resistant colony, was termed variant strain 7/1.

Strain 7/1 was resistant to phage 7 and to the other phages active on the parent strain including the LPS specific phages (E79 and PLS1), suggesting a considerable alteration in the surface receptors for these phages. The pyocin type of both strains was identical,

providing confirmation that strain 7/1 was truly a variant of strain 7 (Table 5).

The resistant colonies were tested with the O-typing sera by slide agglutination. The propagating strain 7 was serotype 06 but the resistant colonies were polyagglutinable, PA, (see Figure 3). The titres of both parent and variant strains with the O-sera are given in Table 6. The variant was strongly PA and reacted with seven O-typing sera (01, 03, 05d, 06, 09, 010 and 013) and was classified as PA, while the parent was agglutinated specifically by serum 06. Strain 7/1 was promptly sensitive to the bactericidal action of normal human serum and a decrease in viable count of 4-5 log was observed after incubation with serum for 1h at 37°C.

Non-typable Variant (NT). During the course of the serological typing of a clinical isolate from a patient with CF it was observed that about 1 in 20 colonies gave a strong agglutination reaction with serum 05d (titre 160) but the other colonies did not react with this antiserum. The non-typable colonies were promptly sensitive to human serum but in repeated tests some colonies emerged which were resistant to serum and expressed antigen 05d.

Two cultures were selected, one serum resistant and the other sensitive, for further study as they represented two serological states within a single clinical isolate. They were termed 18R (resistant) and 18S (sensitive). The two strains were stable after several subcultures and had the same pyocin type (Table 7). Although strain 18S was lysed by an additional typing phage, both were sensitive to the LPS specific phage E79.

TABLE 5

Selection of Pseudomonas aeruginosa variant strain 7/1

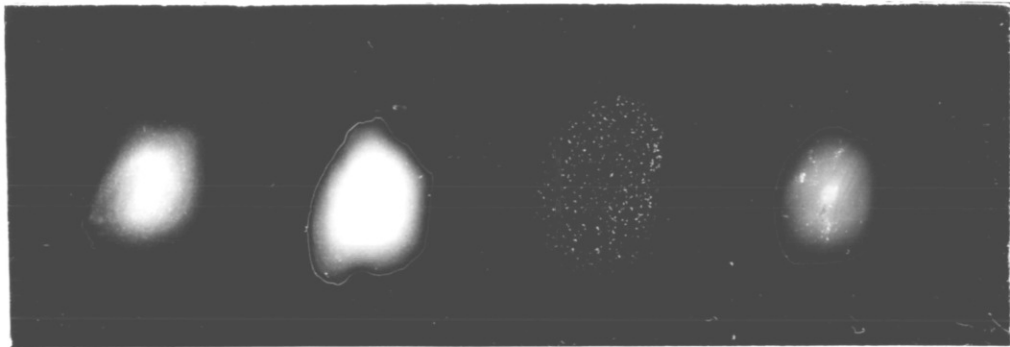
Strain	Description	O-serotype	Serum sensitivity	Pyocin type	Phage typing pattern	Lysis by LPS specific phage	
						E79	PLS1
7	Propagating strain for phage 7	6	R	22/a	7 44 119x Col.11	+	+
7/1	Variant of strain 7 resistant to phage 7	PA	S	22/a	NT	-	-

R = resistant, S = sensitive, to bactericidal action of normal human serum;

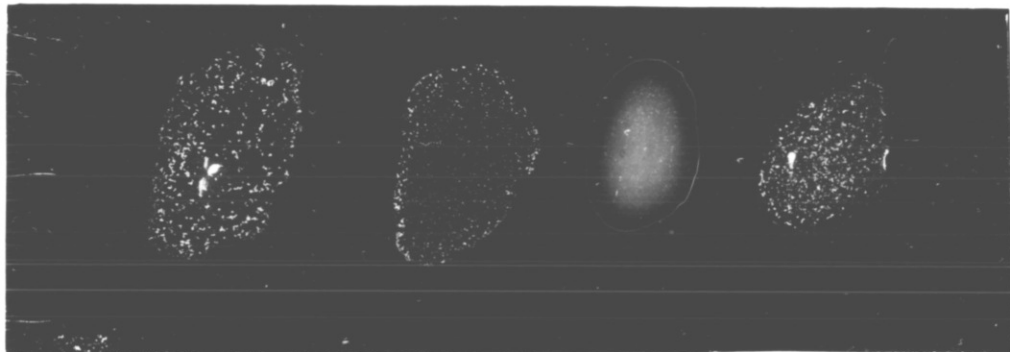
PA = polyagglutinable (with antisera 01, 03, 05d, 06, 09, 010, 013).

NT = Not typable, LPS = lipopolysaccharide.

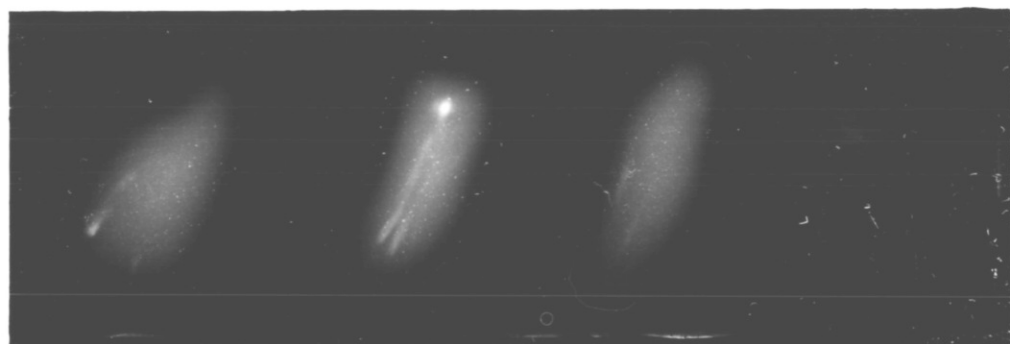
a



b



c



Slide agglutination reactions of *P.aeruginosa* with O-typing sera.

- a O-serotype specific strain
- b Polyagglutinating (PA) strain
- c Non-typable (NT) strain

TABLE 6

Slide agglutination titres of O-typing sera of Pseudomonas aeruginosa with strains 7 and 7/1

Strain	Titre of O-serum						
	01	03	05d	06	09	010	013
7	-	-	-	160	-	-	-
7/1	80	80	80	160	80	80	80

TABLE 7

Selection of Pseudomonas aeruginosa variant strain 18S

Strain	Origin	O-serotype	Serum sensitivity	Pyocin type	Phage-typing pattern	Lysis by LPS specific phage	
						E79	PLS1
18R	Cystic Fibrosis	5d	R	1/c	7 F8 109 352 1214	+	-
18S	Cystic Fibrosis	NT	S	1/c	7 44 F8 109 352 1214	+	-

R = resistant, S = sensitive, to bactericidal action of normal human serum,

NT = not typable, LPS = lipopolysaccharide.

Biochemical characterisation. The four strains produced pyocyanin and fluorescein on King's A agar and were colonial morphological type 1 (Wahba and Darrell 1965). The variants grew less well in overnight broth culture and gave viable counts of approximately 10-fold less than the parent strains. Nutrient broth suspensions of the variant strains became granular on heating at 100°C and were unsuitable for use in agglutination tests without prior washing. This was not observed with tryptone soy broth cultures. All four strains identified as typical P.aeruginosa in 68 biochemical test carried out by the Computer Trials Laboratory.

Antibiotic Sensitivity. The MICs of 13 antibiotics for the four strains are shown in Table 8. The variant strain 7/1 was markedly more sensitive to B-lactam antibiotics than its parent. There was an 8-fold difference in the MIC of azlocillin and carbenicillin for strains 7 and 7/1 and the latter was 16-fold more sensitive to cefsulodin and ceftazidime and was 4-fold more sensitive to moxalactam. The MIC of the aminoglycosides and ciprofloxacin was the same for both strains 7 and 7/1.

The MICs of the 13 antibiotics for strains 18R and 18S were similar with the exception of azlocillin to which 18S was 4-fold more sensitive. Both strains were extremely sensitive to carbenicillin (MIC = 4mg/l).

Serological characterisation of experimental strains. Antisera were raised in rabbits against heated whole cell vaccines of the four strains. The sera were tested by slide agglutination and in

TABLE 8

Minimum inhibitory concentration (mg/L) of 13 antibiotics for
Pseudomonas aeruginosa strains 7, 7/1 and 18R, 18S

Strain	Ami	Azl	Car	Cfs	Cef	Cip	Col	Gen	Mox	Net	Pip	Tob	Thi
7	2	8	128	8	8	.06	4	4	16	1	4	1	8
7/1	2	1	16	0.5	0.5	.06	8	4	4	1	4	2	8
18R	2	8	4	1	2	.12	2	4	32	2	4	1	16
18S	4	2	4	1	2	.12	4	4	32	1	2	1	16

Ami = Amikacin, Azl = Azlocillin, Car = Carbenicillin, Cfs = Cefsulodin,
 Cef = Ceftazidime, Cip = Ciprofloxacin, Col = Colistin, Gen = Gentamicin,
 Mox = Moxalactam, Net = Netilmicin, Pip = Piperacillin, Tob = Tobramycin,
 Thi = Thienamycin (N-formimidoyl)

microtitre trays with the four strains. Table 9 shows that the serum to each of the parent strains contained agglutinins only to the homologous vaccine strain and its variant. The heterologous titres of the four antisera were at least 4-fold lower than their homologous reactions in repeated tests. Antiserum 18S reacted with each of the heterologous strains to the same titre but the slide agglutination reaction was incomplete as a proportion of cells remained in suspension after mixing for five minutes. The titres were reproducible and they did not vary more than two-fold in tests performed on separate occasions.

To examine further the serological relationship between the strains each serum was absorbed with the heterologous strain that it had agglutinated in previous tests. Absorption of antiserum 7 with boiled or unheated cells of strain 7/1 did not markedly reduce its homologous titre (Table 9). In contrast absorption of antiserum 7/1 with strain 7 reduced the homologous titre to 10. Absorption of antiserum 18R with strain 18S resulted in no loss of homologous titre but the homologous reaction of serum 18S was completely removed by absorption with the parent strain 18R.

These results indicated that antigens present in the variants were also present in the parent strains. As higher titres to these antigens were obtained in sera prepared against the variants this suggested that the variant antigens were probably masked on the parent strain.

The O-specificity of the four sera was determined by diluting each serum 1 in 4 and slide agglutination tests were carried out on each of the 17 IATS reference serotype strains. Antiserum 7 reacted

TABLE 9

Agglutination of parent and variant strains of Pseudomonas aeruginosa by homologous and heterologous O-antisera before and after absorption with heterologous strains

Serum	Absorbed with strain	Agglutination titre* with strain			
		7	7/1	18R	18S
7	nil	160	40	-	-
	7/1	80	-	ND	ND
7/1	nil	20	160	-	-
	7	-	-	ND	ND
18R	nil	-	-	80	10
	18S	ND	ND	80	-
18S	nil	20	20	20	80
	18R	-	-	-	-

* Tested by slide agglutination

- = < 10

ND = Not done

specifically with serotype strain 06, which was the serotype of strain 7. Antiserum 7/1 gave weak reactions with the serotype strains 015 and 017 but did not agglutinate any other type strains. Both serum 18R and 18S reacted with the type strain 05d (O-serotype of strain 18R) and showed weak cross-reactions with strains 02 and 016 which are known to be serologically related to type 05d (Liu et al. 1983)

Immunochemical Characterisation of Serological Reactions

Lipopolysaccharide (LPS) is the immunodominant component of the outer membrane of P.aeruginosa and is the chemical basis of O-antigenic specificity. As serological tests had suggested that variant antigens, presumably O-antigens, were expressed in both pairs of strains, the contribution of LPS to these reactions was investigated.

Extraction of LPS

LPS was extracted from acetone-dried cells by phenol-water, TCA and the ethanol extraction method of Darveau and Hancock (1983). Table 10 shows that the yields of LPS obtained by ethanol extraction were appreciably higher for all four strains than by other methods. This was consistent with the report of Darveau and Hancock (1983) who found that this method was efficient for the extraction of both rough (R) and smooth (S) LPS. The poorest yield by ethanol extraction was for LPS from strain 18S (4.6%) but this was still higher than the yield of LPS from strain 7 (3%) prepared by the phenol-water method.

Chemical Characterisation of LPS

A limited chemical analysis of the LPS was carried out by the author to ensure that the freeze dried product was free from contamination by protein and to establish the gross composition in terms of

TABLE 10

Basic chemical analysis of LPS extracted by three methods from
acetone-dried cells of *Pseudomonas aeruginosa*

	Extraction Method	STRAIN			
		7	7/1	18R	18S
YIELD	Phenol-water	3.0	0.7	1.7	0.5
percentage by weight of acetone-dried cells	TCA	7.8	1.8	ND	ND
	Ethanol	10.5	9.5	8.5	4.6
PROTEIN	Phenol-water	19.0	13.8	11.0	15.0
percentage per gram of purified LPS	TCA	6.5	2.6	ND	ND
	Ethanol	3.8	3.3	1.4	1.8
TOTAL CARBOHYDRATE	Phenol-water	24.0	17.0	25.0	39.0
percentage per gram of purified LPS	TCA	23.5	7.8	ND	ND
	Ethanol	68.0	44.0	44.0	38.0
KDO	Phenol-water	ND	3.4	4.0	0.3
percentage per gram of purified carbohydrate	TCA	3.1	0.2	ND	ND
	Ethanol	2.7	3.6	2.4	3.2

TCA = Trichloroacetic acid

ND = Not done

KDO = 2 Keto-3-deoxyoctonate

carbohydrate and KDO content.

LPS prepared by ethanol extraction showed minimal contamination by protein and values ranged from 1.4% (for 18R) to 3.8% (for strain 7)(Table 10). This compared favourably with the protein content of the product obtained by phenol-water extraction and by TCA extraction which for strain 7 was 19% and 6.5% respectively.

The total carbohydrate content of ethanol-extracted LPS from three of the strains was consistently higher than that achieved by other methods but that of 18S LPS (38%) was similar to that obtained by phenol-water extraction. The total carbohydrate content of the LPS of the two variant strains 7/1 and 18S was similar (44% and 38%), but there was a considerable difference in the content of the two parent strains (68% and 44%). This difference was not found with LPS extracted by the phenol-water method.

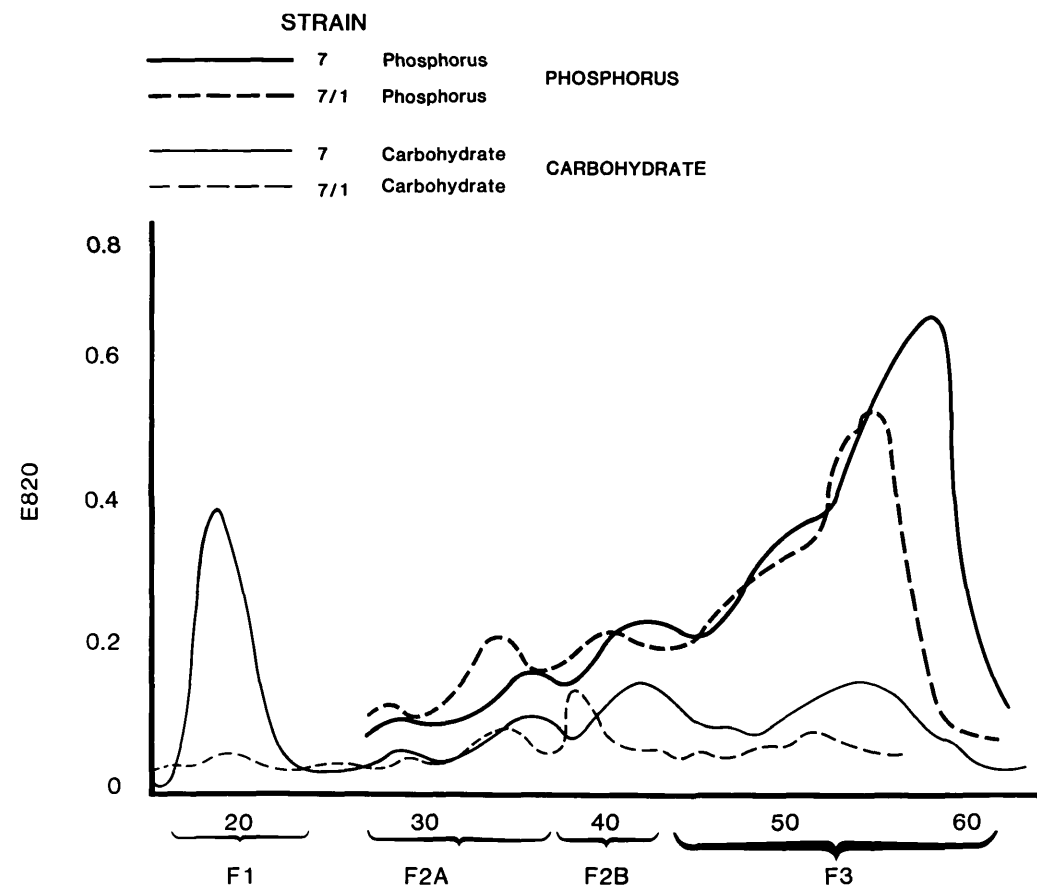
The percentage KDO content of the LPS of variants 7/1 and 18S was higher than that of their parent strains and may have been indicative of a greater proportion of KDO being available for reaction in the thiobarbituric assay in variant strains.

Analytical Chemistry of LPS

LPS extracted from cell walls of strains 7 and 7/1 was kindly analysed by Dr S.G. Wilkinson, Department of Chemistry, University of Hull. The following results are reproduced with his permission. LPS was hydrolysed with 1% acetic acid at 100°C for 1.5 h and the water soluble products were fractionated on a Sephadex G-50 column (Figure 4). Three peaks were obtained, peak F1 corresponded to the high molecular weight components of LPS and peak F2 to core material. Peak F3 was water-soluble, partly degraded polysaccharide which

FIGURE 4

Elution profiles of *Pseudomonas aeruginosa* strains 7 and 7/1 on Sephadex G-50 column



F1 corresponds to high molecular weight material = side chains

F2A) medium to low molecular weight material = core
F2B)

F3 partially degraded polysaccharide

although high in phosphorus was free of KDO and other core components. F2 was fractionated further on a Bio-gel P-4 column to yield two fractions F2A and F2B.

The basic composition of LPS extracted from the cell walls of strains 7 and 7/1 is shown in Table 11. The carbohydrate content of LPS of both strains was similar and LPS 7/1 contained more phosphorus and heptose. The KDO content of LPS 7/1 was about twice that of LPS 7 which reflected the earlier finding with whole cells that the variant strain was richer in this compound than the parent. No significant difference was detected in uronic acid values but the ratio of glucose to rhamnose was markedly reduced in the variant strain; glucosamine and galactosamine were increased in LPS 7/1 but no quinovosamine was detected.

After hydrolysis of the LPS and fractionation by gel filtration the F1 fraction (Figure 4) was analysed (Table 12). The carbohydrate and phosphorus content of the variant was at least twice that of the parent but the glucose:rhamnose ratios were similar. No aminosugars were detected in the F1 fraction of 7/1 but strain 7, contained by weight, about 5% quinovosamine.

Dr Wilkinson commented that the side chains of the LPS of strain 7/1 were atypical in composition but that this was not attributable to a reduction of the 7-type side chain; the F1 of LPS 7/1 was almost entirely a neutral polysaccharide compared to the F1 of strain 7 which had rhamnose, quinovosamine and aminogalactose as specific components.

The composition of the core components (F2 fractions) of the two LPS preparations is given in Table 13. Two fractions, A and B, were

TABLE 11

Analysis of LPS extracted from cell walls of *Pseudomonas aeruginosa*
strains 7 and 7/1

Component by weight	STRAIN	
	7	7/1
Phosphorus (%)	3.4	5.7
Total carbohydrate (%)	26.9	27.2
Heptose (%)	6.4	11.2
KDO (%)	2.4	4.2
Uronic acid (%)	2	2
Glc:Rha ^a	1:1.5	1:0.5
Glucosamine (%)	3.8	6.1
Galactosamine (%)	3.5	7.6
Quinovosamine ^b (%)	1.0	0
Aminogalacturonic acid ^b (%)	+	Tr

^a Ratio of peak areas on g.l.c. of alditol acetates

^b Not confirmed quantitatively.

TABLE 12

Analysis of O side-chain fractions (F1) of parent and variant strains of
Pseudomonas aeruginosa

Component by weight	STRAIN	
	7	7/1
Phosphorus (%)	0.7	1.4
Total carbohydrate (%)	33.4	81.6
Glc:Rha ^a	1:5.5	1:6
Glucosamine (%)	0	Tr
Galactosamine (%)	Tr	Tr
Quinovosamine (%)	5	0
Aminogalacturonic acid (%) ^b	++	0

^a Ratio of peak areas on g.l.c. of alditol acetates

^b Not confirmed quantitatively.

TABLE 13

Analysis of core fractions (F2) of parent and variant strains of
Pseudomonas aeruginosa

Component by weight	STRAIN			
	<u>7</u>		<u>7/1</u>	
	F2A	F2B	F2A	F2B
Phosphorus (%)	4.1	5.4	3.8	5.7
Total carbohydrate (%)	49.7	51.9	50.1	45.3
Heptose (%)	10.6	11.5	9.5	10.5
Glc:Rha ^a	1:0.7	1:0.3	1:0.5	1:0.2
Glucosamine (%)	0	0	Tr	0
Galactosamine (%)	4.3	6.2	4.6	8.9
Quinovosamine (%)	+	0	Tr	0
Aminogalacturonic acid (%) ^b	+	0	Tr	0
Alanine (%)	2	3	Tr	Tr

^a Ratio of peak areas on g.l.c. of alditol acetates

^b Not confirmed quantitatively.

obtained for each LPS and these were similar in terms of carbohydrate, phosphorus, heptose and glucose:rhamnose ratio. The only major difference was a higher value for galactosamine in the F2B fraction of strain 7/1. The results therefore suggested that the cores of the strains were homogeneous.

There were no gross differences in the fatty acid composition of the LPS of the two strains on primary analysis and this was not examined further.

Electrophoretic Characterisation of LPS

Charged molecules in an electric field will migrate in the direction of the current and if a matrix which sorts molecules on the basis of their size and/or shape is employed, such as acrylamide, then 'molecular sieving' will occur. This allows the heterogeneity of molecules in a mixture to be determined. In practice, sodium dodecyl sulphate (SDS) is used to increase the dissociation of proteins and other molecules, thus facilitating their passage in the support matrix. Palva and Mäkelä (1980) showed that LPS of Gram-negative bacteria could be conveniently separated by this method into regions of high, medium and low molecular weight. The high molecular weight components corresponded to the repeating oligosaccharide units and the length of the O-antigen side chain could be directly inferred by the number of distinct bands obtained on electrophoresis. Medium molecular weight material represented core and smaller side chain components while deep core and lipid A moieties migrated fastest. The silver stain detects the cis-glycol components of the carbohydrate which have been oxidised by periodate and they appear as black or brown staining bands in the gel.

LPS profiles of serotype strains

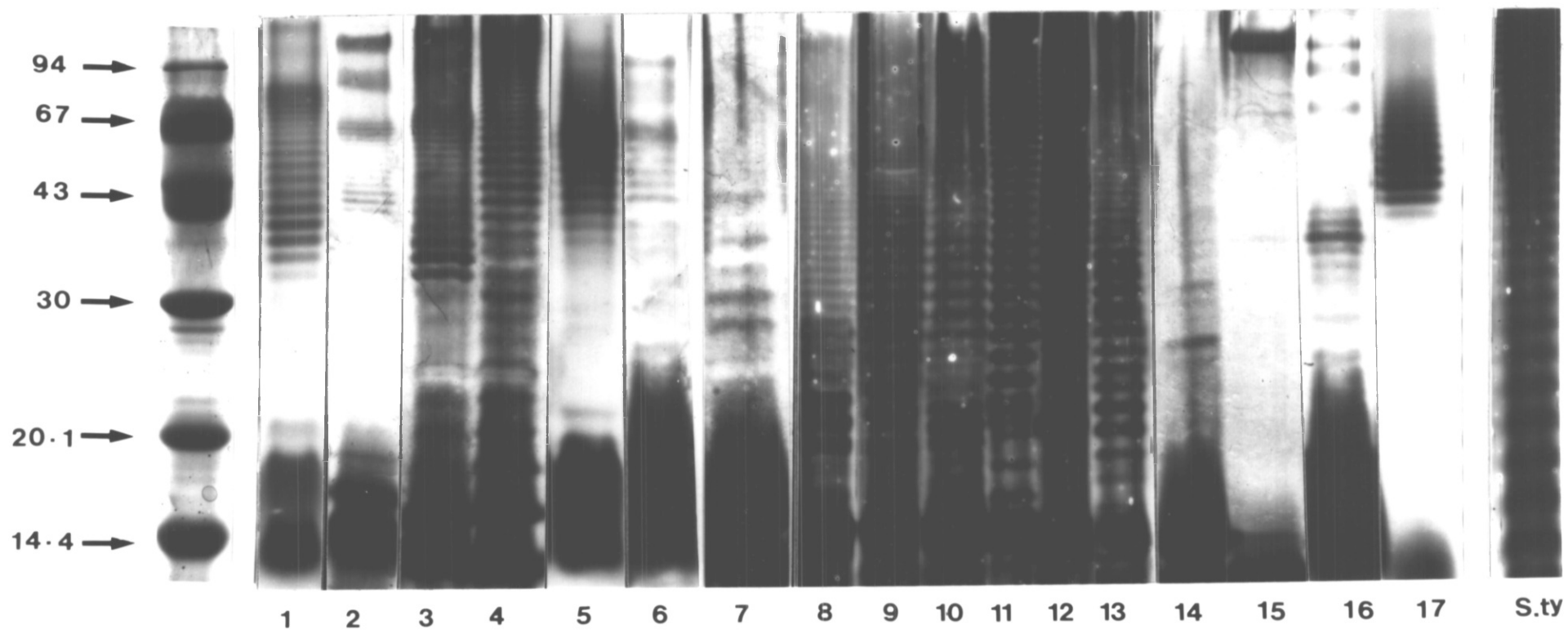
In order to establish the range of LPS profiles occurring in the species P.aeruginosa, the LPS of the 17 serotype strains of the IATS were analysed by SDS-PAGE. Figure 5 shows that there was considerable variation in the banding profiles of the serotype strains. Some of the strains; 03, 04, 011, 012 and 013 showed a 'ladder-like' pattern of repeating sugar units indicating that the O-specific antigens were chemically heterogeneous. This type of LPS profile is common in the enterobacteriaceae and Salmonella sp., although as can be seen in Figure 5 the banding of Salmonella typhimurium extended the whole length of the gel and did not contain the pronounced low molecular weight core region seen in the LPS of strains of P.aeruginosa.

Other strains notably 01, 02, 05, 06, 015 and 017 gave fewer bands in the middle portion of the gel and for strains 02, 06, 015 and 016 the high molecular weight bands were densely staining doublets separated clearly by molecular weight. The absence of 'ladder' formation in LPS of these strains and strain 014 may be related to the lack of periodate sensitive cis-glycol groups or to a different chemical composition of LPS.

The fast moving, core, low molecular weight material was present in the 17 strains and appeared as a densely stained mass. Subsequent gels loaded with less LPS enabled the visualisation of this region and it appeared as a heavier, wide, densely stained band which was common to all serotype strains. In all strains a lightly stained diffuse area or distinct bands were observed below the major band but heterogeneity in this region of the gel was not as marked as in high

FIGURE 5

Heterogeneity of *P.aeruginosa* lipopolysaccharide banding profiles in SDS-PAGE, with reference to the 17 O-serotype strains of the IATS.



Key

S.ty = *Salmonella typhimurium*

molecular weight components.

LPS of Parent and Variant Strains

The LPS of the four experimental strains produced electrophoretic profiles shown in figure 6. LPS 7 (track 2) gave a densely stained zone which apparently consisted of three bands in the gel region corresponding to molecular weights of 10,000-17,000. This probably represented core constituents of the LPS as the lower two bands were common to LPS 7/1 and LPS 18R (tracks 3 and 4 respectively) but only one band was visible in the track of LPS 18S (track 5).

The tracks of LPS 7 and LPS 7/1 exhibited a region of molecular heterogeneity distinguishable as bands forming a 'ladder-like' pattern in the middle area of the gel. The most marked difference between the LPS of strain 7 and its variant 7/1 was the absence of higher molecular weight, densely staining bands in the track of 7/1. The LPS of strain 18R did not exhibit molecular size heterogeneity as evidenced by the lack of multiple banding but it gave a profile similar to the pattern of the LPS of the serotype strain 02 (Figure 5) which is known to be related to 05d, the serotype of 18R.

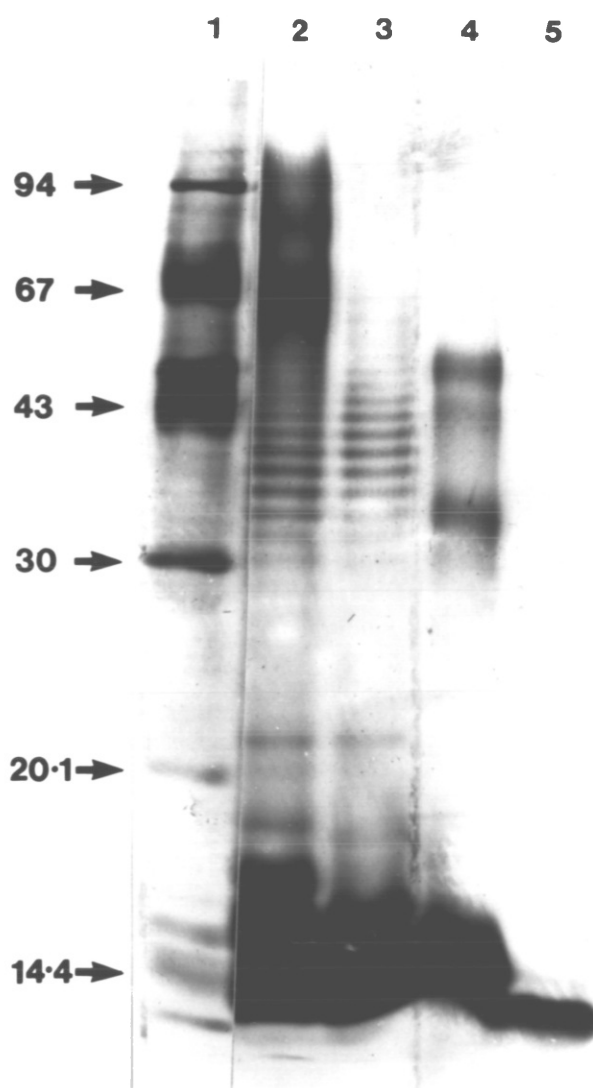
The LPS of strain 18S appeared to be completely lacking in side chain moieties. Only one band common to all 4 strains remained (track 5) suggesting that this variant expressed highly defective LPS.

SDS-PAGE of outer membrane proteins

Outer membrane protein extracts of the four strains were prepared by solubilisation in lauryl sarcosyl and the SDS-PAGE profiles were visualised by staining with Coomassie blue (Figure 7). Each of the six major proteins described by various authors (Mizuno and Kageyama 1978; Hancock and Carey 1979) were presumptively identified by

FIGURE 6

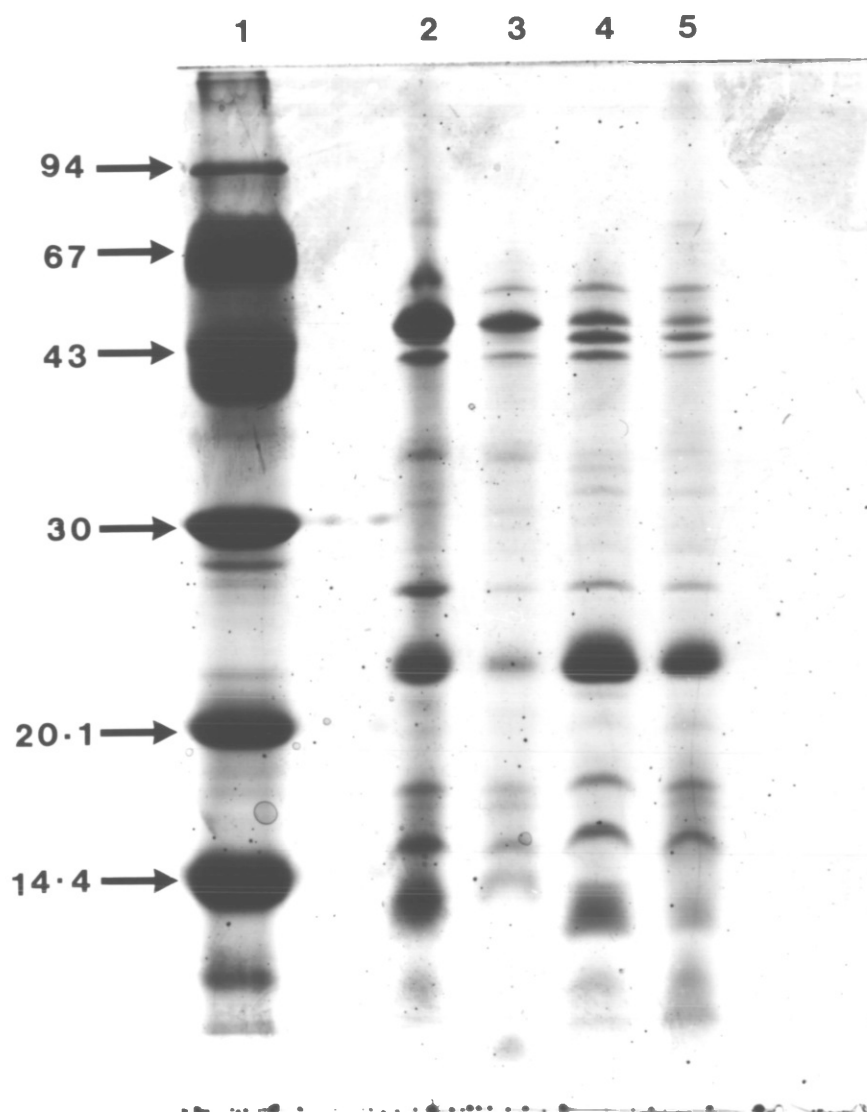
LPS profiles of strains 7 and 7/1, 18R and 18S as shown by SDS-PAGE and Silver Stain.



Track 1 molecular weight markers
Track 2 LPS of strain 7
Track 3 LPS of strain 7/1
Track 4 LPS of strain 18R
Track 5 LPS of strain 18S

FIGURE 7

Electrophoretic profiles of outer membrane proteins of strains; 7 and 7/1, 18R and 18S stained with Coomassie blue.



Track 1 Molecular weight markers
Track 2 OMP of strain 7
Track 3 OMP of strain 7/1
Track 4 OMP of strain 18R
Track 5 OMP of strain 18S

comparison with molecular weight standards. These results indicated that although alteration of the structure of LPS of the strains had occurred, the major proteins remained constant although a protein of molecular weight approximately 14,000 was either absent or in low concentration in the membranes of the variant strains. Apart from this, all four strains gave similar protein profiles.

Precipitation in agar gel

LPS preparations from the four strains were tested by double diffusion in agar gels against each of the four antisera. Serum 7 formed a line of precipitation with LPS 7 and also gave a single line common to LPS 7/1, 18S and 18R; this latter line was partially identical to the homologous line but it was formed nearer to the serum well (Figure 8). Serum 7/1 gave a line of identity between LPS 7/1 and 18S and a similar reaction was seen with serum 18S. Serum 18R was specific in precipitation tests.

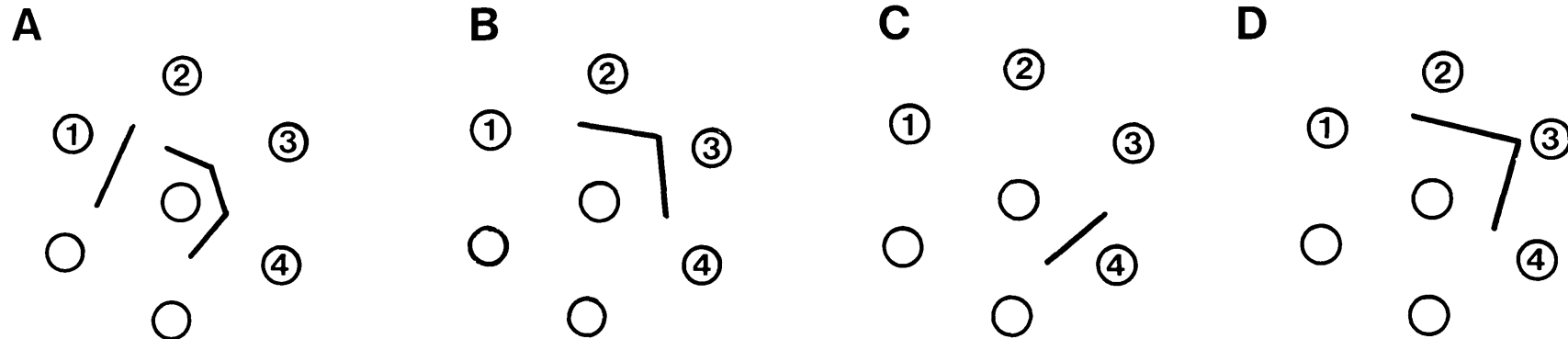
Line drawings of the precipitation reactions are given in the figure as the precipitation lines were often faint and photographed poorly even after staining.

Indirect haemagglutination

The LPS of strain 7 adsorbed efficiently to sheep erythrocytes and high homologous titres of indirect haemagglutination (IHA) were obtained (Table 14) in repeated experiments. The titre was considerably higher (10-fold) than the homologous titres of the other three sera. Adsorption of the LPS of both variants to red cells was less efficient and lysis of the erythrocytes occurred with concentrations of LPS above 2.5mg/ml for 7/1 and 1.25mg/ml for 18S. The degree of lysis appeared to be dependent on the batch of LPS used

FIGURE 8

Tracing of precipitation lines in agar gel from double diffusion tests between LPS extracts of the strains; 7 and 7/1, 18R and 18S (peripheral wells) with homologous and heterologous antisera (central wells).



1 LPS strain 7, 2 LPS strain 7/1, 3 LPS strain 18S, 4 LPS strain 18R,
A antiserum 7, B antiserum 7/1, C antiserum 18R, D antiserum 18S.

in the test. The titres of the two variant LPS were not reproducible and are expressed as the range obtained in repeated tests (Table 14).

The results of indirect haemagglutination agreed in general with those given by agglutination of whole cells (Table 9). LPS 7/1 was distinguishable from the parent and although some cross-reaction was evident the heterologous titre was considerably lower than the homologous. The titres of sera 18R and 18S for LPS 18R were identical but serum 18R was specific for its LPS although earlier 18R exhibited low titre agglutination of strain 18S (see Table 9). Serum 18S agglutinated red cells coated with LPS 7/1 and there was a reciprocal reaction of serum 7/1 with LPS 18S.

The partial serological relationship between the two variants observed in agglutination and precipitation tests was therefore confirmed by IHA.

ELISA

The indirect haemagglutination test is not universally considered to be the most suitable method for the study of the antigenic relationship of smooth and rough LPS because the hydrophobic LPS of rough strains bind poorly to mammalian erythrocytes (Appelmeik et al. 1985). An ELISA procedure was therefore investigated to determine whether previously unrevealed relationships existed between the four LPS preparations.

A comparison of Tables 14 and 15 shows that no substantial differences in the reactivity of the four LPS with each serum was found between IHA and ELISA. Serum 18S gave homologous titres 4-8 fold lower than sera 7 and 7/1 and 18R. Additional reactions that

TABLE 14

Indirect haemagglutination of LPS of parent and variant strains of
Pseudomonas aeruginosa by homologous and heterologous O antisera

Serum	Titre of haemagglutination of LPS of strain			
	7	7/1	18R	18S
7	50,000	40	-	40
7/1	640	40-2560*	-	20
18R	-	-	5120	-
18S	-	40-640*	5120	640

- = <20

* Range of titres in repeated tests.

TABLE 15

Titres of homologous and heterologous O-antisera obtained by ELISA with
LPS of the parent and variant strains of Pseudomonas aeruginosa

Serum	ELISA Titre* with LPS of Strain			
	7	7/1	18R	18S
7	16,800	500	0	2,000
7/1	1,000	9,800	0	500
18R	0	0	8,000	500
18R	125	500	1,000	2,500

* Average of repeated tests

0 = Below background absorbance

had not occurred in IHA tests between serum 18S and LPS 7 and serum 18R and LPS 18S were revealed by ELISA.

As IHA had been compared directly with whole cell agglutination it was necessary to confirm that ELISA reactions obtained with purified LPS and whole cells were complementary. ELISA with whole cells revealed more cross-reactions than those seen with purified LPS preparations (Table 16). The heterologous titres were generally higher and novel reactions were found with serum 18R and cells of 7 and 7/1. Furthermore sera 7 and 7/1 bound to cells of 18R and 18S confirming the previous results in double diffusion tests.

Competitive Inhibition ELISA

It was necessary to determine the contribution of LPS to the ELISA reactions with whole bacterial cells. Whole LPS and fractions prepared from cell walls of strains 7 and 7/1 were incubated in varying weight/volume concentrations, with a single dilution of hyperimmune sera raised against whole cells of the two test strains. The degree of competition of the LPS or its fraction for the epitope recognised by the antibodies in the sera was observed as inhibition of the reaction with either whole cells or LPS of each strain. The optical density values were plotted against the weight of competing antigen and the 50% inhibition value was calculated with comparison to controls.

Table 17 shows that some of the antibody in serum 7 recognised LPS antigens on the whole cells of strain 7 as whole LPS 7 blocked the reaction of this serum with the homologous cells. However, isolated fractions of LPS 7 did not compete for antibodies reactive with the whole cells as no inhibition was observed at concentrations of 20

TABLE 16

Titres of homologous and heterologous O-antisera obtained by ELISA
with whole cells of the parent and variant strains of Pseudomonas aeruginosa

Serum	ELISA Titre* with Whole Cells of Strain			
	7	7/1	18R	18S
7	14,000	4,000	1,000	1,500
7/1	2,000	8,600	4,000	3,000
18R	400	500	9,500	3,000
18S	500	1,000	1,500	2,200

* Average from repeated tests

0 = Below background absorbance.

µg/ml (w/v). In contrast antibody binding of serum 7 to LPS 7 was inhibited by both whole LPS and fractions F1 and F2A of strain 7 but the second core component, fraction F2B, had no activity. These results suggested that antibody to LPS was present in serum 7 and at least the F1 and F2A portions were necessary to inhibit the homologous reaction.

The presence of 7/1-like epitopes on strain 7 was not confirmed by inhibition experiments as 10 µg/ml (w/v) or greater of whole LPS or fractions of 7/1 was required to block the homologous reaction of serum 7 with either whole cell or LPS. The inhibition of the reaction of LPS 7 by fraction F1 of strain 7/1 cannot be explained as one would have expected the whole LPS to have greater activity. A possible explanation may be that core components were not exposed on the whole cells of 7 or on isolated LPS of this strain and as the serum was raised towards whole cells the inner core components may not have been exposed sufficiently to stimulate antibody production. The results also indicated that strain 7/1 contained components similar to those present on strain 7. Support for this reasoning is given by the inhibition of the reaction of serum 7 with both 7/1 cells and LPS, by LPS 7 and its fractions. The F2B fraction of 7 did not compete for the antigen site on 7/1 cells but was highly inhibitory for the interaction of serum 7 with 7/1 LPS. The reaction of serum 7 with LPS 7/1 was almost certainly due to components present in the LPS of both strains as the LPS and F1 component of 7/1 competed for the antibody-antigen binding site.

The target sites for the antibody in 7/1 serum were investigated and Table 18 shows that only LPS 7 and its F1 and F2A fractions inhibited

TABLE 17

Inhibition of the reaction of serum 7 with either whole cells or LPS
of strains 7 and 7/1 by whole LPS and LPS fractions of these strains

Serum 7 Reaction with:	Concentration of antigen ($\mu\text{g/ml}$) required to give 50% inhibition of ELISA Reaction							
	<u>7</u>				<u>7/1</u>			
	LPS	F1	F2A	F2B	LPS	F1	F2A	F2B
7 Whole cell	4	-	-	-	-	-	-	-
7 LPS	2	1.25	1.25	-	-	2	-	-
7/1 Whole cell	4.8	1.25	15	-	-	6	-	-
7/1 LPS	0.9	1.25	1.25	4.1	0.63	1.25	-	6

- greater than 10 $\mu\text{g/ml}$ (w/v) required for 50% inhibition of the reaction.

TABLE 18

Inhibition of the reaction of serum 7/1 with either whole cells or LPS
of strains 7 and 7/1 by whole LPS and LPS fractions of these strains

Serum 7/1 Reaction with:	Concentration of antigen (µg/ml) required to give 50% inhibition of ELISA reaction:							
	<u>7</u>				<u>7/1</u>			
	LPS	F1	F2A	F2B	LPS	F1	F2A	F2B
<u>7</u> Whole cell	-	1.25	1.25	-	-	-	-	-
<u>7</u> LPS	0.63	1.25	1.25	-	-	-	-	-
<u>7/1</u> Whole cell	-	1.25	1.25	-	-	-	-	-
<u>7/1</u> LPS	-	-	-	-	-	-	-	-

- greater than 10 µg/ml (w/v) required for 50% inhibition of the reaction.

the reaction of this serum with either strain. Surprisingly inhibition of the 7/1 serum reaction with whole cells was greater when fractions of LPS 7 were used in preference to whole LPS. This may have been due to the inaccessibility of core epitopes in polymerised or unfractionated LPS. The inability of LPS 7/1 and its fractions to compete for the antibodies in reactions with either whole cells or LPS suggest that either LPS was not the target antigen for antibody in this serum or that there was little anti-core antibody stimulated by injection of rabbits with whole cells of the variant strain. It would seem therefore that the major antibodies of 7/1 serum were directed towards 'parent-like' side chains.

Nevertheless, the inability of either LPS 7 or LPS 7/1 or their fractions to block the ELISA reaction of serum 7/1 with LPS 7/1 is puzzling because, if the epitope recognised by antibody in serum 7/1 was 'parent-like' side chains, one would expect LPS 7 or its F1 fraction to inhibit the reaction. The possibility exists therefore that the epitopes which exhibited blocking may not be identical with those which gave the original reactions in ELISA.

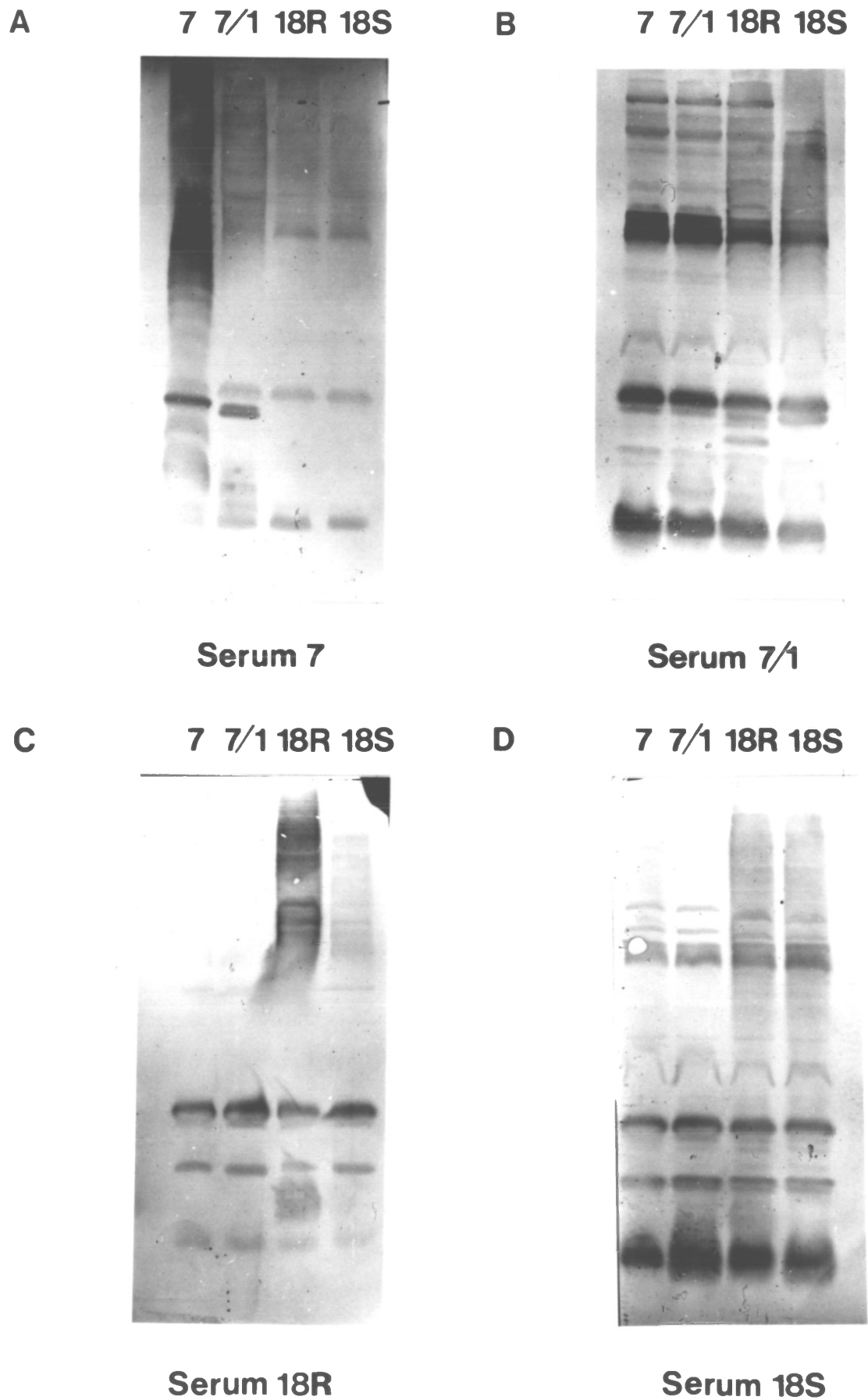
Immunoblotting

In order to identify more accurately the seroreactive components of the four experimental strains, extracts of whole cells and purified LPS were separated by SDS-PAGE and transferred to nitrocellulose sheets. The transferred components were incubated in antisera and the antigen-antibody reactions were developed by ELISA.

Serum 7 contained antibodies which reacted with the whole cell extract of each of the strains. Figure 9 (A) shows that there was considerable variation in the intensity of the blots given with each

FIGURE 9

Immunoblots of whole cell preparations of strains; 7 and 7/1, 18R and 18S with homologous and heterologous antisera.



extract but the homologous reaction was the strongest. Incubation with serum 7 revealed seroreactive components that were distributed throughout the length of the tracks of strain 7 but there was considerably less reactive material in the region of medium molecular weight (20,000-30,000), on strain 7/1. This was shown more clearly in the reaction with whole cells of 18R and 18S where lower molecular weight regions were also excluded from the reaction. Furthermore the profiles of the immunoblot of serum 7 with strains 7 and 7/1 were distinguishable although many bands were common to both.

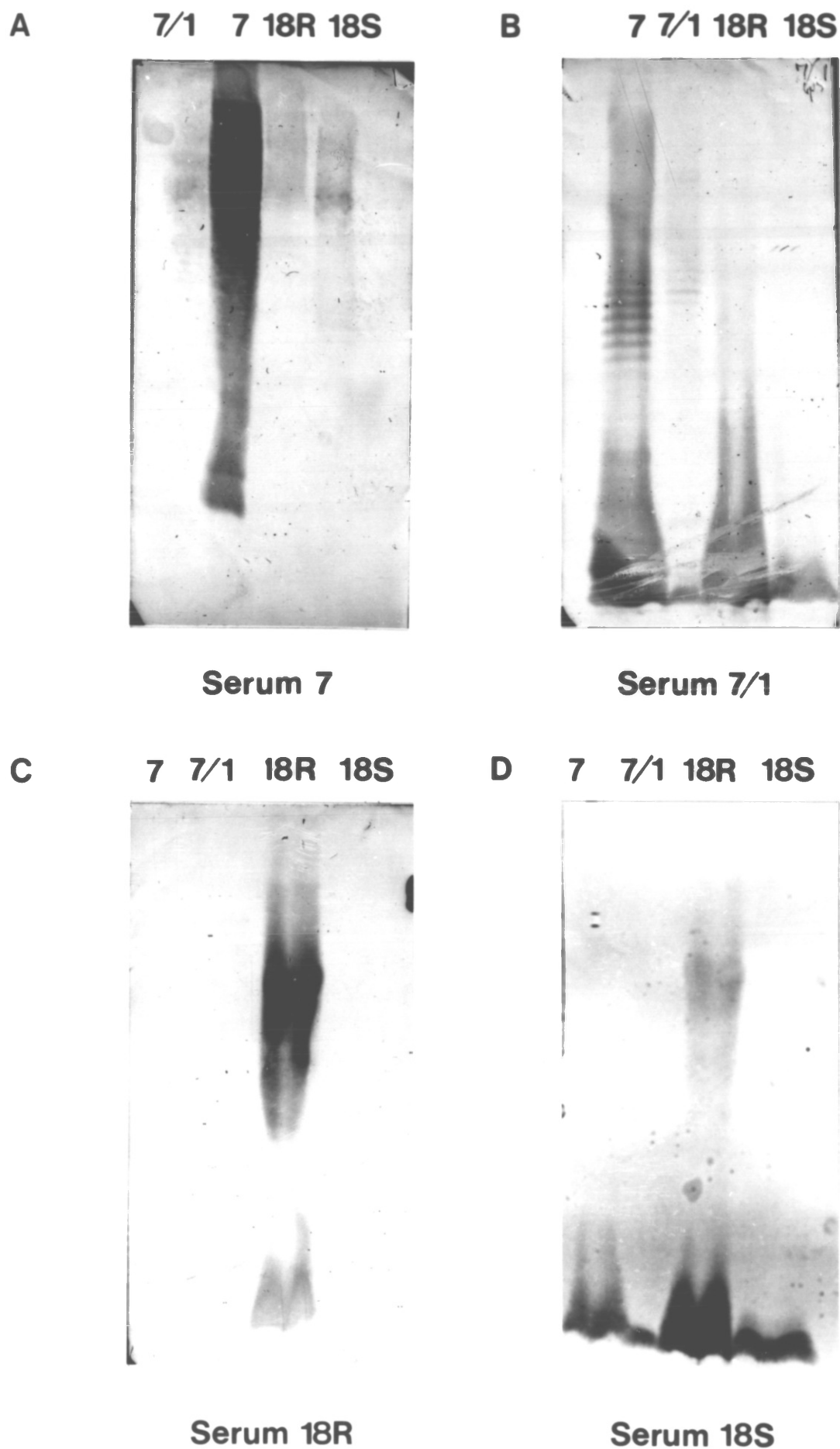
In contrast serum 7/1 (Figure 9B) reacted to an equal intensity with whole cells of all four of the strains indicating the presence of common epitopes in the heterologous strains. Many of the common bands were due to protein moieties as a similar picture was obtained with blots of purified outer membrane proteins. Serum 18R was comparatively specific for whole cell components of the homologous strain (figure 9C), although it also recognised protein epitopes common to all four strains. Serum 18S produced blots similar to those of the other variant, strain 7/1, in that numerous bands presumably including proteins, were developed on each of the four strains, with almost equal intensity.

The similarity of the reactions of antisera 18S and 7/1 may indicate that the loss of smooth LPS in these variant strains resulted in exposure of immunogenic sites, present in all the strains but these were occluded on the parent cells used as vaccines.

When the purified LPS of the four strains was examined by immunoblotting with the sera, the profiles obtained mirrored those found with whole cell preparations. The parent sera, 7 and 18R,

FIGURE 10

Immunoblots of the LPS of strains; 7 and 7/1, 18R and 18S with homologous and heterologous antisera.



generally were specific for the homologous LPS but sera to the variants bound to core LPS of all the strains (Figure 10). There were less bands in the heterologous LPS blots as the outer membrane proteins had been removed in the purification process. Sera to the parent strains reacted strongly with all components throughout the tracks of the homologous LPS but the variant sera recognised only regions of LPS which were common to each of the strains. The profiles shown in Figure 10B (the reaction with serum 7/1) resemble those developed with the silver stain (Figure 6). The banding of LPS 7 blotted with serum 7/1 confirmed the earlier suggestion that antibodies to 'parent-like' side chains were present in the variant antiserum. It appeared that the antibodies in serum 18S were almost totally directed towards low molecular weight core components although some medium to high molecular weight material in LPS 18R was developed by this serum (figure 10D).

Detection of isolates with variant antigens from CF sputum

The foregoing results served to document the defects in the LPS of the two experimental strains. The antisera raised towards these defects were used to detect LPS alterations in strains of P.aeruginosa isolated from CF sputa. As previous studies had shown a high incidence of serum sensitivity in P.aeruginosa from CF, apparently associated with LPS defects, a collection of CF isolates were examined for both serum sensitivity and O-antigen status. A total of 1204 cultures of P.aeruginosa were obtained from 844 sputum samples of 210 CF patients over the course of the two-year study (see Section II). Each was serotyped by agglutination with O-typing sera and the two antisera raised towards the variants 7/1 (PA) and 18S

(NT) and tested for sensitivity to NHS.

Serum resistance

Four categories of resistance or sensitivity to NHS were found among the isolates. Serum resistant cultures grew equally well in NHS and control media. Reduced resistance to serum was shown as a small reduction in viable count (1 log) following incubation in NHS and partially sensitive cultures were those where the viable count in NHS decreased by 2-3 log in comparison with controls. The growth of fully sensitive cultures was completely inhibited by NHS.

Those isolates that were agglutinated by two or more O-typing sera and/or the additional serum 7/1 were termed PA. Similarly those that were not agglutinated by any of the O-typing sera were termed unclassified, and those that reacted with serum 18S were called NT. Isolates that gave a reaction with both PA and NT sera, thus indicating the expression of both variant antigens, were labelled as PA/NT.

Table 19 shows the distribution of cultures classified by both serum resistance and agglutination reactions. Two hundred and one of the 1204 (17%) were fully resistant to serum and of these 92 (46%) were agglutinated specifically by an O-typing serum. There was a slight decrease in O-specific reactions (30 of 93) with isolates that showed reduced resistance to NHS and the frequency of O-specific reactions decreased further for partially sensitive isolates. The greatest proportion 792 of 1204 (65%) were fully sensitive to serum and only 41 of these (5%) could be allocated a specific O-serotype. Statistical analysis by Chi-square test confirmed a significant association between the lack of O-type and sensitivity to serum ($E =$

TABLE 19

Sensitivity to normal human serum and agglutination reactions of
1204 isolates of Pseudomonas aeruginosa from 210 CF patients

Serum Sensitivity	Number (%) of isolates in agglutination category					
	O- typable	u/c	PA	NT	PA/NT	Total (%)
Resistant	92 (51)	42 (19)	48 (10)	8 (7)	11 (5)	201 (17)
Reduced Resistance	30 (16)	17 (8)	30 (7)	4 (4)	12 (5)	93 (8)
Partially Sensitive	18 (10)	23 (11)	53 (12)	8 (7)	16 (6)	118 (10)
Sensitive	41 (23)	134 (62)	327 (71)	84 (82)	206 (84)	792 (65)
Total	181 (15)	216 (18)	458 (38)	104 (9)	245 (20)	1204

u/c = unclassified

PA = agglutinated by two or more antisera and/or PA antiserum (anti 7/1)

NT = agglutinated by NT antiserum (anti 18S)

PA/NT = agglutinated by both PA and NT antisera

$p < 0.001$ (χ^2 test)

263.23, 16 d.f. $p < 0.001$).

The proportion of unclassified cultures within each category of susceptibility to NHS remained constant. For example 42 of 201 (21%) serum resistant cultures were serologically unclassified compared with 134 of 792 (17%) of those which were fully sensitive to NHS. Of all isolates, 216 (18%) were serologically unclassified, it is noteworthy that 80 (37%) of these were mucoid and did not form stable emulsions in saline and therefore could not be typed by agglutination. Nevertheless of the 548 mucoid cultures isolated, 85% reacted with one of the sera.

The incidence of reactions with antisera to the variants (7/1 and 18S) increased as isolates became more sensitive to serum and reaction with the PA serum was found approximately twice as frequently among serum sensitive isolates compared with serum resistant strains.

Overall, only 104 (19%) of the CF isolates gave a reaction with serum 18S (termed NT) and 84 of these (81%) were fully sensitive to serum. About one-quarter of the sensitive cultures reacted with both variant sera and there was a concomitant decline in positive reactions with these sera as resistance to NHS increased.

This suggested that there was no absolute relationship between serum sensitivity and expression of PA or NT antigen but statistically there was a greater likelihood of a serum sensitive strain reacting with antibodies directed towards defective LPS.

Frequency of O-serotypes among isolates

Table 20 shows the distribution of O-serotypes among the 181 CF isolates that could be allocated a specific O-type. Thirteen of the

TABLE 20
Frequency of O-serotypes among 181 O-typable strains of *Pseudomonas*
aeruginosa isolated from 210 CF patients

Serotype*	Number of Isolates	Percentage Frequency
06	71	39
03	30	16
016	21	12
011	12	7
01	11	6
08	8	5
02b	6	3
010	6	3
07	5	3
09	5	3
02a	2	1
04	2	1
05	2	1
13	181	100

* Typing sera used at CPHL corresponds numerically with the IATS (Liu et al. 1983)

21 serotypes tested by the O-typing sera used at C.P.H.L. were represented by this set of isolates. Of these serotypes 06 was the most frequent and accounted for 39% of the isolates. Types 03 and 016 were also common followed by serotype 011 and 01. The distribution of types was similar to that for other clinical sources (Pitt unpublished) with the possible exception of 011 which was not as frequent in CF as it is in non-CF isolates (Pitt 1980).

Lipopolysaccharide of CF variant strains

The results of serum sensitivity and agglutination tests with O-typing sera and sera to apparently LPS-defective strains made it necessary to investigate the constitution of the LPS of CF strains. LPS was extracted from eight selected strains and the CF isolate 18R by the method of Darveau and Hancock (1983). The basic chemical analysis of the LPS preparations in relation to their serological reactions is shown in Table 21. There was a considerable difference between the nine strains in yields of LPS and this did not appear to be related to their agglutination reactions. Four strains gave yields of LPS comparable with that obtained from the control strain, 18R, but three yielded one percent or less LPS per gram of dry cells. Contamination of the preparations with protein was generally low although the LPS of three strains contained approximately four times more protein than the control (18R). Carbohydrate and KDO determinations were not performed on three of the samples because of insufficient material but the values for five of the CF isolates varied considerably. Two of them gave percentage KDO contents of 13.2% per gram of carbohydrate while the other three CF isolates tested gave KDO values of less than 2%.

TABLE 21

Analysis of lipopolysaccharide by ethanol extraction from selected
CF isolates of Pseudomonas aeruginosa

Strain	Serum Sensitivity	O-type	Yield ¹ of LPS	Analysis of LPS		
				Protein ²	Carbohydrate ²	KDO ³
464 ^b	S	PA	9.7	0.8	38.8	1.8
499 ^b	S	PA	8.4	5.3	25.2	13.25
578 ^b	S	NT	1	4.8	34.8	1.32
500 ^b	S	NT	7.06	1.1	25.2	13.2
418 ^b	PS	PA/NT	0.3	2.1	ND	ND
551 ^a	S	PA/NT	9.3	3.6	46	1.2
376 ^b	S	u/c	1	1.8	ND	ND
572	R	u/c	3.43	4.6	ND	ND
18R	R	O5d	8.5	1.4	44	2.4

KDO = 2 Keto-3-deoxyoctonate

1 Percentage of dry weight of acetone-dried whole cells

2 Percentage of purified LPS

3 Percentage of total carbohydrate

ND = Not done

R = resistant, S = sensitive, PS = partially sensitive to bactericidal action of normal human serum.

PA = agglutinated by two or more O-antisera and/or PA antiserum (anti 7/1)

NT = agglutinated by NT antiserum (anti 18S)

PA/NT = agglutinated by both PA and NT antisera

u/c = unclassified

The sensitivity of these isolates to serum was not reflected by the chemical composition of the LPS. The partially sensitive isolate (418b) gave the poorest LPS yield, although it may have been expected to retain some side chain material, and the resistant strain (572) yielded half the amount of LPS obtained from the control strain 18R. It was evident from these results that a comparison of the empirical chemical composition of LPS was an unsatisfactory procedure for the determination of the contribution of LPS to serotype or serum sensitivity reactions. Therefore a larger panel of isolates were selected for analysis by SDS-PAGE.

Analysis of the LPS of CF isolates by SDS-PAGE

A collection of 30 isolates was made to represent each of the five categories of agglutination reaction observed. LPS from these isolates was prepared by proteinase K digestion and their electrophoretic profiles were revealed by silver staining.

Figure 11a illustrates the heterogeneity of LPS profiles of CF isolates which were O-typable. Some isolates, tracks 1 and 2, showed banding which formed a 'ladder-like' pattern and extended the length of the gel. This type of LPS profile was similar to those given by some of the O-serotype strains (Figure 5). The isolate in track 1 was serotype 06 and resistant to NHS and that in track 2 although displaying slightly less high molecular weight material was nevertheless O-typable (09) and resistant to serum. The isolate in track 3 was serotype 06, serum resistant and mucoid and although the banding in the middle portion of the gel was highly resolved there was a loss of resolution between the high molecular weight components. The LPS in track 4 gave bands of medium molecular

weight (20,000 - 30,000) but no repeating bands of higher molecular weight were visible although this area of the gel was densely stained. This strain gave a specific agglutination reaction with the 09 serum and was resistant to NHS. In contrast LPS in tracks 5 and 6 showed only faint staining of high molecular weight material and densely staining core components. These profiles may have been due to less LPS by weight in the sample because of poor extraction by the proteinase K method but this was not confirmed. The presence of defective LPS in these strains was not indicated by their reaction with 0-sera or resistance to NHS.

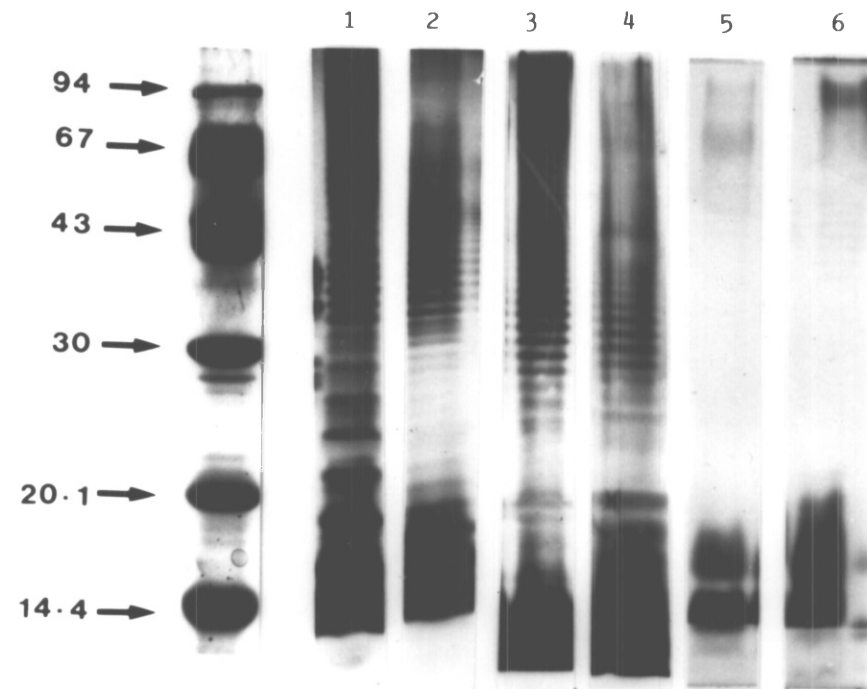
The LPS profiles of isolates which were unclassified in agglutination tests are shown in Figure 11b. All were mucoid on agar. The LPS from a serum resistant isolate (track 1) gave a profile typical of 0-typable strains and this was similar to the LPS in track 2 although less medium molecular weight bands were present in the latter. Two isolates in tracks 3 and 4 respectively showed a progressive loss of LPS constituents and the LPS in track 4 retained only core components. Both these isolates were fully sensitive to NHS. In contrast the LPS in track 5 was also from a fully sensitive isolate and gave a silver stain reaction throughout the length of the gel. The last isolate in this category track 6 was also serum sensitive and it lacked high and medium molecular weight components.

Electrophoresis profiles of LPS from six CF isolates which were agglutinated by both 'variant' antisera i.e. PA/NT, are shown in Figure 11c. Three of the LPS samples (tracks 1, 2 and 3) exhibited a full 'ladder-like' pattern of repeating polysaccharide units. Isolates 1 and 2 were fully resistant to NHS and isolate 3 showed

Figure 11

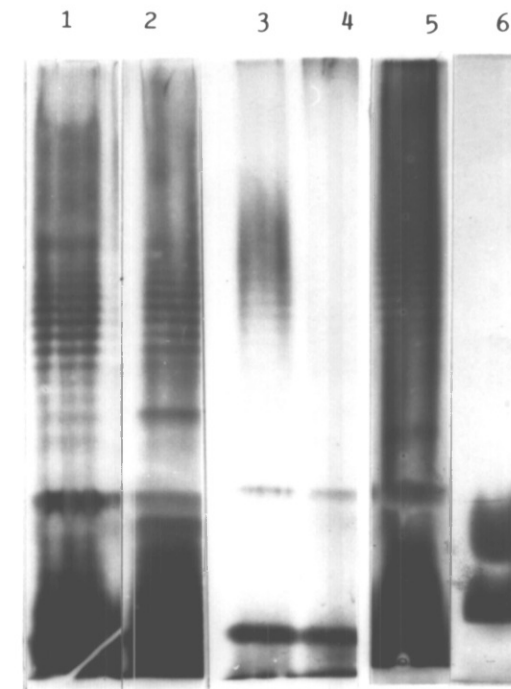
LPS profiles of Pseudomonas aeruginosa isolates from CF divided
by agglutination category

a. O-typable



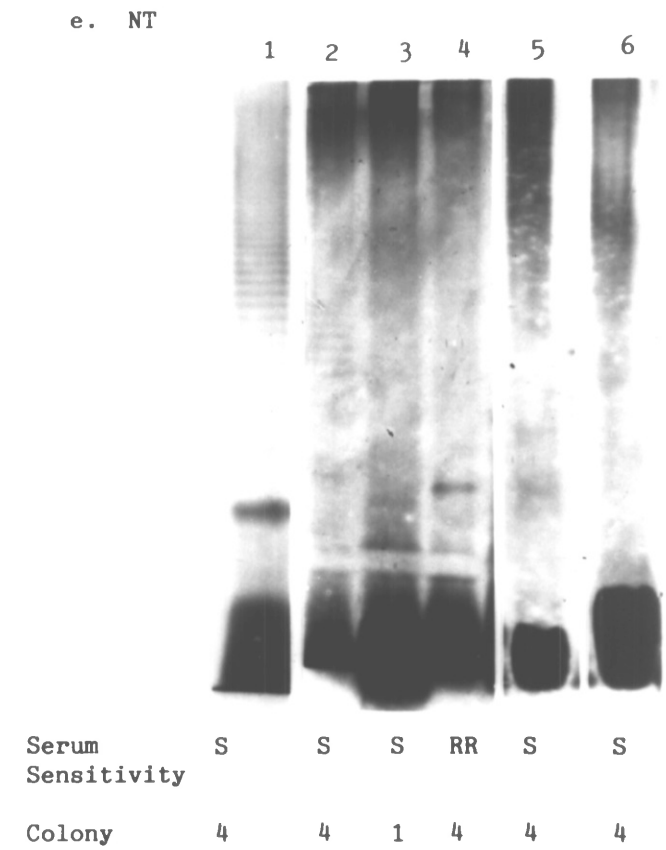
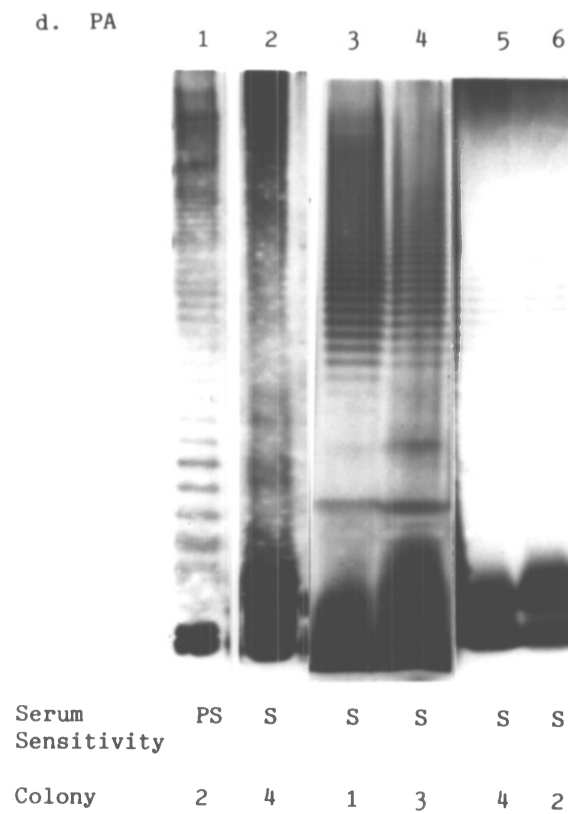
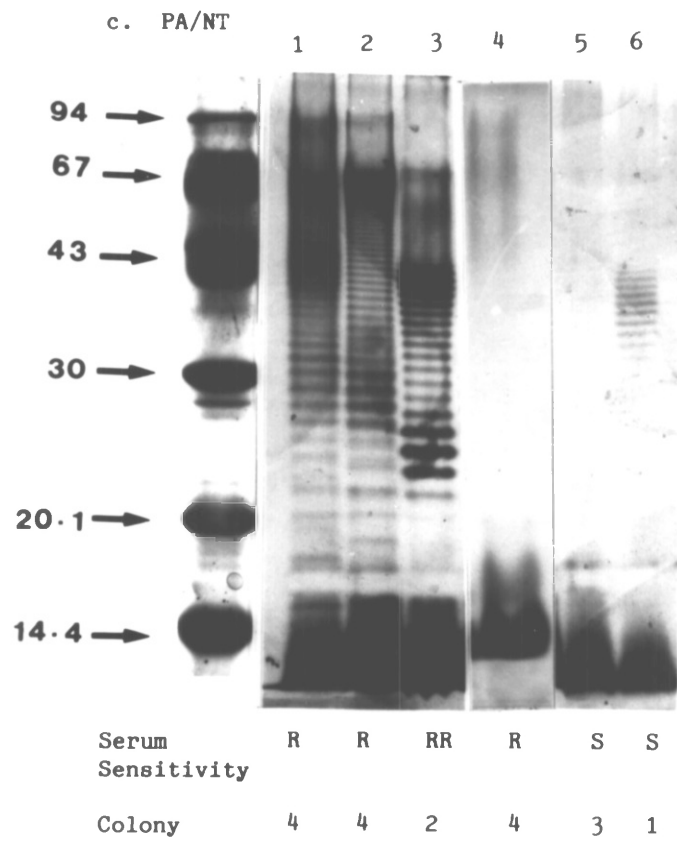
Agglutination category	06	09	06	09	06	011
Serum sensitivity	R	R	R	R	R	RR
Colony	1	1	4	1	1	2

b. Unclassified



Serum sensitivity	R	S	S	S	S	S
Colony	4	4	4	4	4	4

Figure 11 continued



slightly reduced resistance to serum. Isolate 4 was resistant to serum despite the absence of medium and high molecular weight components. The remaining two isolates in this category were both sensitive to NHS and only the LPS of the isolate 6 contained bands corresponding to side chain. The LPS of isolate 5 appeared to be composed entirely of low molecular weight core material.

Figures 11d and 11e respectively show the heterogeneity of LPS profiles among those CF isolates agglutinated by either the PA antiserum or the NT serum. PA isolates showed a similar degree of variation in banding profile as those of other serological phenotypes. The number and distribution of bands did not correlate with the sensitivity of the isolate to serum or the colony type of the isolate. Mucoid PA strains as in the other serotypes contained smooth or rough LPS as determined by their profile in gels. Two of the PA strains (5 and 6, Figure 11d) had semi-rough LPS but three of the strains showed extensive laddering through most regions of the gel.

With the exception of two isolates (1 and 2, Figure 11e), the remainder of the NT isolates were characterised by the presence of diffuse silver staining material along the track. It is doubtful whether this was due to components of LPS and the smearing was probably the result of substances extracted by proteinase K digestion which disassociated in the SDS buffer.

The foregoing results confirmed that the presence of LPS defects can be detected by SDS-PAGE and silver staining but the significance of the presence or absence of components did not correlate with serology or colony form. Furthermore they show that SDS-PAGE is inadequate ,

when used alone, for the classification of CF isolates. However in combination with serology it does provide some corroborative evidence of the molecular basis of the serological reactions of strains.

Immunoblotting of defective LPS

In order to establish which region of LPS reacted with antibodies in the variant sera, the LPS of 15 CF isolates and strains 7/1 and 18S were immunoblotted with 7/1 and 18S antisera at a dilution of 1 in 1000. The immunoblots from this experiment were not photographed but Figure 12 shows a diagrammatic representation of a tracing from the original nitrocellulose sheet.

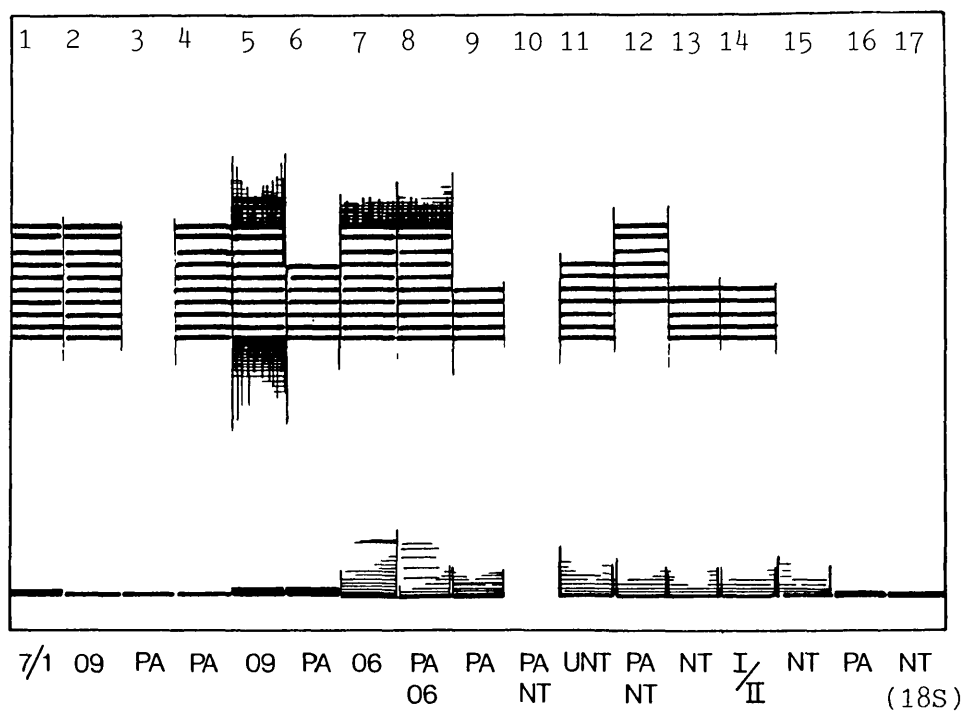
Antiserum 7/1 (the PA antiserum) reacted predominantly with medium molecular weight components of the LPS of 12 of the strains tested. (Figure 12a). This appeared as evenly spaced bands which corresponded with the banding seen in SDS-PAGE of the LPS of strain 7/1 (Figure 6), indicating that antibody in the PA antiserum was directed towards epitopes of LPS which were probably responsible for the agglutination reaction of the isolates with this antiserum. However, the antiserum did not bind with the LPS of the isolates in tracks 3, 10, 15 or 16 although three of them had been agglutinated by this antiserum. The PA antiserum also recognised medium molecular weight repeating units of the two O-typable strains, (tracks 5 and 6). This region was also recognised in the LPS of an isolate which had a specific O reaction but was also agglutinated by PA antiserum, (track 7). Some material in these isolates of higher molecular weight was also seroreactive but did not resolve as clear bands.

There was very little evidence of antibodies to core LPS in the PA

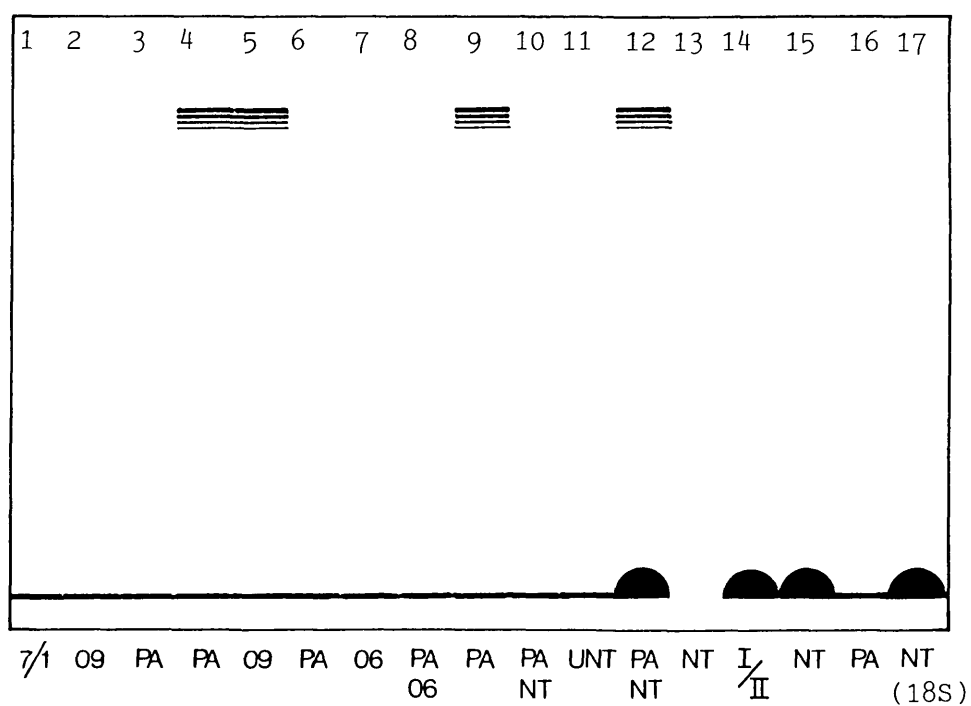
Figure 12

LPS of *Pseudomonas aeruginosa* isolates from CF immunoblotted with the two experimental sera

a) PA antiserum (anti 7/1)



b) NT antiserum (anti 18S)



antiserum. Eight of the isolates showed weak, diffuse binding of low molecular weight constituents indicative of core substance. LPS of strain 18S did not react with the PA antiserum diluted 1 in 1000 but when the serum was used at twice this concentration weak binding in the core region of this strain was evident.

It appeared therefore that the immunodominant component of strain 7/1 was the side chain retained by the mutant and PA antibodies were directed towards this sequence of repeating oligosaccharide units and probably was responsible for the agglutination of CF isolates. There was also some evidence of antibody, albeit in low concentration, towards core-like epitopes. However, the lack of reaction with the LPS of three isolates that had previously been agglutinated by this antiserum suggests that other epitopes possibly protein which were destroyed by the extraction procedure were responsible for their seroreaction. An alternative explanation may be that the configuration of the LPS was altered by SDS-PAGE thus rendering them unreactive with antibody. Detailed studies of the complex immunoreactive components are necessary but were considered outside the scope of this thesis.

The binding of the LPS of the isolates with the 18S antiserum (NT) was very variable, (Figure 12b). In some tracks; 12, 14, 15 and 17 an antigen-antibody reaction corresponding to low molecular weight core-like components was revealed whilst no reactions were given by LPS of ten of the isolates including strain 7/1 and three isolates which had been agglutinated by this antiserum. Some faint banding in the high molecular weight region of the nitrocellulose was seen with four isolates which was unexpected as this region of LPS was not

found in earlier SDS-PAGE profiles of the vaccine strain 18S, (Figure 6), therefore the presence of antibody in the antiserum to these epitopes is difficult to explain.

As the overall intensity of the immunoblots of LPS of isolates with the NT antiserum was so markedly reduced in comparison with the homologous immunoblot with an extract of whole cells (Figure 9D), the presence of antibodies to surface components other than LPS was investigated. Whole cell extracts and outer membrane proteins from selected CF isolates were separated by SDS-PAGE and examined by immunoblotting with serum 18S in a range of dilutions. Numerous seroreactive components were revealed of diverse molecular weights and the profiles were similar to that given with extracts of the vaccine strain (Figure 9D).

The contribution of outer membrane proteins to the reaction of CF isolates with the NT antiserum was therefore confirmed. As strain 18S had markedly reduced LPS this may have led to the exposure of proteins on the cell surface and stimulation of antibody production in the animal. The relevance of these antibodies and the specific epitopes that led to agglutination of CF isolates is worthy of further study as traditionally, antisera prepared against heated cells, as was the case here, are generally considered to be devoid of antibody to heat-labile protein antigens.

DISCUSSION

SECTION I

The occurrence of strains of P.aeruginosa with defective O somatic antigens in CF has been described independently by two groups of workers (Hancock et al. 1983; Penketh et al. 1983^a). Both studies indicated that most isolates of P.aeruginosa were unable to synthesise complete lipopolysaccharides, which confer O-antigenic specificity on the strain.

This study was designed to provide information on the nature of the defect in surface antigens of strains and to characterise these antigens in order to facilitate their identification in strains from sputum samples of CF patients. Alterations in the structure or steric configuration of the LPS molecule result in loss of O-antigen specificity and give rise to variants which are either agglutinated by several O-antisera or have no reaction with any typing sera. Attempts were made to construct reference laboratory strains of P.aeruginosa which exhibited these characteristics.

It was clear from earlier studies that LPS defective variants could be isolated from wild-type strains which possessed a single specific O-antigen determinant by the action of phages that adsorbed to receptors in the O-antigen (Koval and Meadow 1977; Meadow and Wells 1978). The mechanism of selection of variants by phage is thought to be as follows. The phage adsorbs to receptors on O-antigenic LPS side chains and is then able to penetrate the host cell. On multiplication and release of daughter phage particles, lysis and death of the host cell occurs. Variants which lack these primary receptors arise naturally by mutation within a culture and do not adsorb phage. These cells are resistant to infection by the phage and are therefore selected as viable cells.

This approach was used to obtain variants of strains of P.aeruginosa in the laboratory. Alterations in the surface of one of the laboratory variants were indicated by the loss of phage receptors as there was no reaction with any of the phages active on the parent strain. Since the pyocin type of the two strains was identical it was concluded that the variant had arisen from the parent strain. Although pyocins are known to adsorb to somatic structures, including LPS (Govan 1978; Meadow and Wells 1978) the pyocin typing method used determined only the range of pyocins produced by the isolates rather than their sensitivity to these particles (Gillies and Govan 1966). Indeed, Meadow and Wells (1978) found that a phage resistant variant had altered susceptibility to R-pyocins which were active on the wild-type strain.

The other variant, 18S, was a naturally occurring isolate from CF sputum. It was sensitive to the bactericidal action of normal human serum (NHS) and as it was not agglutinated by any of the O-typing sera, it was termed NT. Revertants of this isolate emerged in serum sensitivity tests and these were resistant to serum and O-typable. These revertants were considered to represent the original wild-type state because of the phenotypic similarity to strains of P.aeruginosa from conditions other than CF.

The pair of strains were of identical pyocin type and had a similar phage sensitivity. Considering the apparent alteration in LPS profile of the variant strain illustrated in Figure 6 it was surprising that it was susceptible to apparently LPS-specific phages (Temple et al. 1986^a). Moreover, Meadow and Wells (1978) suggested that one of the LPS specific phages active on both strains, phage

E79, adsorbed to core components of LPS. This indicated that although the 'parent' strain 18R expressed a specific O antigen, probably determined by side chain oligosaccharides, the core receptor for this phage was accessible. In contrast, the other variant, strain 7/1, lost sensitivity to phage E79 as well as to the somatic phages.

Schiller, Hackley and Morrison (1984) also isolated serum resistant colonies from serum sensitive CF strains of P.aeruginosa and showed that the serum resistant forms were of a different O-type from that expressed by the serum sensitive strains. Interestingly their serum sensitive strains were serotype O10 or PA and the resistant strains were either O6 or O9. They did not test the pairs of strains for phage or pyocin susceptibility and therefore could not confirm their identity although they noted that there was no apparent difference in the colonial morphology of the strains.

The increased sensitivity to B-lactam antibiotics of one of the variant strains provided further evidence of an altered outer membrane as alterations in the outer membrane proteins and LPS have been found to influence the permeability of P.aeruginosa to cell wall antibiotics.

Irvin et al. (1981) found that a significant number of isolates from CF sputum were hypersensitive to B-lactams and they identified two additional outer membrane proteins which were not expressed by strains with 'normal' sensitivity to these antibiotics. I found no differences in the outer membrane profiles corresponding to those described by Irvin et al. (1981) on SDS-PAGE analysis of the laboratory constructed variant 7/1 which was hypersensitive to

B-lactam antibiotics.

On the other hand defects in LPS structure are known to result in the increased sensitivity to antibiotics of strains of Salmonella and Pseudomonas (Roantree, Kuo and MacPhee 1977; Shearer and Legakis 1985). Rough mutants of P.aeruginosa such as Zimmerman's mutant (Kropinski, Chan and Milazzo 1978) have gross alterations in their LPS constitution and this mutant in particular is extremely sensitive to the action of B-lactams.

The increased sensitivity of strain 7/1 to B-lactams provided circumstantial additional evidence of a lesion in the surface structures of the cell and subsequent experiments on the constituents of the LPS of this strain supported this conclusion. No differences were found in the sensitivity of the other pair of strains (18R and 18S) to B-lactams although they were both extremely sensitive to carbenicillin. This indicated that hypersensitivity to antibiotics was not determined solely by the integrity of O-antigen.

Agglutination tests showed the presence of antigenic epitopes common to both parent and variant strains. On the parent strain this epitope was not as accessible to antibody as in the variant and therefore was not the immunodominant component of the parent strain, a supposition which explains why the homologous titres of the parent sera were not reduced by absorption with variant cells.

A similar experiment was reported by Meadow et al. (1984) who showed that antiserum to a wild-type strain also reacted with O-variants of this strain and obtained comparable results in absorption tests. This was consistent with the assumption that although the epitopes were present on both parent and variant strains and were immunogenic,

they were not readily accessible on the cell surface to remove the appropriate antibody from a serum. Two of Meadow's mutants (Meadow et al. 1984), unlike 7/1 and 18S, absorbed homologous antibody from the parent antiserum, and likewise parent cells were able to bind antibodies in antisera to the mutants. It is possible that although their mutants lacked O-specificity there was still sufficient type specific LPS available on the cell surface to combine with antibody. Only a limited chemical analysis of the LPS composition of the four strains could be carried out in the Division of Hospital Infection. From this it appeared that the pair of strains 7 and 7/1 contained similar amounts of LPS, but the variant 18S yielded less LPS than its parent. Moreover there was a substantial difference in the total carbohydrate content of the first pair but not between 18R and 18S. These findings confirmed that an alteration had occurred in the composition of the LPS in terms of gross content. A higher ratio of KDO to carbohydrate in the variant strains was indicative of a loss of side chain but that there was little alteration in the core region. Support for this conclusion is given by Hancock et al. (1983) who found that KDO accounted for almost 60% of the LPS from O-defective strains isolated from CF.

Two of the three PA mutants of strain PACIR described by Meadow et al. (1984) yielded high molecular weight fractions in their LPS and one of the mutants contained the repeating O-unit of the parent strain. The LPS of this mutant yielded slightly less rhamnose than the parent and it had a lower molecular weight corroborating an alteration in its structure although the main antigenic determinants were still present and serologically active. All three mutants of

Meadow et al. (1984) yielded low molecular weight components which differed from those of the parent strain either in molecular size or concentration indicating heterogeneity of the core in contrast to strains 7 and 7/1 which had no demonstrable difference in their core composition.

The LPS of the three PA mutants of Meadow et al. (1984) differed markedly in chemical composition implying that the expression of PA was not due solely to an altered O-antigen but to the exposure of novel epitopes on the bacterial surface.

Dr Wilkinson found no gross differences in the fatty acid composition of 7 and 7/1 and Kropinski et al. (1982) showed that five of the seven major fatty acids of the LPS of an antibiotic supersensitive strain were unaltered. Nevertheless a decrease was detected in the concentration of dodecanoic and hexadecanoic acid in fractions of LPS corresponding to the core region.

The chemical analysis provided circumstantial evidence of alterations in the LPS of the variants originally selected by their serological properties. However due to the inherent variability of chemical analysis and the labour and material intensive nature of such work an alternative method was sought whereby the heterogeneity of LPS preparations could be readily identified. Electrophoresis in SDS-PAGE had been used extensively for the analysis of LPS by other workers (Jann, Reske and Jann 1975; Palva and Mäkelä 1980; Goldman and Leive 1980). On electrophoresis LPS resolves as distinct bands, the complexity of which depend on the nature of the LPS.

Lipopolysaccharide from LPS defective strains, rough (R)-LPS, resolves as a single, fast moving band which migrates with the

tracking dye. This band corresponds to the low molecular weight peak (F2) obtained by gel filtration i.e. core material (Goldman and Leive 1980). The slow moving high molecular weight bands are comparable to the F1 fraction and represent the O-side chains of the LPS.

The introduction of the silver staining method of Tsai and Frasch (1982) has provided a simple and sensitive method for the detection of LPS separated by SDS-PAGE. The polysaccharide portion of the LPS is thought to be the reactive component in the silver stain. Lipid A appears not to be stained by this method. The silver reaction is dependent upon the presence of periodate sensitive cis-glycol structures in the core and side chain polysaccharide of LPS molecules. However, Kropinski, Berry and Greenberg (1986) dispute this, as the side chain portion of gels often stains less well than the lower molecular weight units. This may be caused by steric interference of the oxidation of cis-glycol moieties by longer side chains. Kropinski et al. (1986) concluded that the identification of LPS components based solely on silver stained gels may be inaccurate. Hitchcock and Brown (1983) in their studies of heterogeneity in S.minnesota found that differences in LPS as shown by SDS-PAGE and silver stain could be positively correlated with biochemical differences in LPS and that LPS chemotypes had unique migration patterns in SDS-PAGE.

The heterogeneity of banding in the serotype strains of P.aeruginosa LPS reflected the variety of O-specific determinants. It would be unwise at this stage to attempt to interpret banding profiles of strains of P.aeruginosa as an absolute indicator of their serological

characteristics. Nevertheless the banding patterns obtained with the four strains 7, 7/1, 18R and 18S (Figure 6) confirmed that the LPS of the serological variants was defective.

Deep rough mutants of Salmonella sp. and E.coli (Palva and Mäkelä 1980; Goldman and Leive 1980) which were unable to synthesise repeating side chains showed only a double band in the low molecular weight region of gels. A similar profile was obtained with LPS 18S although other probes of cell surface architecture (e.g. phages) had indicated that the outer membrane of this strain was not dissimilar from its parent. An LPS defect in strain 18S was indicated however by the sensitivity of this strain to the lethal action of normal human serum. Exposure of lipid A, a site of complement activation, (Taylor 1983) had probably occurred on this strain due to the absence of protective O side chains thereby rendering it sensitive to serum (Mäkelä et al. 1980). The single band identified in LPS 18S was present in each of the other LPS tracks and might have been an epitope common to all the strains and responsible for the cross reactions of serum 18S in agglutination tests. Alternatively the dramatic reduction in LPS constituents of 18S may have exposed surface protein antigens which stimulated antibody production in the rabbit when this strain was used as a vaccine.

The foregoing evidence taken along with that provided by immunoblotting suggests that protein antigens did not give rise to significant levels of agglutinating antibody when the LPS of a strain was unaltered. The failure of strain 18S to remove homologous antibody from the parent serum, 18R, in absorption tests, is consistent with the fact that as the homologous antibody was directed

towards side chains the absence of these structures in the variant (18S) would not result in the removal of this antibody.

The LPS profile of strain 7/1 was slightly surprising as the track showed numerous bands of higher molecular weight corresponding to part of the profile of the parent strain. Thus a subtle defect in LPS synthesis was probably sufficient to alter the phenotype of strains of P.aeruginosa. However the results of the chemical analysis of the side chains of strain 7/1 did not reflect the electrophoretic picture that suggested simply a reduction in the number of 'parent-type' side chains in the variant strain.

Most serologically atypical isolates of P.aeruginosa from CF gave profiles in SDS-PAGE indicative of a loss of side chain in their LPS but they retained core components (Hancock et al. 1983). Serologically typical isolates, i.e. O-typable, were serum resistant and their LPS resolved as typical ladders of side chain bands.

Hancock et al. (1983) were unable to distinguish between PA and NT isolates by electrophoretic profile alone and unless corroborated by serological typing this method cannot be used to identify unequivocally strains of P.aeruginosa with defective LPS.

Electrotransfer was developed by Southern (1975) for the transfer of electrophoretically fractionated DNA to nitrocellulose filters in order to examine complementary sequences by hybridisation in situ. A modification of this procedure 'Western-blotting' was described by Towbin, Staehelin and Gordon (1979) and relies on the samples fractionated by SDS-PAGE being eluted as anions by the application of an electric current, to nitrocellulose. The modified ELISA method described by Hawkes et al. (1982) enables detection of immunogenic

moieties immobilised on the nitrocellulose. Electrophoresis and transfer apparently do not effect the immunoreactivity of such components.

Immunoblotting with whole cells of the four strains and the four antisera (Figure 9) provided results which were in general agreement with those obtained in agglutination and ELISA. Serum 7 (Figure 9A) reacted most strongly with the LPS of the homologous strain, but its reaction with strain 7/1 was less evidently attributable to LPS. Bands were also obtained with whole cells of 18R and 18S and many of these were due to proteins. Similarly serum 18R gave an intense reaction with homologous whole cells but also revealed low molecular weight proteins common to all four strains.

The two variant antisera reacted similarly with all four strains suggesting that these sera contained antibody to protein epitopes exposed on the vaccine strain as a result of an alteration in LPS. However LPS antibody was also present in these sera as shown by blots with purified LPS (Figure 10). The two parent sera were highly specific for their homologous LPS.

The contribution of purified LPS to the serological reactions of the experimental strains was examined in a variety of test systems. All tests corroborated the fact that the predominant antibody in sera to the parent strains was directed against an epitope of high molecular weight. This determinant was not identical in either of the wild-type strains in electrophoretic profile or in seroreactivity although each determinant conferred quite distinct serotype specificities.

The defective LPS of the variant strains was less immunogenic than

the complete parent molecule and gave rise to antibodies which bound to epitopes shared by the strains. The results of various serological tests indicated that this cross-reactivity was due, at least in part, to the core components of the LPS of the strains.

Competitive-inhibition ELISA with purified LPS probably provided the most accurate picture of the contribution of LPS to the serological reactions of the four strains of P.aeruginosa. The homologous reaction of serum 7 with whole cells could only be inhibited by a high concentration of homologous LPS presumably because the number of combining sites on the surface of the bacterial cell greatly exceeded those in the LPS preparation. It seems reasonable to suppose that the O-specific side chains radiate from the entire bacterial surface and provide numerous sites for the cross linking of antibody molecules. In contrast purified LPS is probably considerably less polymerised on reconstitution than in the native form and contains fewer antibody binding sites. Indeed less LPS was needed to neutralise the reaction of this serum with homologous LPS. Similarly LPS of the variant strain did not block the homologous reaction of the parent serum and whole cells although the results of serological tests had inferred that this strain contained antigenic components present in the parent. Alternatively the failure of homologous purified LPS to compete for antibody reacting with the intact cell may be attributable to the stoichiometric configuration of isolated LPS which results in an alteration of its affinity for these antibodies.

The loss of O-specific side chains by P.aeruginosa results in alterations in many of the properties of the outer membrane. Both

the variants studied here and variants isolated from CF (Penketh et al 1983^a; Pitt et al. 1986) showed increased sensitivity to the action of NHS.

The activation of complement by Gram-negative bacteria can occur via the classical or alternative pathway; the former requires recognition of bacterial surface antigens by antibody in the serum. The alternative pathway however can be activated by bacterial surface polysaccharides independent from antibody. In addition lipid A has been shown to mediate activation of the classical pathway in the absence of detectable specific antibody (Morrison and Kline 1977). Precursor C1 is bound and activated by lipid A due to the specific binding of Cq thus obviating the need for specific antibody.

Many studies on different Gram-negative bacteria have established a link between the presence of O-specific side-chains and sensitivity to NHS (Shafer et al. 1984; Grossman and Leive 1984; Odurmeru, Wiseman and Ronald 1985). If the serum killing of P.aeruginosa from CF is mediated by lipid A the reduction in LPS may expose lipid A and initiate the complement cascade. However the relationship between the length of O-side chain and sensitivity to NHS is not absolute. Meadow et al. (1984) demonstrated that a spontaneous serum resistant mutant lacked O-antigenic side chains and was non-typable. Conversely Hancock et al. (1983) showed that an O-typable CF strain was sensitive to normal human serum. In the series of CF isolates studied here serum resistance although highly statistically associated with O-typability was not absolute as 5% of the serum sensitive strains were of a specific O-type.

It may be that changes in the chemical structure of the LPS have more

effect on the sensitivity of the strain than the actual length of O-side chain retained. The studies of Goldman and Leive (1984) in Salmonella sp. would suggest that this is a factor when activation of the alternative pathway is initiated by LPS itself. However, if the activation of complement in these defective strains of P.aeruginosa is initiated by lipid A the stereochemistry of the molecule may be important. Presumably any variation in the integrity of the LPS molecule, with or without the loss of O-specificity would expose lipid A on the cell surface and enable the activation of complement. The foregoing results confirmed that the laboratory constructed variants were defective in LPS synthesis and the two strains represented at least two distinct categories of LPS alteration. Examination of CF isolates with these antisera indicated that similar alterations in LPS characteristics occurred in CF although several other phenotypes of LPS as judged by SDS-PAGE profiles obviously exist. The sera allowed the rapid identification of O-variants in sputum samples and when used in conjunction with serum sensitivity tests enabled the classification of several isolates. The degree of heterogeneity in LPS profile and serum resistance even within the same specimen showed that the simple division of CF strains into O-typable and O-not typable was insufficient to distinguish between the many variations present in a sample.

It was now necessary to investigate the association of the O-defects of strains of P.aeruginosa from CF with the duration of infection and other clinical parameters in order to assess the significance of these alterations to the course of pulmonary infection in CF.

RESULTS

SECTION II

Longitudinal bacteriological survey

Patients

The total number of patients from whom samples were collected in the two year survey was 210 (Table 22). During the course of the investigation 33 of these patients died. Sixty of the patients did not attend an outpatient clinic on more than one occasion and so 150 patients were sampled more than once during the survey period.

Each patient attended an outpatient's clinic at least twice-yearly and on average eight patients were examined at each of two weekly clinics. One hundred and fifty two of the patients were hospitalised on one or more occasion and samples obtained from them as inpatients were included in the survey. A total of 844 sputum samples were received and the number of samples per patient ranged from 1 to 28 with a mean of 5.0.

The patient population comprised 117 males and 93 females, with an age range of 12-61 years (mean 22.3 years) (Table 23). The age of a patient was taken as the age on first attendance at outpatients at which a sputum sample was collected for the survey. Males ranged in age from 12-61 years (mean 21.8 years) and females from 13-45 years (mean 23 years).

The lung function of the patients, measured at the same time as the collection of the first sputum sample, ranged from 0.7L to 6.5L for FVC and 0.3L to 5.2L for FEV_1 . Table 24 gives a breakdown of the lung function values according to the sex and age of the patients. No lung function data were available for 13 patients at first sampling due either to haemoptysis or other reasons.

TABLE 22

Cystic fibrosis patients attending the Brompton Hospital
September 1983 - September 1985 from whom sputum samples were obtained

Number of patients	210
Males	117
Females	93
Sampled once only	60
Deaths	33
Patients hospitalised ^a	152

^a On at least one occasion

TABLE 23

Age distribution of male and female cystic fibrosis patients in survey

Age of Patient (yrs)	Sex and (number)	
	Male ^a (117)	Female ^b (93)
12 - 20	59	36
21 - 25	36	27
26 - 30	14	20
31 - 40	6	8
41 - 50	0	2
51 - 61	2	0

^a mean = 21.8 years

^b mean = 23.0 years

TABLE 24

Mean lung function values of cystic fibrosis patients
according to age and sex

Age of Patient	Male		Female	
	FVC ^a	FEV ₁ ^b	FVC ^a	FEV ₁ ^b
12 - 20	2.38	1.50	1.88	1.26
21 - 25	2.37	1.32	2.46	1.60
26 - 30	2.37	1.82	1.91	1.18
31 - 40	2.37	1.00	1.67	0.98
41 - 50	-	-	2.67	1.42
51 - 61	2.52	1.35	-	-

^a Forced vital capacity (litres)

^b Forced expiratory volume in 1 second

Bacteriology of Sputum Samples

Of the 844 samples processed, 676 (80%) yielded P.aeruginosa and Staphylococcus sp. was found in 254 (30%) of samples (Table 25). The incidence of H.influenzae was not determined in each specimen but of the 210, patients 142 (67%) were known to harbour H.influenzae at some time during the survey.

Other bacterial species identified included Proteus sp., E.coli and other enterobacteria, Pseudomonas maltophilia, and yeasts. Samples were not screened for the presence of P.cepacia as a suitable selective medium was not available at the start of the investigation. Colonies which may have been P.cepacia were presumptively identified as Pseudomonas sp. and subsequently tested in the API 20 NE system. Most of these cultures were identified as P.maltophilia but on further examination by the Computer Trials Laboratory at Colindale a proportion were tentatively identified as P.cepacia. Later in the survey 20 sputum samples were plated on a selective medium for P.cepacia and only one sample yielded this organism. Due to the lack of this medium in the initial stages of the survey, accurate figures for the incidence of P.cepacia in this patient population could not be obtained.

Staphylococci were divided into coagulase-positive S.aureus and coagulase-negative 'white' staphylococci and were isolated from 204 (24%) and 50 (6%) of the sputum samples respectively to give a total frequency of 30% (Table 25). Proteus sp. was found in 20 samples (2%), E.coli in 18 (2%) and other enterobacteria in 30 (3.5%) and these included Klebsiella sp., Enterobacter agglomerans, Citrobacter freundii and Serratia sp. Organisms identified as Pseudomonas sp.

TABLE 25

Frequency of isolation of bacterial species from 844 sputum samples
from 210 CF patients

Bacterial Species	Number and (percentage) of cultures of stated species	
<u>Pseudomonas aeruginosa</u>	676	(80)
<u>Staphylococcus aureus</u>	204	(24)
<u>Pseudomonas</u> sp.	72	(9)
Staphylococci Coagulase-negative	50	(6)
Enterobacteria	48	(6)
Gram-positive ^a	39	(4)
<u>Proteus</u> sp.	20	(2)
Yeasts	7	(1)
No bacterial growth	14	(2)
<u>Haemophilus influenzae</u> ^b	ND	(-)

^a Not identified

^b Not included in survey but 67% of patients known to be culture
positive for H.influenzae.

which included P.maltophilia were isolated from 72 (9%) of samples and yeasts were found in 7 specimens (1%).

Unidentified Gram-positive bacterial species were recovered from 39 (4%) of samples. The majority of these grew on MacConkey agar as small, pink, lactose-fermenting colonies which did not appear as staphylococci on non-selective media. Fourteen samples (2%) gave no bacterial growth on any of the media used after the 48h incubation period.

The incidence of P.aeruginosa, Staphylococcus sp. and other bacteria in the primary specimen from each patient is given in Table 26. P.aeruginosa was the most prevalent and was present in 164 of 210 samples giving a colonisation rate for all patients of 78%. Staphylococci were more frequently isolated from the primary specimen than from all specimens during the survey (40% vs. 30% Table 25). The isolation rates of other species in the first sample from each patient reflected the overall trend seen with all subsequent specimens (Table 25). All of the primary samples from each of the 210 patients yielded bacterial growth.

Examination of the 168 sputum samples that did not contain P.aeruginosa showed that 14 (8%) were free of other bacterial species and 114 (68%) of samples contained staphylococci either alone or in combination with other species (Table 27). P.maltophilia was identified in 11 sputum samples (7%), but ten of these were sequential samples from the same patient. The incidence of isolation of other species of bacteria was similar to that observed for all specimens and those patients not colonised with staphylococci harboured enterobacteria such as E.coli and Klebsiella sp.

TABLE 26

Frequency of isolation of bacterial species from the first
sputum sample from each of 210 CF patients

<u>Bacterial Species</u>	<u>Number and (percentage) of cultures of stated species</u>	
<u>Pseudomonas aeruginosa</u>	164	(78)
<u>Staphylococcus aureus</u>	84	(40)
Staphylococci Coagulase-negative	35	(16)
<u>Pseudomonas sp.</u>	24	(11)
Enterobacteria	24	(11)
Gram-positive ^a	6	(3)
<u>Proteus sp.</u>	5	(2)
Yeasts	4	(2)
No bacterial growth	0	(0)

^a not identified

TABLE 27

Frequency of isolation of bacterial species from those sputum samples
(168) which were free of Pseudomonas aeruginosa

Bacterial Species	Number and (percentage) of cultures of stated species	
Staphylococcus	114	(68)
Enterobacteria	27	(16)
<u>Pseudomonas</u> sp.	14	(8)
Gram-positive ^a	8	(5)
<u>Proteus</u> sp.	2	(1)
Yeast	4	(2)
No bacterial growth	14	(8)

^a Not identified

Association of clinical data with the isolation of *P.aeruginosa*

The patients were divided into three groups based on the preliminary examination of their sputum samples and information from the Brompton (Table 28).

Group A Those patients that did not harbour *P.aeruginosa* throughout the survey.

Group B Patients that were initially free of *P.aeruginosa* but subsequently became colonised with this organism.

Group C Patients who consistently yielded *P.aeruginosa* throughout the survey.

Group A contained 35 patients (17 males, 18 females) with an age range of 12-61 years (mean 22.6). Group B numbered 42 patients (22 males, 20 females), age range 13-51 years (mean 24.73). Group C was the largest group with 133 of the patients (78 males, 55 females), their ages ranged from 12-45 years (mean 21.3).

Table 28 shows the incidence of staphylococci in each group. Fishers Exact Probability Two-Tailed Test was used to determine the degree of association between the presence or absence of *P.aeruginosa* with the isolation of staphylococci. Staphylococci were more often isolated from those patients free of *P.aeruginosa* (Group A) than from those who either acquired it (Group B) or were persistently colonised with the organism (Group C) ($p < 0.001$). The data were examined further to determine whether the presence of staphylococci was associated with the presence of *P.aeruginosa* in patient groups B and C. Ninety one

TABLE 28

Incidence of staphylococci in CF patients grouped according to the
presence or absence of Pseudomonas aeruginosa in their sputum

Number	Patient Group					
	A		B		C	
Patients	35		42		133	
Male : Female	17	18	22	20	78	55
Mean age (yrs)	22.6		24.73		21.3	
Number and (%) with Staphylococci in first sputum sample	24 (68)		24 (57)		42 (32)	
					p < 0.01*	
					p < 0.001*	

A patients free of Pseudomonas aeruginosa

B patients initially free

C patients persistently colonised

* Fishers Exact Probability Two-Tailed Test

patients in Group C harboured P.aeruginosa but were free of staphylococci compared with 18 in Group B ($p < 0.01$). It appeared therefore that as patients acquired P.aeruginosa the incidence of staphylococci fell.

The bacteriological findings made available by the Brompton Hospital showed that equal numbers of patients from each group were colonised by H.influenzae so no association between the isolation rates of this organism and the presence or absence of P.aeruginosa could be determined.

The range (and mean) of lung function values (FVC and FEV_1) for each of the groups is shown in Table 29. The mean value for FEV_1 was lower for that group of patients who were persistently colonised by P.aeruginosa and highest for those free of the organism but there was no real difference in the mean value for FVC between the three groups.

Other associations were sought between the presence of P.aeruginosa and hospitalisation and/or mortality of the patients (Table 30). Although more of the patients in Group C died this was not associated statistically with the presence of P.aeruginosa. There was a higher incidence of admissions in Group B but this was not statistically related to the presence of P.aeruginosa. However the increased hospitalisation of these patients may suggest that the acquisition of P.aeruginosa is in some way associated with a decline in clinical status.

Furthermore no association between the presence or absence of P.aeruginosa and hypersensitivity reactions was found (Table 31). Although the average age at which the three groups were diagnosed was

TABLE 29

Respiratory function of cystic fibrosis patients grouped according to
the presence or absence of Pseudomonas aeruginosa in their sputum

	Patient Group		
	A	B	C
*FVC ₁	2.27	2.30	2.27
(range) mean	(0.27-5.2)	(0.55-5.05)	(0.28-6.5)
*FEV	1.56	1.33	1.25
(range) mean	(0.3-4.2)	(0.35-3.2)	(0.4-5.2)

A patients free of Pseudomonas aeruginosa

B patients initially free

C patients persistently colonised

* litres

TABLE 30

Hospitalisation and mortality of cystic fibrosis patients grouped according to the presence or absence of *Pseudomonas aeruginosa* in their sputum

Number	Patient Group		
	A	B	C
Patients admitted during trial (%)	23 (66)	34 (81)	95 (71)
	NS*		NS*
	NS*		
Average admissions per patient	0.97	1.88	1.34
Deaths (%)	2 (6)	6 (14)	21 (16)

A patients free of *Pseudomonas aeruginosa*

B patients initially free

C patients persistently colonised

* Fishers Exact Probability Two-Tailed Test

TABLE 31

Clinical information on cystic fibrosis patients grouped according to
the presence or absence of Pseudomonas aeruginosa in their sputum

	Patient Group		
	A	B	C
Average age diagnosed (yrs)	3.7	3.8	3.7
First chest symptoms recorded (yrs)	6.8	6.1	5.4
Average weight (kg)	44.9	41.8	44.59
Number hypersensitive ^a	23 (66)	26 (62)	83 (62)

A patients free of Pseudomonas aeruginosa

B patients initially free

C patients persistently colonised

a Reaction in skin test

very similar, those patients in Group C tended to present with chest symptoms earlier (5.4 years compared with 6.8 years Group A). There was no apparent difference between the weights of patients in each group.

The antibiotics received by patients in each group were examined under the broad categories of antistaphylococcal agents, antipseudomonal and antihaemophilus agents. Associations were sought between the presence or absence of P.aeruginosa and treatment with these antibiotics.

Table 32 shows the numbers of patients in each group receiving antistaphylococcal agents. There was no statistical association between the use of these antibiotics and the subsequent isolation of pseudomonas and a similar proportion of patients from each group had received antistaphylococcal drugs. A similar result was obtained for the use of antihaemophilus agents (Table 33). However there was a statistical association between the use of antipseudomonal agents and the presence of P.aeruginosa ($p < 0.01$), which was apparent when comparing Groups A and B ($p < 0.01$) (Table 34). This was consistent with the fact that antipseudomonal agents were generally not used prophylactically in patients who were not colonised by P.aeruginosa (e.g. group A).

The presence of mucoid P.aeruginosa

The emergence of the mucoid phenotype of P.aeruginosa has frequently been recorded in CF (Doggett 1969; Penketh et al. 1983^a). The number of patients in Groups B and C harbouring the mucoid form of the organism was calculated and an association between emergence of this colonial form and length of colonisation with P.aeruginosa was

TABLE 32

Association between treatment with antistaphylococcal antibiotics and colonisation with *Pseudomonas aeruginosa* in cystic fibrosis patients grouped according to the presence or absence of *Pseudomonas aeruginosa* in their sputum

Antibiotic	Patient group		
	A	B	C
Erythromycin	0	1	5
Flucloxacillin	10	10	39
Methicillin	1	0	0
Fucidin	(2 ^a)	(1 ^a)	4
Septrin	1	1	2
Number receiving 'antistaphylococcal' agents (%)	12 (34)	12 (28)	50 (36)
Number receiving no 'antistaphylococcal' agents (%)	23 (66)	30 (71)	83 (62)

A patients free of *Pseudomonas aeruginosa*

B patients initially free

C patients persistently colonised

^a in conjunction with flucloxacillin

TABLE 33

Association between treatment with antihaemophilus antibiotics and colonisation with Pseudomonas aeruginosa in cystic fibrosis patients grouped according to the presence or absence of Pseudomonas aeruginosa in their sputum

Antibiotic	Patient Group		
	A	B	C
Amoxycillin	6	6	30
Augmentin	2	4	4
Chloramphenicol	0	1	3
Number receiving 'antihaemophilus' agents (%)	8 (23)	11 (26)	37 (28)
Number receiving no 'antihaemophilus' agents (%)	27 (77)	31 (74)	96 (72)

A patients free of Pseudomonas aeruginosa

B patients initially free

C patients persistently colonised

TABLE 34

Association between treatment with antipseudomonal antibiotics and colonisation with Pseudomonas aeruginosa in cystic fibrosis patients grouped according to the presence or absence of Pseudomonas aeruginosa in their sputum

Antibiotic	Patient Group		
	A	B	C
Azlocillin/gentamicin	6	1	5
Carbenicillin/gentamicin	0	8	18
Ceftazidime	1	2	4
Colistin	0	3	9
Ticarcillin/gentamicin	1	5	14
Azlocillin/tobramycin	1	0	10
Carbenicillin/tobramycin	0	1	0
*Ciprofloxacin 'trial':	-	-	10
Total treated with 'anti-pseudomonal' agents (%)	9 (26)	20 (47)	70 (53)
Total not treated with 'antipseudomonal' agents (%)	26 (74)	22 (53)	63 (47)
	$\underbrace{\hspace{10em}}_{\substack{b \\ p < .01}}$		

A patients free of Pseudomonas aeruginosa

B patients initially free

C patients persistently colonised

* double blind trial carried out simultaneously with this trial

^b Fishers Exact Probability Two-Tailed Test.

sought. No such relationship existed, 19 patients in Group C (14%) were free of the mucoid form but single samples only were received from 11 of these patients, and more frequent sampling may have revealed isolates with type 4 morphology. In Group B 7 (16%) of the patients were free of mucoid P.aeruginosa and only one yielded multiple samples containing P.aeruginosa which were nonmucoid.

Classification of P.aeruginosa

The amount of growth of P.aeruginosa on primary plates was assessed visually (Figure 13). Pilot experiments (Table 35) had given approximate colony counts per ml of sputum for the degree of growth by comparison with that obtained from a loopful (5mm) of an overnight broth of known concentration. The approximate values obtained were as below;

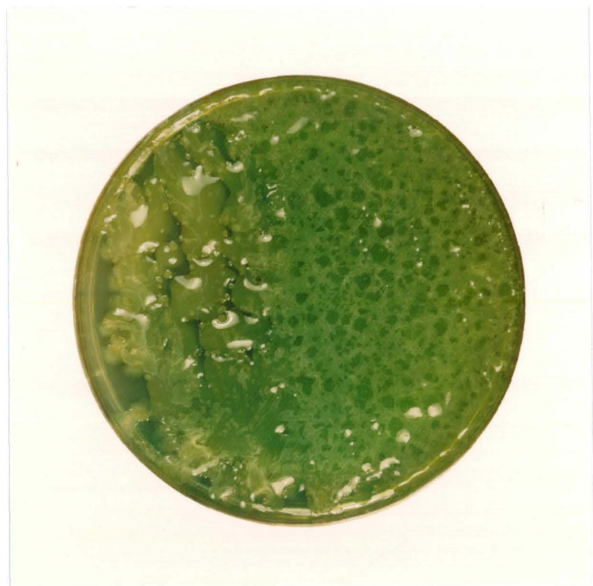
$$\begin{aligned} 3+ \text{ growth} &= 1.0 \times 10^8 - 1.0 \times 10^9 \text{ cfu/ml} \\ 2+ \text{ growth} &= 1.0 \times 10^6 - 1.0 \times 10^7 \text{ cfu/ml} \\ 1+ \text{ growth} &= 1.0 \times 10^4 - 1.0 \times 10^5 \text{ cfu/ml} \end{aligned}$$

The growth of mucoid isolates was markedly reduced on PIA presumably due to the hypersensitivity of these strains to the selective agent, Irgasan, in this medium. The concentration of Irgasan in PIA was 25mg/L which was inhibitory for about 20% of CF isolates (Fonseca et al. 1986).

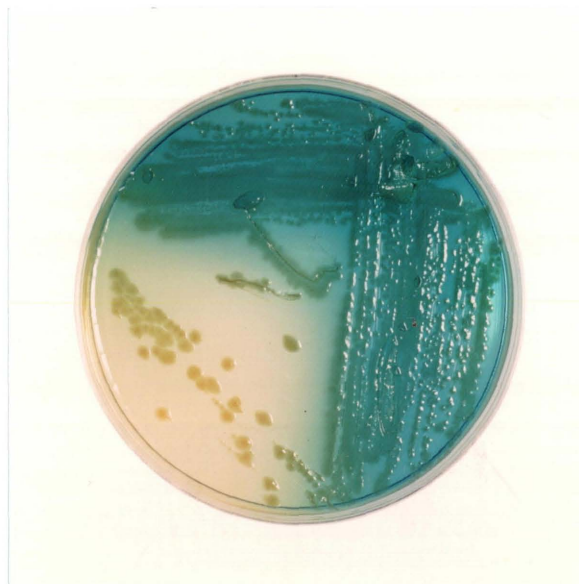
The scoring of mucoid growth as 3+ did not always reflect the number of colony forming units in the sample as strains varied considerably in the amount of mucoid material produced and only rough estimates based on the area of agar covered by growth or mucoid

Figure 13

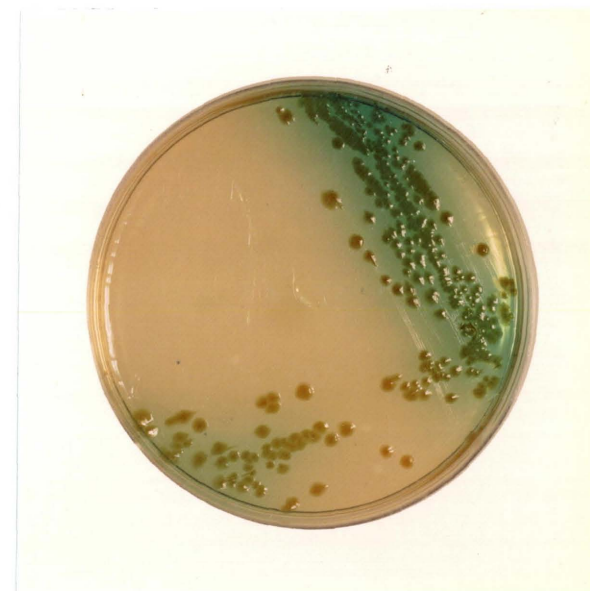
Growth of Pseudomonas aeruginosa from sputum on primary plates after 48h. at 37°C.



3+



2+



1+

TABLE 35

Estimation of the number of colony forming units per ml (cfu/ml) of
Pseudomonas aeruginosa on primary plates of CF sputum

^a Visual Score	^b cfu/ml	Average	Average
1+ mucoid	1.2×10^5	8.5×10^4	1.0×10^5
1+ mucoid	5.0×10^4		
1+ nonmucoid	1.0×10^5	1.15×10^5	
1+ nonmucoid	1.3×10^5		
2+ mucoid	1.2×10^7	8.5×10^6	1.0×10^7
2+ mucoid	5.0×10^6		
2+ nonmucoid	1.0×10^7	1.15×10^7	
2+ nonmucoid	1.3×10^7		
3+ mucoid	1.2×10^9	8.5×10^8	1.0×10^9
3+ mucoid	5.0×10^8		
3+ nonmucoid	1.0×10^9	1.15×10^9	
3+ nonmucoid	1.3×10^9		

^a visual score on King's A agar after 48h at 37°C

^b by comparison with the 48h growth from a loopful of an overnight broth of known cfu/ml

exopolysaccharide were made. Therefore the actual number of bacteria may have been equivalent to a count obtained with nonmucoid isolates recorded as 2+ growth.

Heterogeneity of *P.aeruginosa* from CF sputum

In the course of the two-year survey 1204 isolates of *P.aeruginosa* were recovered from 844 sputum samples from 210 CF patients. The variation in the number of isolates in all samples made analysis difficult and therefore an indication of the variability of the isolates was obtained by studying the first sample only from each patient in the survey. A comparison of the bacteriology of first and last samples from each patient showed that the former was representative of the variation in all isolates. Furthermore as there was considerable variation in the number of samples received from each patient, analysis of all 1204 isolates might influence the incidence of a particular phenotype. For instance 28 samples were received from a single patient which yielded 64 isolates of *P.aeruginosa* and only one sample was received from each of 60 patients. The following criteria were adopted to identify the heterogeneity of phenotypes of *P.aeruginosa*. They were; colonial morphology i.e. nonmucoid/mucoid, pigment production, reaction with O-typing sera and serum sensitivity and hypersensitivity to antibiotics.

Colonial form. All but one of the six varieties of colony form of *P.aeruginosa* described by Wahba and Darrell (1965) and Phillips (1969) were found in the first sputum sample from 162 patients (Table 36). Two hundred and eighty isolates were recovered from the primary plates and of these, 141 (50%) were mucoid. No attempt was

TABLE 36

Frequency of colonial types of 280 isolates of *Pseudomonas aeruginosa*
from the first sputum sample of 162 CF patients

	Colony Type ^a					
	1	2	3	4	5	6
Number of Isolates	52	76	9	141	0	2
Percentage	19	27	3	50	0	1

^a On King's A agar after 48h at 37°C; after Wahba and Darrell (1965)

made to distinguish between mucoid forms although it was evident that these varied from a wet glistening colony to those with a gum arabic consistency as illustrated in Figure 14. In agglutination tests mucoid colonies were often difficult to emulsify and formed stringy suspensions in saline. The nonmucoid isolates were either of type 1 or type 2 morphology (19% and 27% respectively) and only nine cultures gave dry friable colonies (type 3) on sub-culture. The two 'dwarf' isolates (type 6) grew poorly on the first day of incubation and at 48h, the diameter of the colonies was 1.0 - 2.0mm.

Pigment production. A variety of colours were produced by the growth of isolates on King's A agar (Table 37). These ranged from a frankly green extracellular pigment surrounding the mass of the bacterial growth to non-pigmented 'beige' colonies without discernible colouring of the surrounding agar. Nearly half the isolates did not produce pigment (138 of 280). The greater proportion of these (56%) were mucoid and the high isolation rate of non-pigmented forms was probably influenced by the isolation of mucoid and nonmucoid colonies from the same specimen. Furthermore non-pigmented colonies from the same specimen were often of different colonial morphology (Figure 14).

The majority of the pigmented cultures produced a green colour due to the expression of pyocyanin (blue) and yellow fluorescein. Some cultures obviously produced more fluorescein than pyocyanin as they were yellow on agar. Six cultures elaborated a brown or red-brown pigment which was presumed to be pyorubrin but this was not confirmed by culture on furunculosis agar (Ogunnariwo and Hamilton-Miller 1975).

TABLE 37

Frequency and colours of pigment produced by 280 isolates of *Pseudomonas*
aeruginosa from the first sputum sample of 162 CF patients

	Colour of pigment ^a				
	Green	Yellow/Green	Yellow	Red/Brown	No pigment
Number of isolates	114	8	14	6	138
Percentage	41	3	5	2	49

^a On King's A agar after 48h incubation at 37°C

Figure 14

The variety of different colonial phenotypes isolated from a single sputum sample from a CF patient.



O-Serology. The isolates were serotyped by agglutination tests with the standard O-antisera and the two antisera prepared against PA and NT variants described in Section 1. Five categories of agglutination reactions were recorded among the collection of isolates (Table 38). Sixty one (22%) of the cultures could be allocated to a single O-serogroup but a similar number were not agglutinated by any of the typing sera and were termed unclassified. Seventy five cultures were PA in that they were either agglutinated by two or more O-typing sera and/or the experimental PA antiserum (anti-7/1). Ten per cent of the isolates reacted with serum 18S (NT) alone but 17% of the total number of cultures were agglutinated by both experimental sera. Of those cultures which were O-serotypable 22 (36%) were group 06 (Table 39) and the next most frequent serotypes were; 03 (20%), 016 (11%) and 01 (7%). Serotypes 08 and 010 were represented by single cultures only.

Serum Sensitivity. Four categories of response to the bactericidal action of normal human serum were found (Table 40). Nearly two-thirds of the isolates (178 of 280) were fully sensitive to serum and a further 30 exhibited partial sensitivity. One-quarter of the cultures were either fully resistant or showed a slight reduction in their resistance to serum.

Hypersensitivity to antibiotics. In section I it was found that one of the variants, 7/1, was extremely sensitive to semi-synthetic penicillins in particular, carbenicillin. It was therefore decided to investigate whether O-defective isolates from CF were generally

TABLE 38

Reactions in agglutination tests of 280 isolates of *Pseudomonas aeruginosa*
from the first sputum sample of 162 CF patients

	Agglutination category				
	O-typable	u/c	PA	NT	PA/NT
Number of Isolates	61	67	75	29	48
Percentage	22	24	27	10	17

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera.

TABLE 39

Frequency of each O-serotype in the 61 O-typable strains of Pseudomonas
aeruginosa isolated from the first sputum samples of 162 CF patients

Serotype*	Number of isolates	Percentage frequency
06	22	36
03	12	20
016	7	11
01	4	7
02b	3	5
09	3	5
04	2	3
05	2	3
07	2	3
011	2	3
08	1	2
010	1	2
12	61	100

* Typing sera used at CPHL corresponds numerically with the IATS
(Liu et al. 1983)

TABLE 40

Response to the bactericidal action of normal human serum of 280 isolates
of Pseudomonas aeruginosa from the first sputum sample of 162 CF patients

	Category of serum resistance			
	Resistant	Reduced Resistance	Partially Sensitive	Sensitive
Number of isolates	53	19	30	178
Percentage	19	7	11	63

hypersensitive to B-lactam antibiotics. Preliminary studies not shown in this thesis had established that most isolates with a zone size of 30mm diameter or greater for azlocillin, carbenicillin, piperacillin and ticarcillin grew on agar containing 1mg/L but not on 10mg/L concentrations of these antibiotics. These isolates were termed hypersensitive.

One hundred and sixty three strains were tested by disc diffusion and the distribution of hypersensitive isolates within each agglutination category is shown in Table 41. Hypersensitivity was not restricted to those isolates with defective LPS. Four of 33 (12%) O-typable strains were also hypersensitive to antibiotics but most of the 48 isolates which were hypersensitive were O-defective. The converse was not always true as 79 of 105 (75%) of isolates with normal sensitivity to antibiotics were either unclassified, PA and/or NT and were thus termed O-defective.

The influence of mucoid phenotype on the sensitivity of the isolates to these antibiotics was determined. Table 42 shows that there was an equal number of mucoid and nonmucoid hypersensitive isolates, but fewer resistant strains were mucoid.

Hypersensitivity to the third generation cephalosporins was not shown by isolates with increased susceptibility to the semi-synthetic penicillins although 15 other isolates gave zone diameters in excess of 30mm which corresponded with a MIC for these drugs of 0.5-1mg/L.

The heterogeneity of antibiotic resistance phenotypes within single sputum samples was evident as isolates with different antibiotic sensitivities were often cultured from the same sputum sample.

TABLE 41

Sensitivity of 163 isolates of *Pseudomonas aeruginosa* to B-lactam
antibiotics and their agglutination reaction

Antibiotic* category	Number (%) of isolates in agglutination category					Total (%)
	O-typable	u/c	PA	NT	PA/NT	
Hypersensitive	4 (12)	14 (32)	19 (39)	3 (20)	8 (36)	48 (29)
Normal Sensitivity	26 (78)	28 (64)	27 (55)	12 (80)	12 (55)	105 (65)
Resistant	3 (10)	2 (4)	3 (6)	0	2 (9)	10 (6)
Total	33	44	49	15	22	163

* Sensitivity to azlocillin, carbenicillin, piperacillin and ticarcillin tested by disc diffusion

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera.

TABLE 42

Sensitivity of 163 isolates of *Pseudomonas aeruginosa* to B-lactam antibiotics
and mucoid phenotype

Antibiotic* category	Number (%) of isolates and phenotypes		Total (%)
	Mucoid	Non-mucoid	
Hypersensitive	24 (30)	24 (29)	48 (29)
Normal Sensitivity	54 (67)	51 (61)	105 (65)
Resistant	2 (3)	8 (10)	10 (6)
Total	80	83	163

* sensitivity to azlocillin, carbenicillin, piperacillin and ticarcillin
 tested by disc diffusion

Association of pigment production, colony form and O-serotype reaction

Twenty-five of 61 (41%) of the isolates which were O-specific produced green colouration on King's A agar and a similar number were PA (Table 43). The distribution of non-pigmented cultures in each agglutination category was similar to that for green pigmented isolates although nearly twice as many serologically unclassified cultures were non-pigmented as were green. This was influenced by the fact that 30 mucoid isolates did not produce pigment and could not be emulsified in saline.

Nevertheless, 41% of the O-typable isolates were mucoid and a similar proportion of mucoid forms were PA (Table 44). In total it was possible to allocate 66% of the mucoid isolates to an agglutination category. None of the nonmucoid colony forms was restricted to a particular serotype reaction. The two experimental sera were included to detect LPS-defective forms of P.aeruginosa and they agglutinated all morphological varieties of colonies.

Association of serum resistance and O-serotype reactions

The highly statistically significant relationship between O-specificity and resistance to normal human serum observed in Section 1 (Table 19) was also found with this selection of isolates. Table 45 shows that the proportion of isolates with an O-specific agglutination reaction decreased markedly as sensitivity to serum increased. Chi-square test $E = 63.41$, 12.d.f. $p < 0.001$. Twenty-eight of the 61 O-typable isolates (46%) were resistant to serum, compared with 13 of 67 unclassified isolates (19%) and 8 of 75 which were PA. Only four (14%) of the cultures which reacted with

TABLE 43

Association of pigment production and agglutination reactions of 280
isolates of Pseudomonas aeruginosa from the first sputum samples of
162 CF patients

Pigment	Number (%) of isolates in agglutination category					Total
	O-typable	u/c	PA	NT	PA/NT	
Green	25 (41)	21 (32)	29 (39)	16 (55)	23 (48)	114 (41)
Yellow-green	5 (8)	5 (7)	1 (1)	1 (3)	2 (4)	14 (5)
Yellow	4 (6)	2 (3)	0	2 (7)	0	8 (3)
Red-brown	3 (5)	0	2 (3)	0	1 (2)	6 (2)
Non pigmented	24 (40)	39 (38)	43 (57)	10 (35)	22 (46)	138 (49)
Total	61	67	75	29	48	280

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera.

TABLE 44

The association of colonial morphology and agglutination reactions of
280 isolates of Pseudomonas aeruginosa from the first sputum sample of
162 CF patients

Colonial morphology	Number (%) of isolates in agglutination category					Total
	O-typable	u/c	PA	NT	PA/NT	
1	16 (26)	4 (6)	16 (21)	4 (14)	9 (19)	52 (27)
2	20 (33)	10 (15)	20 (27)	6 (21)	21 (44)	76 (19)
3	0	3 (4)	2 (3)	1 (3)	3 (6)	9 (3)
4	25 (41)	49 (73)	36 (48)	18 (62)	15 (31)	141 (50)
6	0	1 (2)	1 (1)	0	0	2 (1)
Total	61	67	75	29	48	280

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera.

TABLE 45

Association of resistance to normal human serum and agglutination
reactions of 280 isolates of Pseudomonas aeruginosa from the first
sputum sample of 162 CF patients

Serum Sensitivity	Number (%) of isolates in agglutination category					Total
	0-typable	u/c	PA	NT	PA/NT	
Resistant	28 ^a (46)	13 (19)	8 (11)	4 (14)	0	53 (19)
Reduced Resistance	10 (16)	3 (5)	3 (5)	1 (3)	2 (4)	19 (7)
Partially Sensitive	5 (8)	8 (12)	9 (12)	4 (14)	4 (8)	30 (11)
Sensitive	18 (30)	43 (64)	55 ^b (73)	20 (69)	42 (88)	178 (63)
Total	61	67	75	29	48	280

u/c = unclassified

PA = agglutinated by two or more 0-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

^a } $p < 0.001$ (χ^2 test)
^b }

NT antiserum were resistant to serum but all of the PA/NT isolates showed either reduced resistance or were sensitive to serum. Of the 19 isolates that showed a reduced resistance to serum 10 (53%) were 0-typable and the remainder were distributed among the other agglutination categories. Among the partially or fully serum sensitive isolates only 23 of 208 (11%) were 0-typable and 110 (53%) were agglutinated by the PA antiserum or both experimental antisera (i.e. PA/NT). Forty three of 67 (64%) serologically unclassified isolates were fully serum sensitive and so were 42 of 48 (87%) of the PA/NT strains. Together these serocategories accounted for nearly half (48%) of the total serum sensitive isolates. Eleven per cent (20 of 178) of the serum sensitive strains were agglutinated by NT antiserum alone.

Association of phage type and 0-serotype

Phage typing was used to provide further evidence of the alteration of the outer membrane of CF isolates. One hundred and thirty seven of the 280 isolates were phage typed. Approximately 50% were sensitive to one or more of the typing phages and the remainder were resistant to lysis by any of the phages. The loss of 0-specific antigens correlated highly with sensitivity to the typing phages, the majority of which adsorb to somatic antigen receptors (Temple et al. 1986^a). Table 46 shows that only four of the phage resistant cultures were 0-typable in contrast to nearly half (30 of 68) of the 0-typable strains which were sensitive to phage (Chi-square test, $E = 42.4$, 4 d.f., $p < 0.001$) Thirty two of the serologically unclassified cultures were not lysed by the phages, perhaps due to their mucoid phenotype. Although approximately twice the number of PA strains

TABLE 46

Phage typability of 137 isolates of Pseudomonas aeruginosa of various
agglutination categories from CF

Sensitivity to phage	Number (%) of isolates in agglutination category					Total (%)
	0-typable	u/c	PA	PA/NT	NT	
Sensitive	30 ^a (88)	6 (16)	11 (35)	13 (65)	8 (62)	68 (50)
Resistant	4 ^b (12)	32 (84)	21 (65)	7 (35)	5 (38)	69 (50)
Total	34	38	33	20	13	138

u/c = unclassified

PA = agglutinated by two or more typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera.

^a }
^b } $p < 0.001$ (X^2 test)

were phage resistant than sensitive, those agglutinated by either the NT serum alone or PA/NT did not differ markedly in their susceptibility to the typing phages.

Association of phenotypes of P.aeruginosa with clinical condition of CF patients

The contribution of the number of different phenotypes of P.aeruginosa found in their first sputum sample to the clinical status of the patients was examined. Lung function values (FVC, FEV₁) were obtained at the same time as the sputum sample and phenotypes were distinguished by colonial morphology, pigment, O-typability and serum sensitivity.

Figure 15 shows the number of different isolates in the first sample. Nearly half (46%) of the 162 patients had a single phenotype in pure culture in their sputum and 38% harboured two distinct phenotypes. Only three per cent of patients yielded four different pseudomonas isolates. There was a progressive decrease in lung function of patients as the number of phenotypes increased (Table 47) and this association was statistically significant by regression analysis (FVC, $r = -0.97$, $p < 0.05$ and FEV₁, $r = -0.94$, $p < 0.05$).

However analysis of the association of phenotypes and lung function in sequential samples from individual patients did not confirm this trend. Figure 16 shows examples of lung function in two patients over a period of 6 and 12 months respectively and the distribution of phenotypes in sputum samples received at the same time. The FVC of patient number 69 (Figure 16a) was 1.45L at the time of the first sputum sample which contained two different phenotypes of P.aeruginosa and lung function improved gradually over the next six

Figure 15

Number of distinct isolates of Pseudomonas aeruginosa from the first sputum sample of 162 CF patients

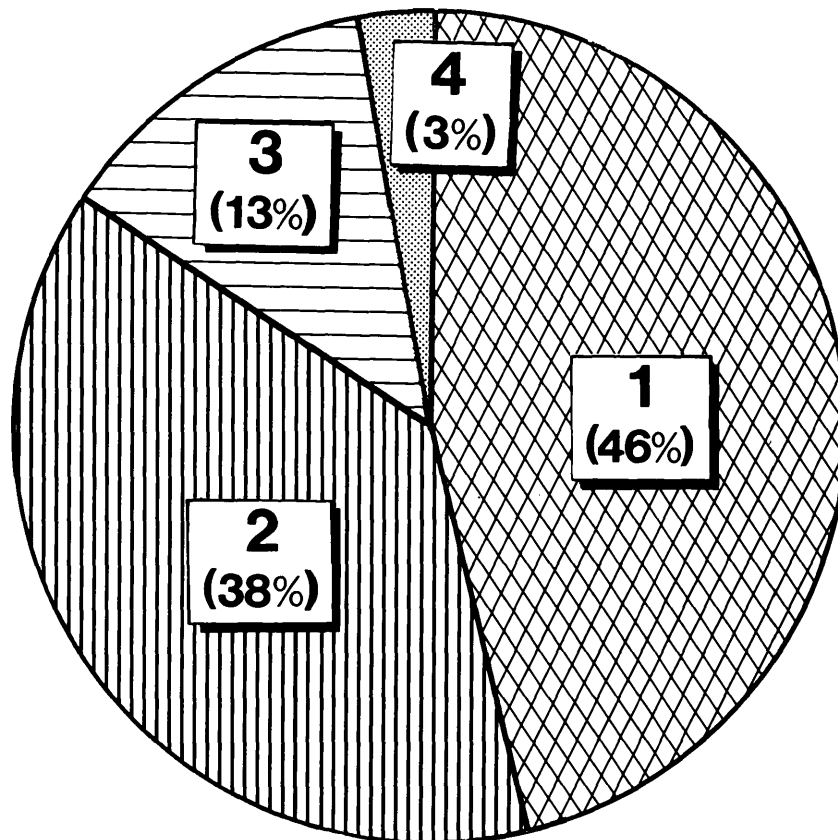


TABLE 47

Lung function values of patients at the time of sputum collection:
association with number of phenotypes of Pseudomonas aeruginosa

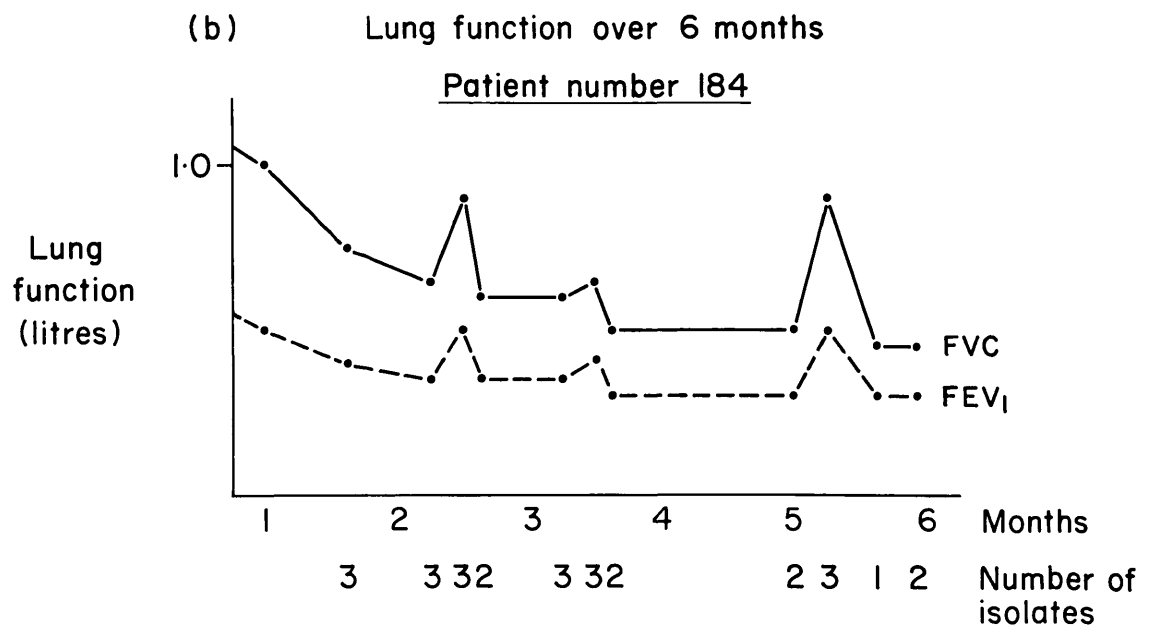
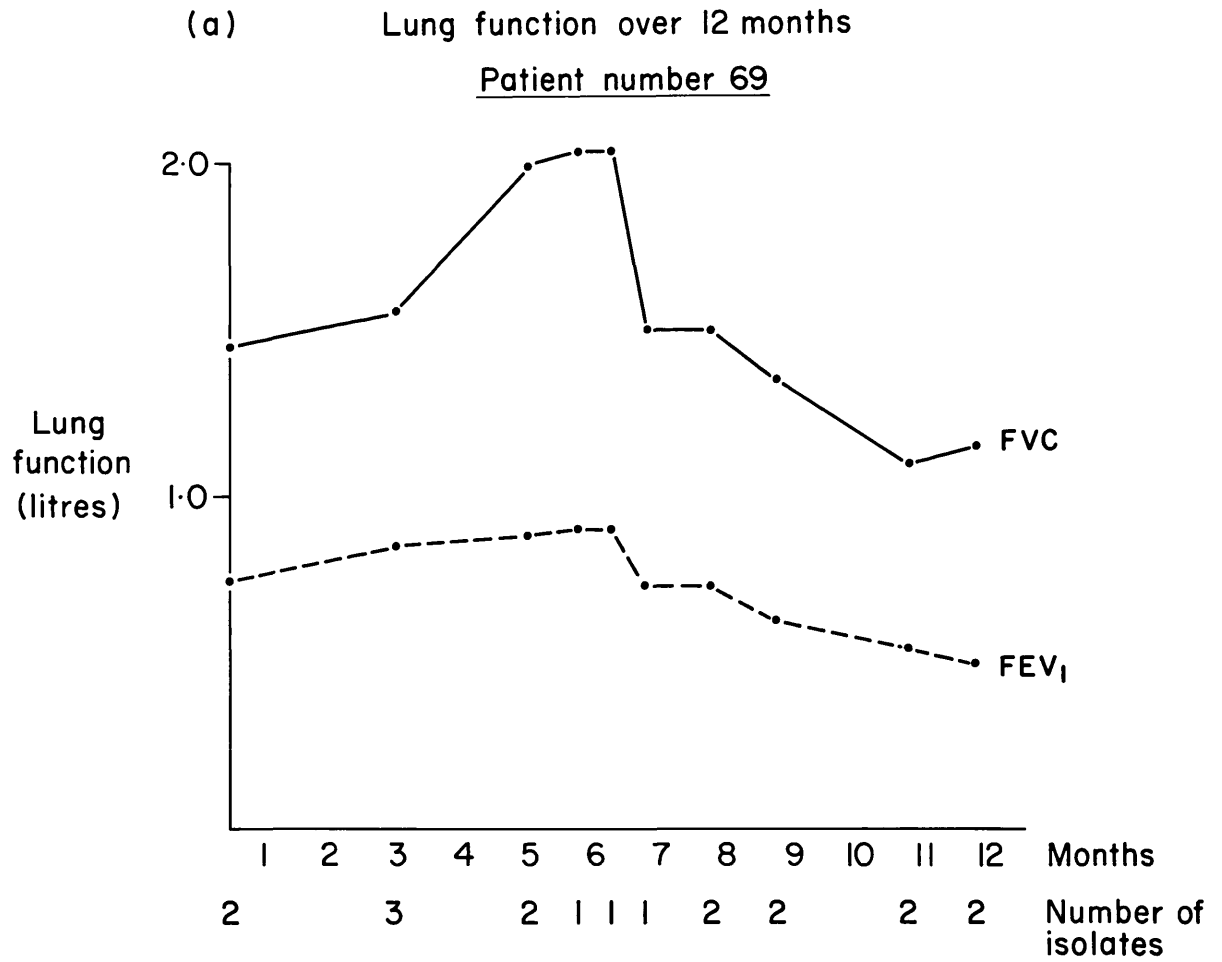
	Number of different phenotypes			
	1	2	3	4
Number (%) of patients	75 (46)	61 (38)	21 (13)	5 (3)
*FVC	2.36	2.24	2.13	1.86 ^a
*FEV ₁	1.40	1.39	1.24	1.02 ^a

Figures calculated from the first sputum sample received from each patient
n = 162.

* mean value in litres

^a $p < 0.05$ (Regression analysis)

Figure 16.



months. Samples taken during this period yielded three, two or one phenotypes of P.aeruginosa. As lung function declined two samples, separated by one month, yielded a pure growth of a single isolate and at the end of the survey (12 months) two different phenotypes were consistently isolated. Patient number 184 (Figure 16b) had a very poor FVC which did not vary markedly over six months. Nevertheless the number of different phenotypes fluctuated between three and one isolates per sample of sputum.

As the number of different phenotypes in a sample did not directly reflect the clinical status of individual patients, the properties of the isolates were examined to determine whether a particular phenotype was associated with a decline in lung function. An association was sought between the presence of O-typable or O-defective strains in the first sputum sample and the lung function of the patients (Table 48). It appeared that patients harbouring a single O-typable phenotype of P.aeruginosa had slightly better lung function than those harbouring a single O-defective strain.

In the group which harboured two isolates, lung function was marginally better if both of these were O-typable. If both isolates were O-defective the FEV_1 was markedly worse. The trend of a decline in lung function continued for patients with three or more isolates but the number of variable in phenotype characteristics made analysis difficult because each of the 26 patients in this category yielded isolates with different combinations of phenotypes.

In Table 49 the contribution of each of the O-defects in CF isolates revealed by agglutination reactions with the two antisera PA and NT to lung function was examined within a group of 37 patients who

harboured single O-defective strains which reacted with these antisera i.e. 37 of the original 60 O-defective phenotypes (Table 48). Although the majority of isolates were agglutinated by the PA antiserum rather than the NT antiserum there was no significant difference between the mean values for either the FVC or FEV₁ of these patients.

As many of the pseudomonas isolates from CF were serum sensitive the lung function of patients harbouring a single serum sensitive or resistant phenotype was examined (Table 50). Almost twice the number of patients harboured serum sensitive strains than serum resistant, but there was no marked difference between the lung function values obtained for the two groups.

A further division of phenotypes to assess the contribution of various agglutination categories and serum resistance to lung function was complicated by the number of phenotypic variables within individual patients. It was concluded that there was no obvious association between the incidence of different phenotypes or combinations of phenotypes with lung function in the total patient group. Therefore an analysis was made of sequential isolates from individual patients.

Of the 42 patients who were not persistently colonised with P.aeruginosa multiple samples (three or greater) were available for 21 of them. An additional complication of this group was that although they did not have pseudomonas immediately before the trial or in their first sputum sample, some of them had harboured pseudomonas intermittently in the past. This reduced the actual number of new pseudomonas positive patients to 13.

TABLE 48

Association between the presence of O-typable or O-defective strains of
Pseudomonas aeruginosa and the lung function of 162 CF patients

	Number of different phenotypes and agglutination category					
	1		2		3	
	O-typable	O-defective ^a	O-typable	O-defective ^a	Mixture	Mixture
Patient number	15	60	4	45	12	26
FVC *	2.50	2.32	2.74	2.20	2.23	2.00
FEV ₁ *	1.38	1.37	2.10	1.33	1.39	1.19

*mean value in litres

^a O defective - unclassified or agglutinated by PA and/or NT antiserum

TABLE 49

Association of agglutination by variant antisera of *Pseudomonas aeruginosa*
with the lung function of 37 CF patients harbouring a single phenotype

	Number of isolates in agglutination category		
	PA	NT	PA/NT
Patient number	21	7	9
FVC*	2.05	2.65	2.47
FEV ₁ *	1.32	1.33	1.71

* mean values in litres

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

TABLE 50

Association of resistance to normal human serum of Pseudomonas
aeruginosa with the lung function of 75 CF patients harbouring a single
phenotype

	Reaction of isolate with human serum	
	R	S
Patient number	26	49
FVC*	2.54	2.65
FEV ₁ *	1.52	1.57

*mean value in litres

R = resistant or reduced resistance to normal human serum

S = sensitive or partially sensitive to normal human serum

Figure 17 shows the occurrence of mucoid P.aeruginosa in sequential samples from these patients. Six of the thirteen patients harboured mucoid strains in their first sample and three of these also had nonmucoid isolates, the remaining patients grew only nonmucoid pseudomonas initially. It may have been that the patients who presented with mucoid strains had been colonised earlier but did not attend the outpatient clinic. No patients consistently yielded nonmucoid isolates and a mixture of mucoid and nonmucoid was normally found. In the majority of patients the mucoid variety was isolated subsequently to the initial nonmucoid strain but patient number 48 was an exception to this rule. Both patients who died gave pure cultures of mucoid pseudomonas in their terminal samples and one of them, patient number 76, was not colonised by a nonmucoid variety during the survey period.

The antigenic characteristics of isolates were sorted into O-typable and O-defective on the basis of their serological reactions in agglutination tests (Figure 18). In eight of the patients the initial sample contained an O-typable strain, although in two of these an O-defective isolate was also present. The majority of patients yielded samples containing O-defective isolates and the occurrence of O-typable P.aeruginosa decreased as the infection progressed. Nevertheless five patients had a pure growth of O-defective P.aeruginosa in their initial sample and O-typable isolates were never recovered from two. Of the two patients that died, one, patient number 3, was colonised in the terminal stages by O-defective phenotypes only, while the other (number 76) harboured intermittently O-typable and O-defective forms of P.aeruginosa.

Figure 17.

Incidence of mucoid phenotype in sequential sputum samples from CF patients recently colonised with Pseudomonas aeruginosa

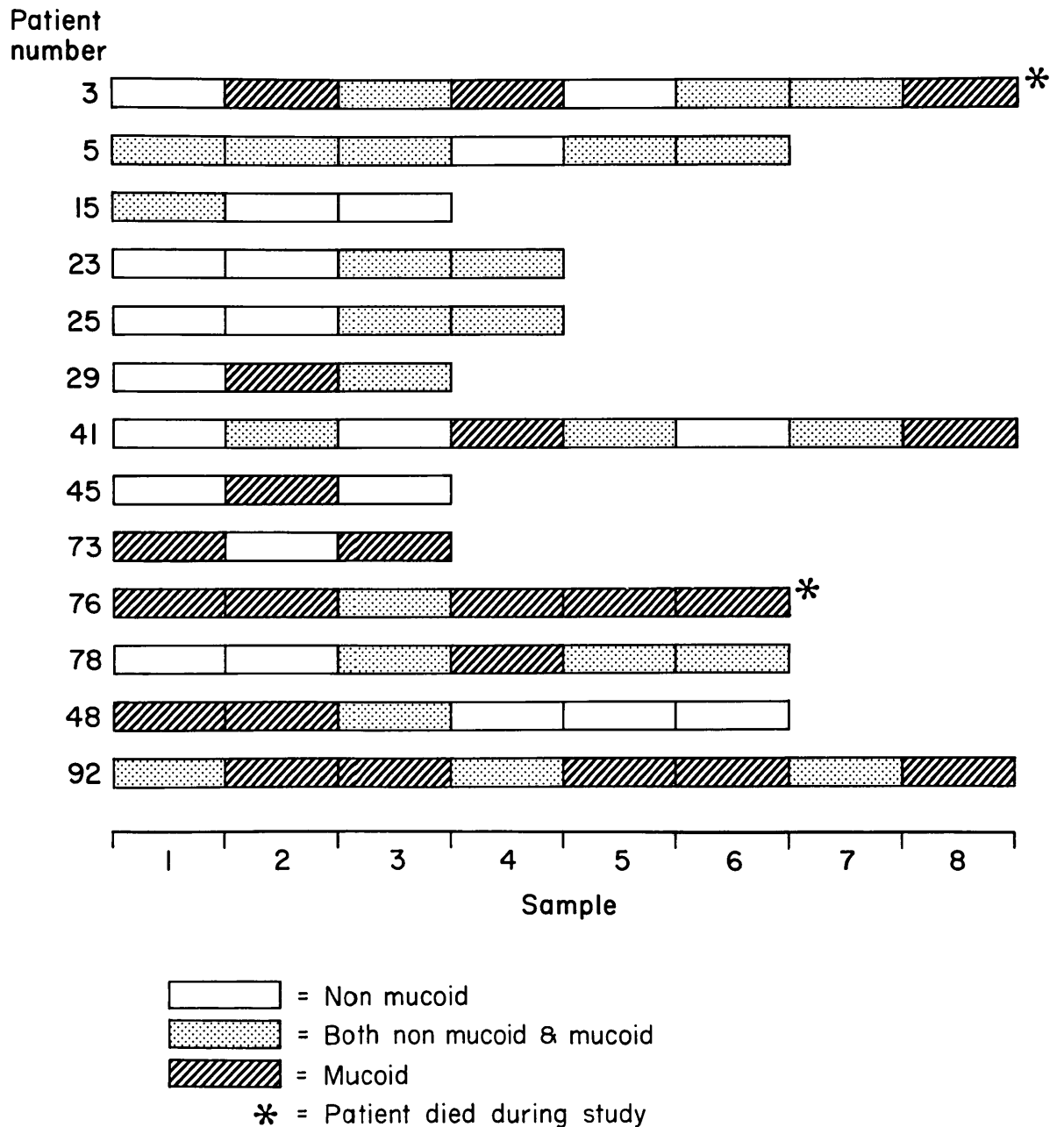
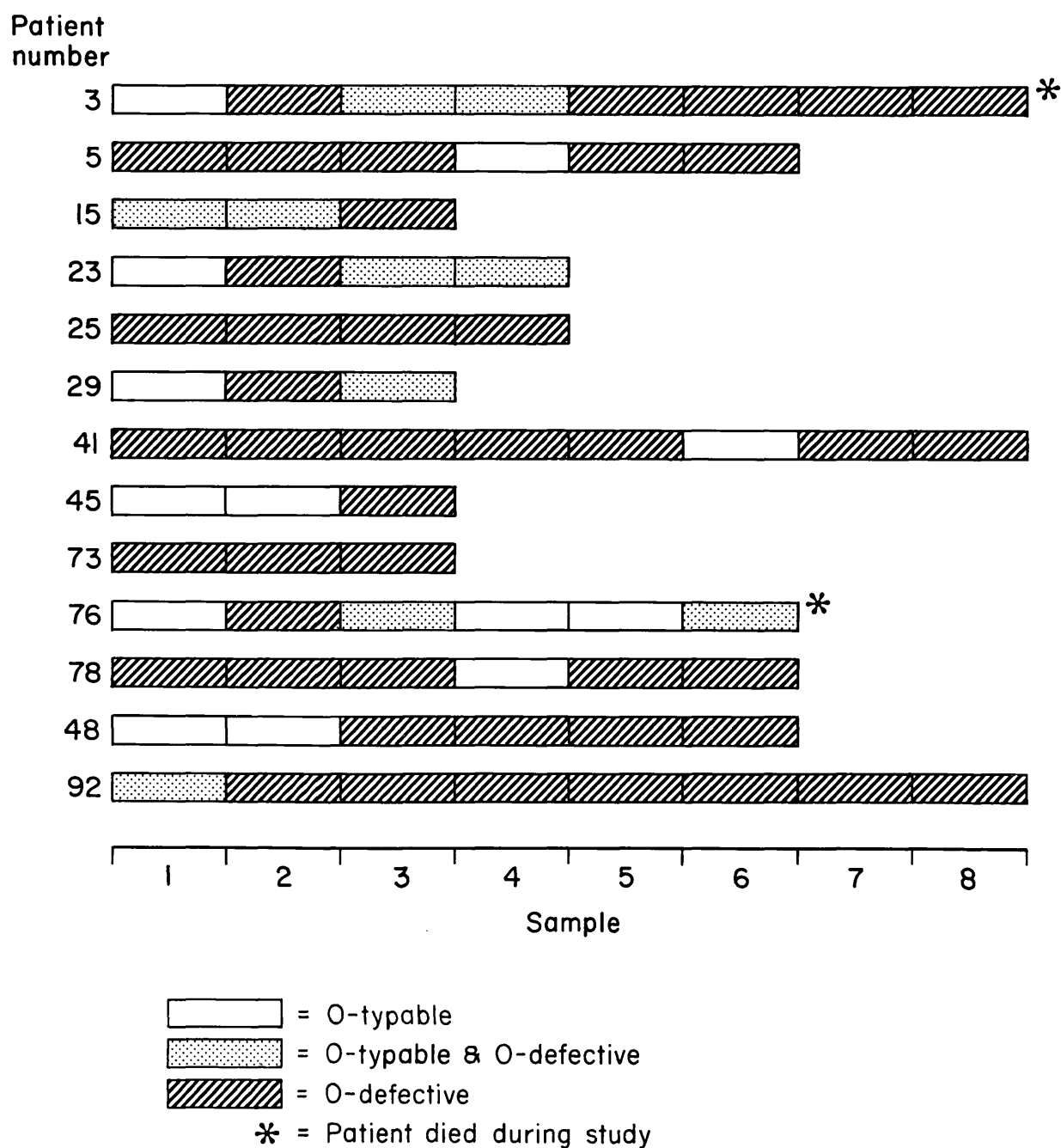


Figure 18.

Incidence of Pseudomonas aeruginosa with atypical serological reactions in sequential sputum samples from recently colonised CF patients



Association of atypical phenotypes of P.aeruginosa and clinical status in individual patients

The different phenotypes of P.aeruginosa isolated from sequential sputum samples of two recently colonised CF patients are shown in Tables 51 and 52. The first patient (number 3) on first attendance in the survey was colonised by an O-typable, nonmucoid, serum resistant strain i.e. a typical wild-type strain. Within one month the isolate showed a reaction with an O-defective antiserum and had reduced resistance to serum but remained nonmucoid. Four months later a mucoid isolate was recovered and this persisted intermittently until the patient's death. In the interim she was colonised by a variety of different phenotypes and the O-typable strain disappeared from the sputum eight months after the initial colonisation. The lung function was relatively stable for the first seven months of colonisation but deteriorated rapidly, apparently concurrently with the establishment of mucoid and O-defective phenotypes.

In contrast CF patient number 76 (Table 52) was colonised initially by a mucoid variety of pseudomonas which was O-typable and serum resistant. Lung function was poor from the onset of colonisation and decreased further over the next year. Nonmucoid revertants appeared in a single specimen approximately one year after initial colonisation and a mixture of O-defective, serum sensitive and O-typable, serum resistant strains were recovered throughout the course of the infection. The final sample contained a mixture of phenotypes.

In both these patients the number of P.aeruginosa present in the sputum increased to 3+ growth as the pulmonary function decreased

TABLE 51

Phenotypes of *Pseudomonas aeruginosa* isolated from sequential sputum samples of a 'recently colonised' CF patient number 3 who died during survey

Date	Lung function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Morphology Growth		Agglutination category	Serum sensitivity
10.10.84	49	47	1+	non-mucoid	09	R
6.11.84	36	39	1+	mucoid	PA	RR
12.3.85	57	61	2+	mucoid non-mucoid	PA/NT 09	R PS
15.5.85	36	44	3+	mucoid mucoid	PA 09	S RR
19.6.85	26	30	3+	non-mucoid	PA	PS
3.7.85	26	30	1+	mucoid non-mucoid	u/c PA	PS R
24.7.85	38	35	3+	mucoid mucoid non-mucoid non-mucoid	u/c u/c PA/NT PA	S R RR PS
21.8.85	17	14	3+	mucoid mucoid	u/c u/c	PS RR

died 21.9.85

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

R = resistant, RR = reduced resistance, PS = partially sensitive,

S = sensitive to the action of normal human serum

TABLE 52

Phenotypes of Pseudomonas aeruginosa isolated from sequential sputum
samples of a 'recently colonised' CF patient number 76 who died during survey

Date	Lung function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Morphology Growth		Agglutination Category	Serum Sensitivity
18.11.83	37	30	2+	muroid	03	R
21.2.84	41	33	3+	muroid	PA	S
9.1.85	28	22	2+	muroid	PA	S
				muroid	03	R
				non- muroid	PA	S
				non- muroid	03	R
13.3.85	22	16	3+	muroid	03	R
22.5.85	32	25	3+	muroid	03	R
				muroid	03	R
30.8.85	19	17	3+	muroid	u/c	S
				muroid	03	R

died 28.11.85

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

R = resistant, S = sensitive to the action of normal human serum

despite intensive antipseudomonal therapy.

The bacteriological data illustrated in Tables 53 and 54 are from two patients who had recently acquired P.aeruginosa and were still alive at the end of the trial. Patient number 49, had an initial strain of P.aeruginosa which although O-typable and serum resistant was mucoid. This patient's lung function was moderate and remained stable throughout the trial despite the emergence of serological variants of P.aeruginosa. These variants were generally present as pure growth of low count and on the single occasion the number of P.aeruginosa increased there was a slight decrease in the FEV₁ value to 26% of that predicted. In contrast the number of P.aeruginosa from patient number 23 increased from 1+ to 3+ (10^4 to 10^9 cfu/ml) with a concurrent decrease in lung function and the simultaneous emergence of multiple phenotypes of P.aeruginosa. These included a serotype 06 strain which replaced the initial 03 strain and probably represented a new episode of colonisation and might explain the marked decrease in lung function.

There were 133 patients identified as persistently colonised with P.aeruginosa throughout the survey and sequential samples were available for 113 of them. Bacteriological analysis revealed a great variation and combinations of phenotypes which proved difficult to correlate with lung function data. Data are shown (Table 55 and 56) which illustrates the variation in phenotypes and lung function in two patients who were persistently colonised throughout the trial. Samples were received from a male patient (number 69) (Table 55) for a period of approximately one year. His lung function values, although poor, were comparable at the beginning and end of the study,

TABLE 53

Phenotypes of *Pseudomonas aeruginosa* isolated from sequential sputum
samples of a 'recently colonised' CF patient number 48

Date	Lung Function		Isolates			
	FVC per cent	FEV ₁ predicted	Growth	Morphology	Agglutination Category	Serum sensitivity
28.10.83	49	32	1+	muroid	01	R
18.11.83	46	32	2+	muroid	01	R
31.10.84	46	32	1+	muroid nonmuroid	PA/NT PA/NT	RR S
4.11.84	42	26	3+	nonmuroid	PA/NT	R
21.11.84	47	32	1+	nonmuroid	PA	S
16.01.85	46	36	1+	nonmuroid	PA/NT	S

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

R = resistant, RR = reduced resistance, S = sensitive to the bactericidal action of normal human serum

TABLE 54

Phenotypes of Pseudomonas aeruginosa isolated from sequential sputum
samples of a 'recently colonised' CF patient number 23

Date	Lung Function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Growth	Morphology	Agglutination Category	Serum Sensitivity
24.10.84	42	22	1+	nonmucoid	03	RR
25.1.85	25	13	3+	nonmucoid nonmucoid nonmucoid	PA PA u/c	S PS PS
3.4.85	30	14	3+	mucoid nonmucoid nonmucoid	06 06 PA	R R S
10.4.85	30	13	3+	mucoid mucoid nonmucoid	u/c 06 06	RR R R

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

R = resistant, RR = reduced resistance, PS = partially sensitive,

S = sensitive to the action of normal human serum

TABLE 55

Phenotypes of *Pseudomonas aeruginosa* isolated from sequential sputum
 samples of a 'persistently colonised' CF patient number 69

Date	Lung function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Growth	Morphology	Agglutination category	Serum Sensitivity
13.9.83	32	21	2+	muroid nonmuroid	06 06	R RR
10.1.84	44	24	3+	muroid nonmuroid	PA 06	R R
7.2.84	45	25	3+	muroid	011	R
21.2.84	45	25	3+	muroid	PA	S
6.3.84	33	20	2+	muroid	06	R
1.5.84	29	20	3+	muroid nonmuroid	PA u/c	R RR
5.10.84	25	14	3+	muroid nonmuroid	PA 011	R R

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

R = resistant, RR = reduced resistance, S = sensitive to the action of
 normal human serum

TABLE 56

Phenotypes of *Pseudomonas aeruginosa* isolated from sequential sputum
samples of a 'persistently colonised' CF patient number 94

Date	Lung Function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Growth	Morphology	Agglutination Category	Serum Sensitivity
9.11.83	33	17	3+	nonmucoid nonmucoid	PA/NT PA/NT	PS S
31.10.84	39	23	3+	nonmucoid nonmucoid	PA/NT PA/NT	S S
5.2.85	33	20	3+	mucoid nonmucoid	PA PA	S PS
3.4.85	43	26	3+	nonmucoid nonmucoid nonmucoid	PA/NT PA PA	S S S
31.7.85	28	20	3+	nonmucoid nonmucoid	PA PA	S S

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

PA/NT = agglutinated by both PA and NT antisera

PS = partially sensitive, S = sensitive to the action of normal human serum

but at one stage his FVC improved slightly. Mucoid and nonmucoid isolates were found in the first sample and these were O-typable and serum resistant. A polyagglutinable strain was found in the next specimen but one month later a new strain (serotype 011) appeared in the sputum. Subsequent specimens gave a mixture of the initial typical strain and O-defective variants but the latter may have arisen from either of the O-typable strains. The number of bacteria (10^8 to 10^9) in the sputum remained relatively constant over the period of the study.

The sputum of CF patient number 94 consistently grew O-defective variants of P.aeruginosa which were serum sensitive. A mucoid form was present in a single specimen only (Table 56). Lung function did not improve from its initial low values and there was no obvious correlation between either the forms or numbers of P.aeruginosa isolated and the patient's clinical condition.

The data from two patients who were persistently colonised and died during the trial are shown in Tables 57 and 58. An O-typable strain was not recovered at any time from CF patient number 106 (Table 57). The first two isolates were a mixture of mucoid and nonmucoid which were both O-defective and serum sensitive. Other serological phenotypes arose over a 14 month period and mucoid strains occurred intermittently. At one point the lung function improved dramatically despite an increased bacterial load (FVC = 60%; FEV₁ = 39% predicted) and two nonmucoid revertants distinguishable by their serological reactions were present. He had received a course of chloramphenicol as an inpatient and this might have accounted for the improvement in lung function but surprisingly the number of bacteria

TABLE 57

Phenotypes of Pseudomonas aeruginosa isolated from sequential sputum
samples of a 'persistently colonised' CF patient number 106 who died
during survey

Date	Lung Function		Isolates			
	FVC per cent predicted	FEV ₁ per cent predicted	Growth	Morphology	Agglutination Category	Serum Sensitivity
13.9.83	36	19	2+	muroid nonmuroid	u/c PA	S S
5.10.83	30	16	3+	muroid nonmuroid nonmuroid	u/c NT PA/NT	S S S
10.2.84	45	46	3+	muroid muroid nonmuroid	PA PA PA	RR S S
15.2.84	45	46	1+	muroid	PA	R
28.2.84	35	16	2+	muroid nonmuroid	PA PA	S S
10.10.84	60	39	3+	nonmuroid nonmuroid	PA/NT NT	S S
13.2.84	17	11	3+	muroid nonmuroid	PA/NT PA	S S

Died 28.12.84

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

R = resistant, RR = reduced resistance, S= sensitive to the action of normal human serum

increased. Subsequently lung function showed a rapid decline and despite intensive antipseudomonal therapy the trend could not be reversed and the patient died.

Of the two patients who died CF patient number 138 (Table 58) had marginally better lung function at entry to the survey despite colonisation by serologically distinguishable mucoid forms. An O-typable revertant appeared two months later and this was nonmucoid and serum resistant. Samples taken two years subsequently contained a variety of atypical strains in one specimen; four phenotypes including the O-typable form were identified. The last specimen before death yielded three O-defective isolates one of which was mucoid. The number of bacteria in this patient's sputum remained high throughout the survey despite the use of both intravenous and nebulised antipseudomonal antibiotics.

Although there was not a direct correlation between the presence of different phenotypes of P.aeruginosa and the lung function of the patient the following points emerged. The initial strains were most often nonmucoid, O-typable and resistant to serum and often the numbers of P.aeruginosa in the sputum (cfu/ml) were low. The number of different phenotypes increased with progressive pulmonary disease. Sometimes a major decline in lung function appeared to occur simultaneously with the increased isolation of different phenotypes. The mucoid form of the organism was more frequent as respiratory disease progressed and was present in all four patients illustrated here who died. However two of the four who survived also harboured mucoid forms so the relationship was not absolute.

TABLE 58

Phenotypes of Pseudomonas aeruginosa isolated from sequential sputum
samples of a 'persistently colonised' CF patient number 138 who died
during survey

Date	Lung function		Isolates			
	FVC	FEV ₁	Growth	Morphology	Agglutination Category	Serum Sensitivity
	Per cent predicted					
6.9.83	69	25	3+	mucoid mucoid	PA u/c	S S
1.11.83	73	28	3+	mucoid nonmucoid	PA 01	S R
13.2.85	58	25	3+	mucoid nonmucoid nonmucoid	PA PA PA	S R R
20.3.85	67	22	3+	mucoid mucoid nonmucoid nonmucoid	PA u/c u/c 01	R S S R
8.5.85	48	19	3+	mucoid nonmucoid nonmucoid	PA PA PA	S S S

Died 8.5.85

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

R = resistant, S = sensitive to the action of normal human serum

DISCUSSION

SECTION II

Despite the number of published reports of the frequency of P.aeruginosa in CF, few systematic longitudinal studies of the acquisition and persistence of this species in a large number of patients have been performed. To this end a study was made over a two year period of the incidence of pseudomonas and the presence of other pathogens to determine their association with the clinical status of patients.

A correlation between colonisation with P.aeruginosa and age was reported by Corey et al. (1984) who found that 70% of their older patients harboured P.aeruginosa in contrast to 50% of patients below ten years. A similar figure was given for adult patients at the Brompton (Hodson 1984) and therefore it was not surprising that 63% of the patients in our series (adults) were colonised by P.aeruginosa. As the incidence of P.aeruginosa apparently increases with age it was surprising that those persistently colonised formed the youngest group.

These patients also tended to present with chest symptoms earlier than those not persistently colonised which may be indicative of a predisposition to infection by pulmonary pathogens. The average age on diagnosis for each of the groups was similar and if prophylactic antimicrobial therapy was begun at this time it did not appear to influence the acquisition of pseudomonas.

There was a higher incidence of staphylococci in the group of patients free of P.aeruginosa which was in keeping with the finding of Mearns et al. (1972) and Corey et al. (1984) that the incidence of staphylococci decreased as the isolation of P.aeruginosa increased. There was however no statistical difference between the numbers in

each group treated with antistaphylococcal agents. It may have been that colonisation with staphylococci and P.aeruginosa occurred simultaneously but effective antibiotic treatment eradicated the former. Alternatively patients may have been treated prophylactically to prevent staphylococcal infections without confirmation of staphylococci in the sputum and thus presented initially with pseudomonas.

The incidence of H.influenzae was similar in each group and reflected the number with treated 'antihaemophilus' antibiotics. No conclusions were drawn in this survey as to the clinical significance of H.influenzae as its culture was not performed by the author and it was necessary to rely on routine bacteriological reports. Examination of these reports did not support an intermediary role for H.influenzae infection between the isolation of staphylococci and the onset of pseudomonas colonisation which was suggested by Burns and May (1968). May et al. (1972) found that the incidence of serum precipitins to H.influenzae increased with age. They reported an incidence of 80% in patients over 21 years which is similar to that rate recorded for these adult patients.

The majority of patients gave hypersensitivity reactions in skin tests with house-dust mite, pollen and aspergillus. However, there was no apparent association between hypersensitivity and colonisation with P.aeruginosa. This was in contrast with the study of Wilmott, Tyson and Matthew (1985) who found in CF children that positive allergy skin tests occurred significantly more often in those patients chronically colonised with P.aeruginosa. However a previous study by this group (Pitcher-Wilmott et al. 1982) had shown

that the incidence of positive skin tests was influenced by age which may explain the lack of association of allergy and pseudomonas colonisation in the adult population reported here.

There was no statistically significant association between colonisation with P.aeruginosa and morbidity and mortality amongst these patients although patients who acquired P.aeruginosa during the trial were more frequently admitted to hospital than those in the first and third categories. This may reflect a deterioration in clinical condition at the time of initial infection or as the clinical condition of the patient declined concurrent opportunist colonisation with P.aeruginosa took place.

Published reports attest to the fact that isolates of P.aeruginosa from CF differ from the 'typical' wild-type strains seen in other infections (Doggett 1969; Govan and Fyfe 1978; Penketh et al. 1983^a). The most commonly reported alterations in phenotype are the production of mucoid exopolysaccharide, hypersensitivity to B-lactam antibiotics, loss of O-specificity and increased sensitivity to normal human serum. These variants were often cultured from the same sputum sample as wild-type strains and this led Penketh et al. (1983^a) and others to suggest that the organism undergoes an environmental adaptation from the wild-type to one or many phenotypic variants. These phenotypes may express mucoid exopolysaccharide, produce unusual pigments and be hypersensitive to antibiotics in addition to alterations in the somatic antigens and in their sensitivity to serum.

The appearance of mucoid P.aeruginosa is virtually diagnostic of CF, but its clinical significance is obscure although it has been

demonstrated to form a matrix in which microcolonies mature in the lung (Baker and Marcus 1982). Most patients (76%) in this series harboured mucoid strains at some time during the survey and this figure is comparable with other published reports (Doggett 1969; Høiby 1975). However of the total number of isolates recovered from all sputum samples only 44% were mucoid as nonmucoid varieties often out-numbered the mucoid form on primary culture.

The high number of unpigmented isolates found was probably due to the lack of expression of pigment by mucoid colonies. Nevertheless in comparison with P.aeruginosa from other sources (Pitt unpublished) there was a considerable lack of pigment producing strains in adult CF. The ciliotoxic (Wilson et al. 1986) and antibacterial properties of pyocyanin may be a contributory factor to initial colonisation of the respiratory tract with pseudomonas. The absence of pigment production in 49% of the isolates of P.aeruginosa was therefore puzzling. An explanation may be that as the pseudomonas have achieved colonisation of the respiratory tract the production of pigment may be superfluous to the continued survival of some strains. A considerable amount of direct and circumstantial experimental evidence suggested that the isolates of P.aeruginosa from CF had altered outer membranes. The loss of O-specific antigen i.e. LPS components, rendered the isolates sensitive to the bactericidal action of NHS. Although this relationship was not absolute it was statistically highly significant. The mechanism of serum sensitivity was not investigated fully but the reduction in LPS may expose lipid A and core components usually embedded deeper in the outer membrane, which act as targets for the activation of complement

in serum (Taylor 1983; Hancock et al. 1983).

Phage susceptibility typing which was used as an indirect probe of the bacterial surface showed that those isolates with complete O-antigens were significantly more sensitive to typing phages than those with defective O-antigens. The majority of the phages adsorbed to LPS receptor sites and the loss of phage receptors was associated with a loss of O-specificity. This finding has considerable bearing on the use of O-serotypes and phage typing on the identification of distinct types of P.aeruginosa in cystic fibrosis. If, as the data clearly shows, the majority of isolates were not only O-defective but also resistant to phages one must question the use of these methods for the establishment of cross-infection amongst CF patients (Højby and Rosendal 1980). These authors suggested that two distinct strains were present in a population of CF children attending a central clinic in Denmark. These strains were, respectively, one which was lysed weakly or not at all by a single phage and was serotype 03 and the other which exhibited cross reactions with three antisera 03, 06 and 09 and some were lysed by phage 109. The seroreactions described by the Danish workers are typical of the PA isolates found in this and other studies and should not be allocated the status of a serotype.

The data in the literature regarding the antibiotic susceptibility of strains of P.aeruginosa from CF is conflicting. Muroid forms have been reported to be hypersensitive to semi-synthetic penicillins (Govan et al. 1983) and have also been found to have increased resistance to all antibiotics. Furthermore, Irvin et al. (1981) showed that there was considerable variation of the MIC of B-lactam

antibiotics for isolates derived from the same specimen and recorded differences of 100-fold between MIC values in the same sample.

Hypersensitivity of the CF isolates was examined by disc diffusion tests and large zone diameters given by B-lactams were taken as evidence of increased susceptibility. It was not within the scope of this investigation to define categorically the sensitivity or resistance of CF isolates but it was necessary to show whether hypersensitivity was associated with outer membrane defects. This association was not absolute which suggests that other factors influence the increased sensitivity of CF isolates to the semi-synthetic penicillins.

Replacement of a serotype by a strain of another serotype was rarely seen. Patients whose first sample yielded a typable strain consistently harboured this strain or variants that apparently arose from this strain. Infection with two strains of specific serotypes was also uncommon and in most cases one or other of the types disappeared from subsequent specimens. Thus once colonisation was established a typable strain was often demonstrable but in some cases patients who acquired pseudomonas during the trial presented with defective strains. This may have been due to the time lapse between initial infection viz- with a typable strain, and the collection of the sample.

A scenario can be envisaged in which patients are first colonised by wild-type, O-typable, serum resistant strains of P.aeruginosa. Support for this is shown by the fact that the serotypes of the O-typable strains isolated from CF were similar to the types of P.aeruginosa found in other clinical conditions. While there was

considerable fluctuation in the relative frequencies of individual types, type 06 which predominates in non CF populations was also the most common in CF. Thirteen of the 17 IATS serotypes were identified amongst CF isolates and the four types not encountered are also extremely rare in non-CF specimens (Pitt 1981). O-typing of P.aeruginosa from CF patients therefore suggests that infection is primarily initiated by strains commonly found in the environment and not by a 'cystic' strain of P.aeruginosa advocated by Zierdt and Williams (1975) who found that the majority of their isolates fell into a single serogroup.

In this series 011 was not as frequent as in general clinical material where it represents 15-20% of isolates (Pitt 1981). There is considerable evidence to suggest that strains of serotype 011 are more often associated with hospital common source outbreaks than other serotypes (Farmer et al. 1982) and in addition it has been commonly associated with water borne infections (Kush and Hoadley 1980) such as folliculitis.

As O-typability of CF isolates was poor (19%) this method was unsuitable for the discrimination of distinct strains in these patients. In routine typing of P.aeruginosa from all clinical sources correspondence of the O-type of strains from an individual incident of infection is taken as evidence of possible cross-infection (Pitt 1980). Most of the patient samples were obtained from the out patient department and therefore it was unlikely that interchange of strains had occurred. No evidence of sharing strains during the same clinic visit by groups of patients was found, except that many harboured PA isolates. The assumption

by Pedersen et al. (1986) that all polyagglutinating isolates are one and the same is therefore erroneous as most unrelated patients carried this phenotype. The PA serological reaction is due to the loss of O-specific antigens and illustrates only the presence of macromolecules common to all P.aeruginosa. Furthermore PA strains of P.aeruginosa are frequently found in chronic urinary tract infections of non CF patients and to suggest an association between these patients would be entirely misleading. Few sibling pairs attended the Brompton clinic and most were persistently colonised with O-defective isolates and thus the possibility of cross-infection could not be confirmed by O-serology. It is possible that pyocin typing which seems an efficient method for the typing of CF strains may have provided further proof of the similarity or distinguishability of strains in this population (Kelly et al. 1982). In sequential samples there was a gradual emergence of strains with atypical serological reactions and these strains persisted as the dominant organisms during long term colonisation. The mechanism by which this change occurs is of course only speculative but the following is proposed. The cystic fibrosis patient has high specific humoral immunity towards P.aeruginosa (Høiby 1982; Speert 1985) and in particular antibody directed towards O-specific antigens is readily demonstrable in high titres (Pitt et al. 1985). The major part of the population of the wild-type strain is killed by a combination of humoral and cellular factors. Within the population however a minority of cells, probably at a frequency of only 10^6 - 10^9 (Pitt and Fonseca unpublished) are able to survive as they are naturally occurring O-defective variants. The loss of O-antigens

renders the cell sensitive to complement but within the favourable environment of the CF lung proliferation takes place and these strains form the dominant flora.

Penketh et al. (1983^a) were able to show a statistical correlation between the clinical status of the patient and the phenotypic characteristics of P.aeruginosa in the sputum. An association between the emergence of phenotypic variants and the course of pulmonary infection was sought in this group of 210 patients. The proportion of phenotypically variant strains in the last samples obtained was similar to the first and it was not possible to ascertain if the clinical condition of patients overall deteriorated over the two years or if the rate of isolation of variant strains increased. This information was only available for individual patients. The observation that patients in a stable clinical condition harboured strains that were of either the wild-type or completely atypical form was in agreement with Penketh et al. (1983^a). However, the impression gained was that the number of different isolates present in the patient's sputum was inversely proportional to clinical status, the greater the variety of phenotypes, the poorer the lung function.

It appeared that hospitalisation and intensive antibiotic therapy influenced the rate of isolation of phenotypic variants from the sputum. The variant forms were more often hypersensitive to semi-synthetic penicillins and although they occasionally disappeared from the sputum, they would invariably be found in subsequent samples from the patient.

Penketh et al. (1983^a) suggested that during the period of transition

from typical to atypical forms of P.aeruginosa the condition of some patients deteriorated. Patients who survived this intermediate period may remain in a relatively stable condition for some time. This would explain the very poor lung function, yet stable condition of those patients who harboured atypical forms of P.aeruginosa. Alteration in phenotype may have aggravated host defences and led to an exacerbation of pulmonary disease with its resultant poor lung function.

It was clear in patients whose condition declined very rapidly that alterations in the serological phenotype of the organism occurred over a short time scale. The host was probably less well able to recognise and combat these rapid changes than when the transition of strains occurred over a longer time period. It follows that if antigenic variation was a feature of P.aeruginosa from CF a longer time course would enable the host to mount an adequate response to the altered forms of the organism.

Antigenic variation may be defined as the process by which an infecting organism changes its antigenic structure sufficiently to avoid elimination by the host's immune system and thus gains a selective advantage. Infections caused initially by an apparently homogeneous population of a pathogen can give rise to a population immunologically distinct from the parent strain. Variation can be induced in microorganisms by the development of specific immunity by the host population (herd immunity) as is the case with influenza virus, or within the individual host as is seen in trypanosomiasis (Turner 1980).

Antigenic variation of bacteria has been recognised previously with

the urinary tract pathogen E.coli (Jodal et al. 1974; Lindberg et al. 1975) and this situation would appear to be analogous to that proposed here for P.aeruginosa in CF. Lindberg et al. (1975) showed that strains of E.coli isolated from recurrent asymptomatic bacteriuria frequently lost their O-antigen specificity and became sensitive to the action of normal human serum. They suggested that as the organism was localised in the bladder, selective pressures i.e. antibodies and antibiotics, resulted in the eradication of the original strain.

In contrast to E.coli which apparently reverted to wild-type forms during exacerbations of infection, P.aeruginosa from CF appears to remain in its atypical state. Extension into the blood stream and pneumonia are extremely rare in CF despite the formidable load of organisms resident in the lung. Serum sensitive strains could not by definition exist in the blood system and hence colonisation remains local.

Similar changes were described by Zak et al. (1984) in Neisseria gonorrhoea. Symptomatic patients although able to recognise strains from other sources did not make antibody towards the strain they harboured, as this organism frequently altered its antigenic profile (Heckels 1986).

Studies on the CF immune system suggest that the systemic and local humoral responses appear qualitatively intact and appropriate (Moss and Lewiston 1984). Moreover patients with CF have no higher incidence of infectious diseases than other patient groups (Batten and Matthew 1983).

Pseudomonas is a versatile organism and is capable of adapting to

many environments. The evidence presented here suggests that antigenic variation occurs in the CF lung and may be accompanied by deterioration in the clinical condition of the patient. It was therefore necessary to investigate the antibody response of patients towards both serologically typical strains and variants of them.

RESULTS

SECTION III

Introduction

CF patients suffer from recurrent pulmonary infections which are recognised as the major complications of the disease. These occur despite the presence of an intact humoral antibody response and most patients have elevated titres of antibody towards bacterial products (Doggett and Harrison 1972). If the antigenic variation of P.aeruginosa described in the foregoing sections occurs in vivo, the host may not make adequate amounts of antibody against those bacteria with a new antigenic profile. It was necessary to evaluate the patients immune response towards both their O-typable (wild-type) isolates and O-defective variants in order to establish the specificity of the antibody response.

Comparison of immunogenicity

The ability of O-typable and O-defective strains to stimulate antibody in rabbits was compared. Four strains of P.aeruginosa were used. Strains 7 and 7/1, and an O-typable (473^a, serotype 03) and O-defective (473^b, PA) pair isolated from the same CF sputum sample. Ten vaccine preparations were made and the cell concentration of each is shown in Table 59. The first five vaccines were strain 7 or 7/1 alone or combinations of these strains in equal proportions and one-tenth the concentration of each other. The vaccines of the pair of CF isolates were prepared similarly. Rabbits were injected over the course of 18 days as described in Materials and Methods for the production of hyperimmune sera.

The antisera were tested by slide agglutination with the strains contained in the vaccines. Serum raised against vaccine 1 (Table 60) agglutinated the vaccine strain to a titre of 160 which was

TABLE 59

Proportions of parent and variant cells of *Pseudomonas aeruginosa* in
vaccines used to produce hyperimmune sera in rabbits

Strain (serotype) and viable count (cfu/ml) of preparation					

Vaccine	7 (06)	7/1 (PA)	Vaccine	473 ^a (03)	473 ^b (PA)

1	3.4×10^{10}	0	6	1×10^{10}	0
2	0	4×10^{10}	7	0	1×10^{10}
3	5×10^9	5×10^9	8	2×10^9	2×10^{10}
4	2.5×10^{10}	3×10^9	9	1×10^9	1×10^9
5	3×10^9	3×10^{10}	10	1×10^9	1×10^{10}

TABLE 60

Homologous and heterologous slide agglutination titres of antisera
raised towards different proportions of strain 7 (0+) and 7/1 (0-)
of *Pseudomonas aeruginosa*

Antiserum (ratio of 7:7/1)	Agglutination titre with	
	7	7/1
1 (1 : 0)	160	160
2 (0 : 1)	-	80
3 (1 : 1)	20	160
4 (10 : 1)	20	20
5 (1 : 10)	10	80

- titre less than 5

comparable with the titres observed in agglutination tests with serotype strains of P.aeruginosa. As expected from previous results serum 7 cross reacted with variant 7/1. The rabbit injected with strain 7/1 alone gave a similar titre with homologous cells but also contained antibody to the parent form. Equal proportions of parent and variant strains resulted in a serum titre for 7/1 which was 8-fold higher than that for the parent and when strain 7 exceeded the variant in the vaccine, titres of equal magnitude were obtained. The serum from the vaccine containing 10-fold greater variant than parent cells also reacted to a higher titre with the variant strain. These results were unexpected and did not appear to be consistent with the assumption that O-defective cells were poorer immunogens than the typical forms. A similar pattern of agglutination response was observed with isolates derived from CF (Table 61). The O-typable CF strain was poorly immunogenic as a very low homologous titre was present in the serum raised against this strain alone. This could have been due to the lack of response in the rabbit used in the experiment as when the strain because when the O+ strain was combined with an equal number of O- cells, the titre, albeit poor, was slightly higher. Vaccination of an animal with 10-fold less variant cells did not result in an appreciable response. Overall the titres of these sera were disappointing and no conclusions could be made as to the relative immunogenicity of the pair of strains. Due to the poor results of agglutination, all sera were examined in a whole cell ELISA. The titres of the sera to combinations of both pairs of strains were much higher in this test (Tables 62 and 63) but they did not reflect the proportion of cells contained in the

TABLE 61

Homologous and heterologous slide agglutination titres of antisera raised
towards different proportions of an O-typable (O+) and O-defective (O-)
pair of Pseudomonas aeruginosa from CF

Antiserum (O+ : O-)	Agglutination titre with	
	473 ^a	473 ^b
6 (1 : 0)	20	10
7 (0 : 1)	80	80
8 (1 : 1)	40	160
9 (10 : 1)	40	5
10 (1 : 10)	40	40

TABLE 62

Homologous and heterologous ELISA titres of antisera raised towards
different proportions of strain 7(0+) and strain 7/1(0-) of
Pseudomonas aeruginosa

Antiserum (7 : 7/1)	ELISA titre with	
	7	7/1
1 (1 : 0)	400	200
2 (0 : 1)	400	600
3 (1 : 1)	400	300
4 (10 : 1)	800	300
5 (1 : 10)	800	800

TABLE 63

Homologous and heterologous ELISA titres of antisera raised towards
different proportions of an O-typable and O-defective (O-) pair of

Pseudomonas aeruginosa from CF

Antiserum (O+ : O-)	ELISA titre with	
	473 ^a	473 ^b
6 (1 : 0)	1600	800
7 (0 : 1)	800	1600
8 (1 : 1)	800	400
9 (10 : 1)	800	400
10 (1 : 10)	1000	500

vaccines. Titres of the sera to 7 and 7/1 varied by only one doubling dilution or less and it was not possible to predict from the titres which vaccine had been used to raise an antiserum (Table 62). Although the titres given by the pairs of CF isolates were marginally higher than those of the two laboratory strains the titres of sera towards each vaccine component were similar. The ELISA did demonstrate that titres to O-typable forms were generally higher, by about 2-fold, and this method was adopted to study the responses of patients to their strains of pseudomonas.

Measurement of patients' antibody

Only one sample of serum from each of 54 CF patients who were colonised with P.aeruginosa was available for tests. On average six isolates from each patient were used in ELISA with each serum sample. The isolates were recovered from sputum samples taken as near as possible to the date of serum collection. The isolates were divided into O-typable and O-defective by agglutination reactions and the average titre of each serum with their respective isolates was determined.

The patients were sorted into categories reflecting the duration of colonisation and Table 64 shows the titres obtained against both varieties of pseudomonas for each patient group. Overall 52 of 54 patients harboured serologically atypical strains and 21 of 54 were also colonised with O-typable forms. The titres in ELISA ranged from extremely low or negative antibody response to a maximum of 50,000 (50% maximal absorbance) with the O-typable isolates and the highest mean titres were given by patients who had been persistently colonised. These patients also formed high levels of antibody

TABLE 64

ELISA titres of serum samples from 54 CF patients towards O-typable
and O-defective isolates of Pseudomonas aeruginosa

Patient Group (number)	*ELISA titre (range)	
	Typable isolates	Variant isolates
All patients (54)	9 000 (1 000 - 50,000)	7 500 (500 - 48,000)
Patients recently colonised (16)	3 500 (1 000 - 4 000)	4 500 (1 000 - 7 000)
Patients persistently colonised (38)	11 000 (1 500 - 50,000)	9 000 (500 - 48,000)

* mean value for patients with these isolates

$p < 0.005$ (Students t-test)

towards their O-defective strains but patients recently colonised had titres approximately one-third to one-half of the titre with serologically typical strains. The ELISA titres for all patients showed little difference towards both types of strains, but this was statistically different by Student's t-test, $t = 0.32$, 4 d.f., $p < 0.05$.

Antibody response of patients

The foregoing results showed that serum from CF patients had a high titre of antibody towards P.aeruginosa of both O-typable and O-variant profiles. There was a slight difference in the magnitude of the titre of those patients persistently colonised and those who had recently acquired the organism but it was necessary to investigate individual patients to confirm this.

Serum specimens from three patients were chosen, one had been recently colonised with P.aeruginosa, another persistently colonised but relatively well and the third who had an exacerbation of infection.

Five isolates from four sputum samples of the recently colonised, patient A, were selected and two of them were serotype 09 and the remainder were PA in agglutination tests.

The ELISA titre towards whole cells ranged from 500 to 4000 (Table 65) and the highest titre was given by an O-defective, mucoid isolate (No. 2a). The titres to the O-typable strains varied by four-fold but as the second typable variety was isolated three months after the serum date the difference in titre may have been due to subtle variation in the O-antigens although they were still of a specific O-type. The type specific nature of this patient's response was underlined by her reaction with the control serotype strain 09 and

TABLE 65

ELISA titres of patient's serum towards five isolates of
Pseudomonas aeruginosa cultured from successive sputum samples

Date	Isolate	Agglutination Category	Serum Resistance	Titre	Lung function	
					FVC	FEV ₁ (litres) ¹
6.1.84	1	09	R	2 000	2.1	1.65
*27.7.84	2a mucoid	PA	S	4 000	2.0	1.65
	2b	PA	S	500		
10.10.84	3	09	R	500	2.05	1.55
6.11.84	4	PA	RR	500	1.5	1.3
	Serotype strain	09		1 500	Control	

* Serum obtained on this date

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)
 R = resistant, RR = reduced resistance, S = sensitive to the action of
 normal human serum.

furthermore suggests that isolate No.3 was not entirely typical of this serotype.

Further evidence of the O-antigen nature of the strains was determined by electrophoresis of purified LPS in SDS-PAGE. Electrophoresis revealed high molecular weight bands in extracts of the two typable strains as well as medium and low molecular weight components (Figure 19a). The PA strains did not give bands of high molecular weight (tracks 2, 3, and 5) but one of them contained medium and low molecular weight components similar to the O-typable strains.

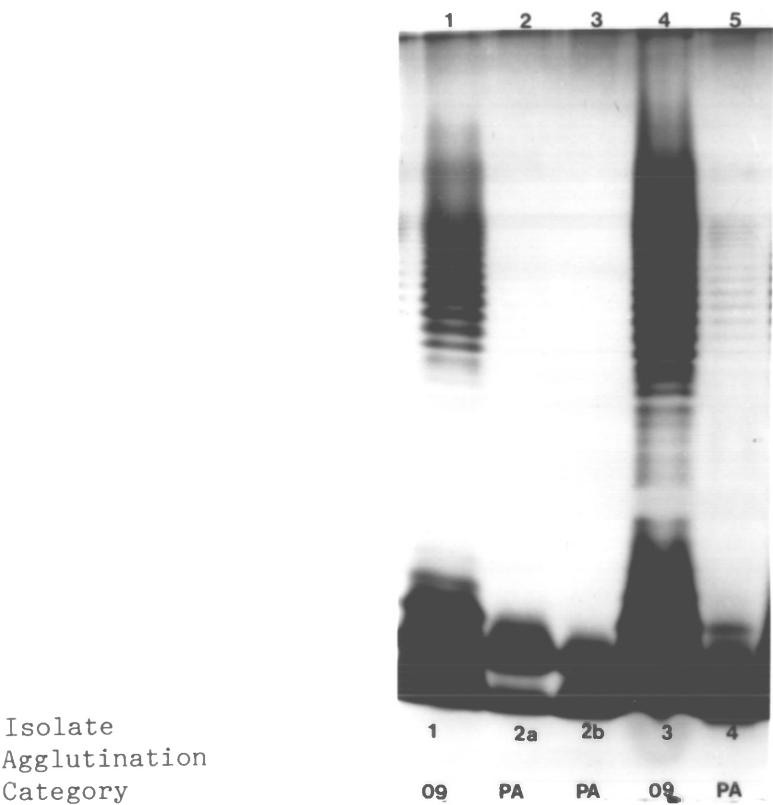
The corresponding immunoblot with the patient's serum is shown in Figure 19b. Antibody binding to high and medium molecular weight bands of the O-typable isolates was clearly visible and no reaction was seen in the low molecular weight core region of these strains (tracks 1 and 4). The antibody of this serum did not bind with two of the three PA isolates but antigen was detected in the higher molecular weight region of the track of the third O-defective isolate (track 5).

In this patient therefore defective LPS did not appear to stimulate an antibody response of the same magnitude as that towards the O-specific units. Furthermore lung function values obtained were independent of the results of the ELISA. The FVC of this patient fluctuated between 1.5 and 2 litres and although the lowest value was associated with the presence of a PA isolate it was probably solely due to a decline in clinical status.

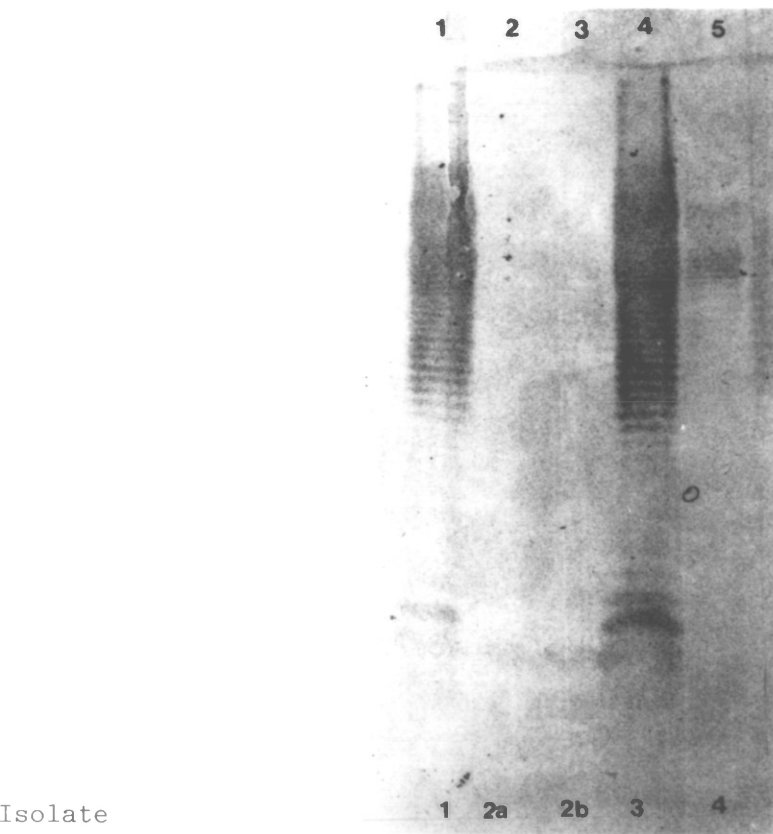
Patient B was chosen to represent the group who had been persistently colonised but who remained clinically relatively stable. The

Figure 19

a) SDS-PAGE and silver stain of purified LPS from Pseudomonas aeruginosa isolates in sequential sputum samples from patient A.



b) Immunoblot of purified LPS with patient's serum diluted 1 in 1000



initial infection of this 21 year old patient with P.aeruginosa had occurred seven years previously. He harboured a variety of phenotypes but in the first samples collected had predominantly an O-typable strain, 06, which was occasionally isolated in the mucoid form. This typable strain continued to be present throughout the one year period of sampling. The serum was taken approximately eight months after the first sample and at this time the patient was colonised with the 06 strain and an O-defective form.

The whole cell ELISA titres for the typable strain ranged from 1500 to 16000 and the antibody response varied towards the same phenotype in different samples, for example the titre with isolate 1a (06, mucoid, serum resistant) was approximately ten-fold lower than that with isolate 3 which was indistinguishable phenotypically from 1a. Variation in antigen binding was also observed with O-defective isolates from this patient and titres as divergent as 64000 and 4000 were given by two isolates (5a and 5b) from the same specimen (Table 66). Interestingly the higher titre was recorded towards the mucoid isolate, 5a.

The lung function of this patient declined over the period of sampling, although at one point there was a marked improvement in FVC (from 1.55L to 2.03L). Although the highest antibody titres were given by isolates cultured at the time of improved lung function there was no obvious association between serum titre and lung function. The emergence of O-defective isolates however did seem to be associated with declining respiratory function.

Due to the variety of antibody titres obtained it was not possible to distinguish a trend of increasing response with prolonged

TABLE 66

ELISA titres of patient's serum towards 14 isolates of
Pseudomonas aeruginosa cultured from successive sputum samples

Date	Isolate	Agglutination Category	Serum Resistance	Titre	Lung function FVC FEV ₁ (litres)	
15.11.83	1a mucoid	06	R	1 500	1.55	0.85
	1b	06	R	2 000		
	1c	06	PS	8 000		
10.11.84	2a mucoid	06	R	6 000	1.99	0.88
	2b	06	R	3 000		
7.2.84	3 mucoid	06	R	16 000	2.03	0.9
6.3.84	4 mucoid	06	R	8 000	2.03	0.9
3.4.84	5a mucoid	PA	R	64 000	1.85	0.7
	5b	PA/NT	PS	4 000		
1.5.84	6a mucoid	PA	R	30 000	1.35	0.63
	6b	u/c	RR	4 000		
*3.7.84	7a	06	R	12 000	1.1	0.55
	7b	PA/NT	R	6 000		
5.10.84	8a mucoid	PA	R	3 000	1.15	0.5
	8b	06	R	6 000		

* Date serum obtained

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

PA/NT = agglutinated by both PA and NT antisera

R = resistant, RR = reduced resistance, PS = partially sensitive to the action of normal human serum

colonisation. Nevertheless the titres of this patient with persistent colonisation were markedly higher than those of the patient A, who had been recently colonised.

The LPS profiles shown in figure 20a are those of isolates from patient B. In general the bands given by most isolates indicated the presence of high and medium molecular weight substances and there was good correspondence in the mobilities of individual bands. It was evident though that the serological nature of the strain as defined by agglutination could not be determined by its LPS profile. One strain which was PA/NT (5b track 6), was completely devoid of high and medium molecular weight LPS components but another isolate of the same serocategory (7b track 10) gave bands throughout the low and middle parts of the track.

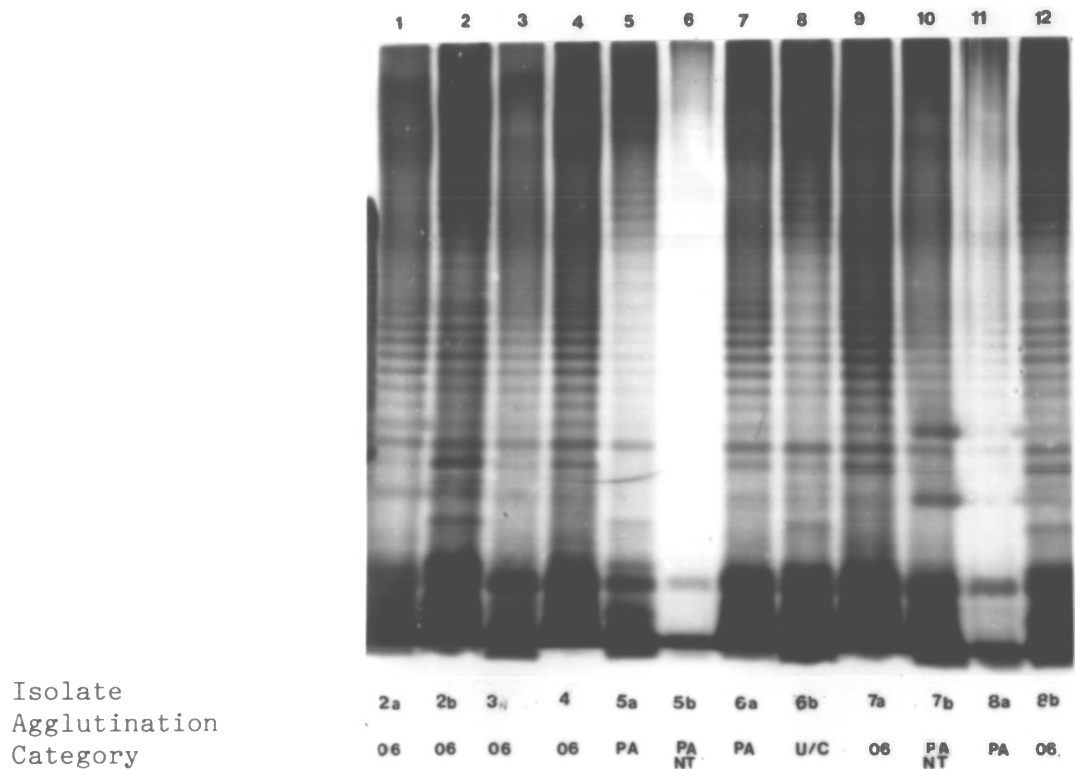
The results of immunoblotting these profiles with the patient's serum diluted 1 in 1000 are shown in Figure 20b. The patient's serum contained antibody which bound with antigen in both the low and high molecular weight regions of LPS. Some of the lower medium molecular weight components were not recognised by antibody in this serum as evidenced by a gap in the immunoblot tracks. This patient had a reduced antibody response towards the LPS of the O-defective isolates in tracks 5 and 6. A faint reaction was seen with components in the higher molecular weight region of the LPS of isolate 5a (track 5).

The third patient examined in serum tests was also persistently colonised with P.aeruginosa but was suffering from an exacerbation of infection. *Pseudomonas* had been present for about seven years and the patient was aged 24 at the time of the survey.

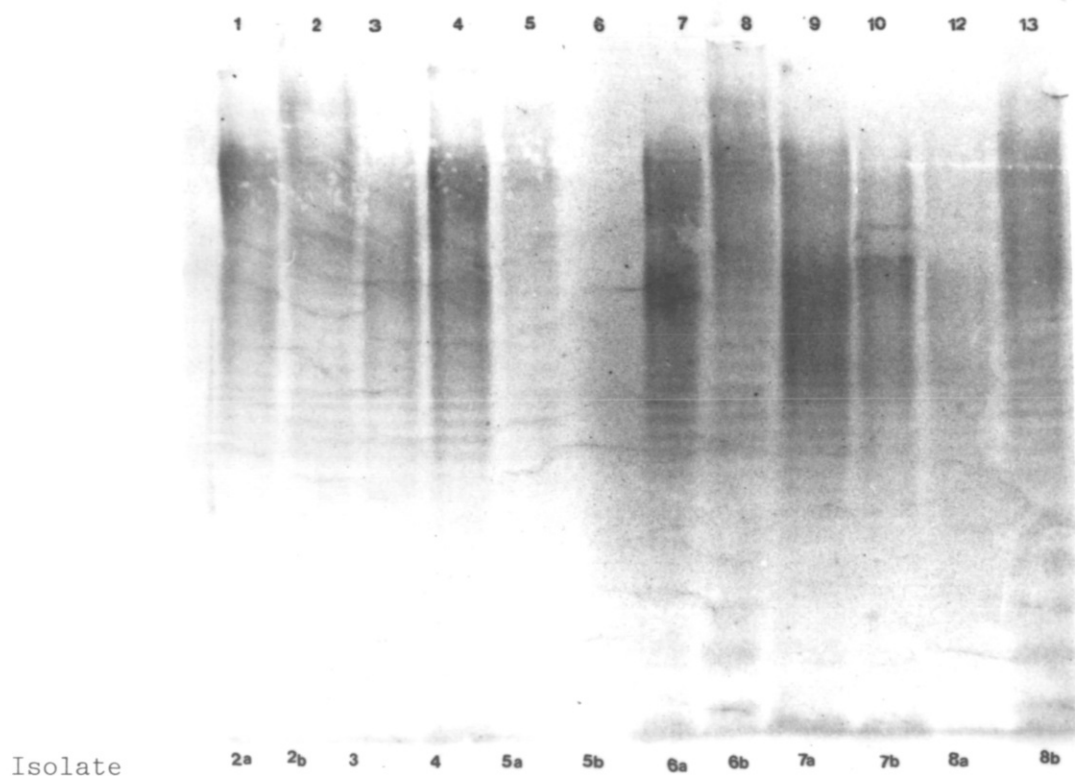
All the isolates recovered from five sputum samples over the three

Figure 20

- a) SDS-PAGE and silver stain of purified LPS from Pseudomonas aeruginosa isolates in sequential sputum samples from patient B.



- b) Immunoblot of purified LPS with patient's serum diluted 1 in 1000.



months were serologically atypical. Most were reactive with the NT serum and at least four phenotypes were distinguishable by serology, mucoid form and serum sensitivity. The serum sample was taken between the first and second sputum samples.

The antibody response to each isolate ranged from high (8000 or greater) to low titres of 1500. This patient had lower titres than the other patient with persistent colonisation and again no association of serum response with phenotype was found (Table 67).

This patient had very low lung function values and on two occasions was so weak that no recordings could be obtained. In this case neither the phenotypes of the isolates nor the serum titres appeared to be associated with lung function.

Silver staining (Figure 21a) revealed the absence of high and medium molecular weight material in most of these isolates. The smears seen in tracks 2, 4, 8 and 10 were probably due to side chains that were poorly reduced in the gel and migrated as a complex.

When these LPS profiles were immunoblotted with the patient's serum, (Figure 21b) the presence of antibody to side chains was apparent for four of the LPS samples (tracks 1, 6, 12 and 13). The major seroreactive component of the LPS as determined by the intensity of colour appeared to be associated with the side chains. There was a noticeable decrease in intensity of the reaction in the low molecular weight core regions. The immunoblot of LPS of isolate 3b in track 7 (corresponding to track 8 in the gel), showed that the uppermost band in the core region was highly seroreactive as was the bulk of the core material in the adjacent track. The LPS of isolate 5c which appeared on silver staining (track 15) to contain only core

TABLE 67

ELISA titres patient's serum towards 15 isolates of

Pseudomonas aeruginosa cultured from successive sputum samples

Date	Isolate	Agglutination Category	Serum Resistance	Titre	Lung function FVC FEV ₁ (litres)
29.2.84	1a mucoid	PA/NT	S	1 500	1.1 0.6
	1b	NT	S	6 000	
	1c	NT	S	4 000	
* 28.3.84	2a mucoid	PA	S	8 000	Unable to blow
	2b	PA	S	10 000	
	2c	PA	S	8 000	
11.4.84	3a mucoid	PA/NT	S	10 000	1.0 0.5
	3b	PA/NT	PS	3 000	
	3c	PA	R	4 000	
18.4.84	4a	PA/NT	S	4 000	1.35 0.68
	4b	NT	S	4 000	
	4c	NT	S	8 000	
15.5.84	5a mucoid	PA	S	2 000	Unable to blow
	5b	NT	S	2 000	
	5c	NT	S	2 000	

* Serum obtained on this date

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1)

NT = agglutinated by NT serum (anti 18S)

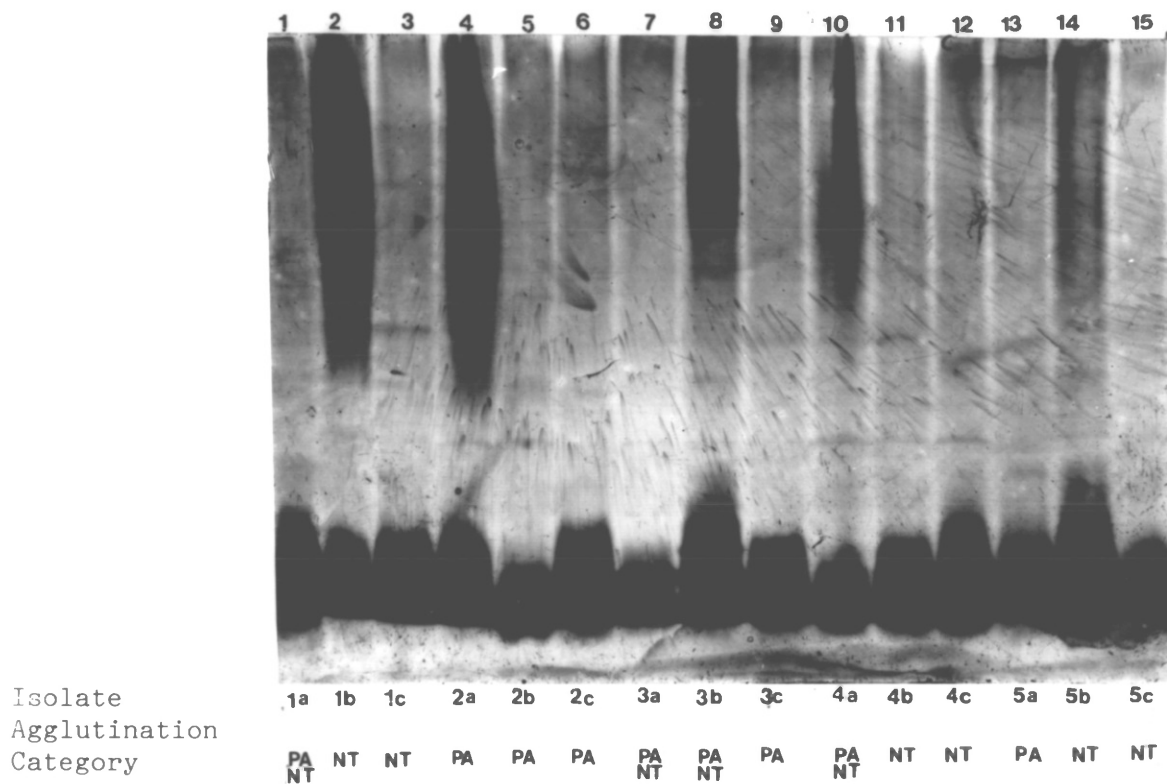
PA/NT = agglutinated by both PA and NT antisera

R = resistant, PS = partially sensitive,

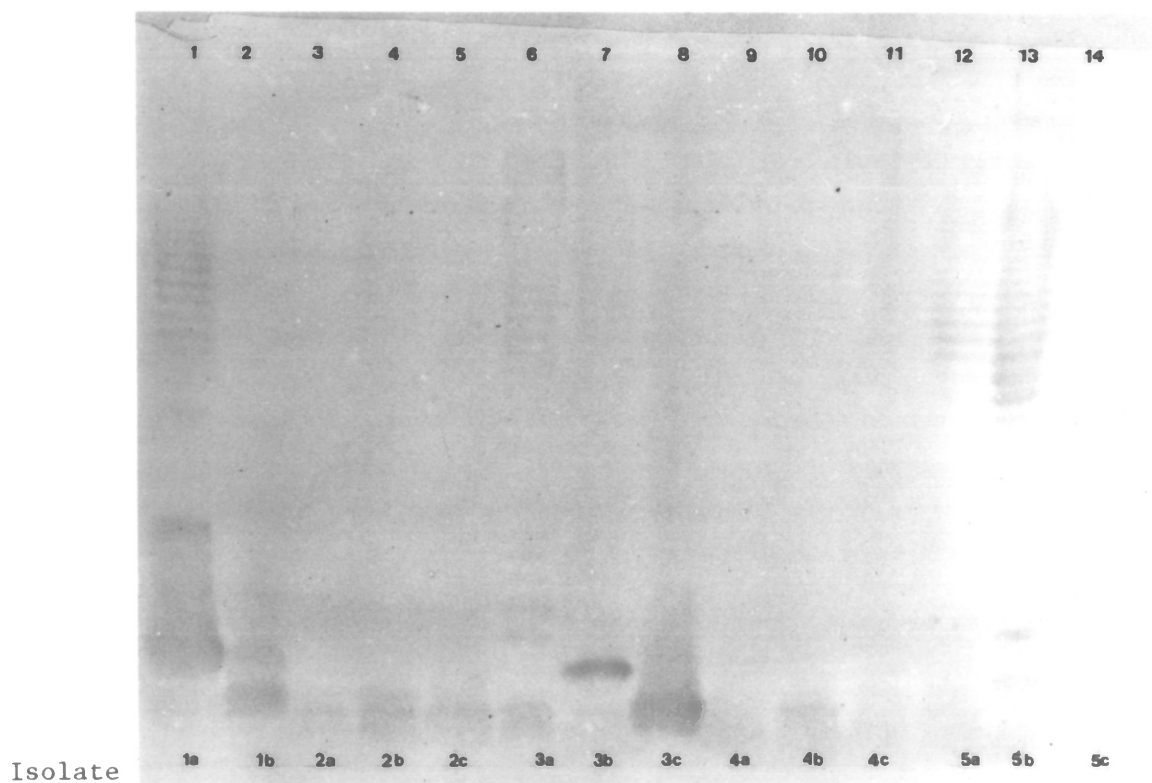
S = sensitive to the action of normal human serum

Figure 21

- a) SDS-PAGE and silver stain of purified LPS from isolates of Pseudomonas aeruginosa in sequential sputum samples from patient C.



- b) Immunoblot of purified LPS with patient's serum diluted 1 in 1000.



components did not bind with antibody. The LPS of isolate 2a (track 3) was similarly unreactive.

Detection of local antibody production

From the foregoing results there appeared to be a good systemic antibody response to the majority of the phenotypes of P.aeruginosa harboured by CF patients. To determine whether antibody was present in bronchial secretions the sol phase of sputum samples of ten CF patients was examined in a 'dot-blot' ELISA system with whole cells of the bacteria isolated from the same sample. Three different conjugates; anti-total immunoglobulins, anti IgG and anti IgA were used and the sol phase was diluted 1 in 10, 50 and 250.

The antibody-antigen reaction detected by anti IgA conjugate gave the most intense reaction with samples from all patients but IgG was also detected. (Table 68). Patients 1, 2 and 4 gave the strongest reaction with their resident pseudomonas but they did not react with strain 7 which was included as a nonspecific control in each batch of tests.

The two patients F and H who harboured yeasts had no detectable antibody towards them but the patient from whom S.aureus was isolated showed an IgA response towards this organism. No reaction was detected with the anti IgG conjugate. Interestingly only two of the patients harbouring P.aeruginosa (patients H and I) reacted with strain 7 and the highest intensity of colour was obtained with the anti IgA conjugate. Although no P.aeruginosa was isolated from the sputum sample of patient J, the sol phase contained antibody to both experimental strains 7 and 7/1 and the reaction with the variant strain was the stronger of the two.

TABLE 68

'Dot blot' ELISA of the sol phase of sputum from ten CF patients with the
Pseudomonas aeruginosa and other organisms isolated from the sputum

Patient	Organism (agglutination category)	Immunoglobulins detected:								
		Total Ig			IgA			IgG		
		dilution			dilution			dilution		
		10	50	250	10	50	250	10	50	250
A	<u>P.aeruginosa</u> (PA)	+	+	+	+	+	+	+	+	+
B	<u>P.aeruginosa</u> (PA)	ND	+	+	ND	+	+	ND	+	+
C	<u>P.aeruginosa</u> (O3)	+	+	-	+	+	-	+	-	-
	<u>S.aureus</u>	-	-	-	+	+	-	-	-	-
D	<u>P.aeruginosa</u> (O3)	+	+	+	+	+	+	+	+	+
	(PA)	+	+	+	+	+	+	+	+	+
E	<u>P.aeruginosa</u> (PA)	+	-	-	+	+	-	+	-	-
	(PA)	+	-	-	+	-	-	+	-	-
F	<u>P.aeruginosa</u> (uc)	ND	+	+	ND	+	+	ND	+	+
	yeast	ND	-	-	ND	-	-	ND	-	-
G	<u>P.aeruginosa</u> (PA/NT)	ND	+	+	ND	+	+	ND	+	+
	O6	ND	+	+	ND	+	+	ND	+	+
H	<u>P.aeruginosa</u> (PA)	+	+	+	+	+	+	+	+	+
	yeast	-	-	-	-	-	-	-	-	-
	b ₇	+	-	-	+	+	+	+	+	+
I	<u>P.aeruginosa</u> (uc)	+	+	-	+	+	-	+	+	-
	b ₇	+	+	-	+	-	-	+	-	-
a _J	7	+	-	-	+	-	-	+	-	-
	7/1	+	+	-	+	+	-	+	+	-

a) no organisms in sputum

b) laboratory strain 7 included for all patients only recorded if positive

PA = agglutinated by two or more O-typing sera and/or PA antiserum (anti-7/1),
 PA/NT = agglutinated by both PA and NT antisera, uc = unclassified,
 ND = not done

Each of the ten patients were inpatients at the time of the sputum sample and their lung function was recorded at that time. The antibody-antigen reactions developed by this method were not quantitative but the lung functions of patients C and E, who had the lowest titre of antibody in terms of colour intensity, were the lowest of the patient group. Patient E was undergoing pleuradesis and died shortly after this sample was taken, while patient C had a FVC of 1.1L and FEV₁ of 0.8L which was the poorest recorded for the ten patients.

Comparison of antibody activity in sputum and serum

The previous experiment had shown the presence of antibody in the sputum which could be detected by a modified ELISA technique. An attempt was made to quantify the amount of antibody by standard ELISA techniques. Whole cell preparations of the P.aeruginosa from the sputum sample were coated on to ELISA plates and the level of antibody in the sputum sol and serum were compared (Table 69).

Serum titres were consistently higher than those of the sol phase. Sputum titres were higher towards the two experimental strains than towards the patients' isolates. The lowest titres of antibody in sol samples were, as previously, given by the two patients who had the lowest values for lung function (patients K and O). These results were completely reproducible in both 'dot-blots' and immunoblots which although qualitative, gave an indication of the degree of antibody activity.

The few experiments performed with sol phase showed that it may be a more reliable indicator of the relative immunogenicity of different phenotypes of P.aeruginosa than serum antibody levels. Also a lack

TABLE 69

ELISA titres of sol phase and serum towards *Pseudomonas aeruginosa*
isolated from the sputum sample of CF patients

Patient	Isolate	Agglutination Category	Titre	
			Sol	Serum
K	1a	(PA)	10	160
	muroid 1b	(u/c)	10	320
	7		160	ND
	7/1		0	ND
L	muroid 2a	(u/c)	10	1280
	2b	(PA)	40	1280
	2c	(PA/NT)	160	1280
	7		160	ND
	7/1		160	ND
M	<u>S.aureus</u>		10	40
	7		160	ND
	7/1		160	ND
N	muroid 4a	u/c	20	ND
	4b	PA/NT	20	ND
	7		160	ND
	7/1		160	ND
O	muroid 5a	u/c	20	ND
	5b	NT	10	ND
	5c	PA	20	ND
	7		40	ND
	7/1		80	ND

u/c = unclassified

PA = agglutinated by two or more O-typing sera and/or the PA antiserum (anti-7/1)

NT = agglutinated by NT antiserum (anti-18S)

PA/NT = agglutinated by both PA and NT antisera

ND = not done

of specific antibody to the patients' own isolates accompanied a worsening of clinical condition.

Detection of antibodies to MEP in CF serum

The production of mucoid exopolysaccharide by strains of P.aeruginosa from CF is well documented. The clinical significance of this production is not clear but recent reports suggest that it may be immunogenic in CF patients.

The study of Bryan, Kureishi and Rabin (1983) measured antibody in CF sera to MEP from P.aeruginosa and commercial alginate in an ELISA system. They found antibody in serum from control non-CF patients and CF patients which reacted with both MEP and commercial alginate. Sera from 29 CF patients (13 not colonised and 16 colonised with P.aeruginosa) were tested by ELISA with MEP extracted from a mucoid strain 217M and commercially prepared alginate. Whole cell extracts of strain 217M and the laboratory produced O-defective variant 7/1 were also included. The serum titres are shown in Table 70. Only one patient (number xxii) failed to produce antibody to strain 217M, but antibody to purified MEP in low titre was found in only five of the patients. Generally titres to the two strains of P.aeruginosa were higher in the colonised group but this may have been due to non-specific antibody as many patients who did not harbour pseudomonas had high titres to these strains.

The lack of response towards purified MEP in the presence of high serum titres to whole cell extracts emphasised the highly purified nature of the mucoid substance and that the reaction was not due to other cellular constituents. Only one patient had a serum titre towards commercial alginate in the absence of antibody to MEP, while

TABLE 70

Serum titres of CF patients towards purified MEP of *Pseudomonas aeruginosa* and commercial alginate
by ELISA

Patients non colonised	ELISA titre				Patients with <i>P.aeruginosa</i>	ELISA titre			
	MEP	Commercial alginate	217M*	7/1		MEP	Commercial alginate	217M*	7/1
i	0	0	400	800	v	0	0	800	800
ii	0	0	1 200	400	vi	0	0	400	1 600
iii	0	0	200	400	vii	0	0	6 400	6 400
iv	0	0	1 600	100	viii	0	0	400	800
ix	0	200	6 400	6 400	xii	10	0	1 600	3 200
x	0	0	400	800	xiii	0	0	6 400	6 400
xi	0	0	1 600	1 300	xiv	0	0	6 400	6 400
xvi	0	0	400	400	xv	0	0	6 400	1 600
xvii	0	0	400	400	xviii	0	0	40	80
xix	200	0	800	1 200	xx	0	0	100	400
xxvii	0	0	400	400	xxi	100	0	6 400	6 400
xxviii	0	0	800	800	xxii	0	0	0	0
xxix	0	0	400	400	xxiii	0	0	800	6 400
					xxiv	0	0	6 400	6 400
					xxv	200	100	6 400	6 400
					xxvi	0	0	800	800

* mucoid strain MEP prepared from

four of the five MEP positive patients had no antibody towards the commercially prepared substance. One patient with very high serum titres to P.aeruginosa recognised both the MEP and the seaweed alginate.

From these results it appeared that the sera of CF patients did not contain antibody towards the purified MEP of strain 217M although other surface components of this strain were recognised. The MEP of the patients' mucoid isolates may have been immunogenic, in vivo, but as no antibody to MEP was detected in this experiment the response to autologous polysaccharide might be specific and its link with colonisation remains unclear.

Production of hyperimmune serum to MEP

An attempt was made to raise antiserum to MEP in mice as described in Materials and Methods. MEP gave a stringy precipitate in saline and no reaction with antibody was seen in double diffusion tests or ELISA. Therefore no further attempts to raise hyperimmune serum to MEP were made.

DISCUSSION

SECTION III

It was not possible to obtain sequential serum samples from patients and indeed very few specimens were available. This was due in the main to the reluctance of the outpatient staff to take blood samples on a visit unless there was a specific clinical reason for the sample to be taken. Unfortunately as a result sequential sera corresponding to the sputum samples were not available for any of the patients.

CF patients have relatively normal levels of circulating immunoglobulins but recently Fick et al. (1986) showed an increase in the IgG subclasses 2 and 3 in the serum of CF patients with chronic P.aeruginosa infection. This was associated with a concomittant decrease in serum IgG1 and a similar pattern was observed for sputum. In most infections caused by P.aeruginosa the appearance and subsequent increase of type-specific antibody and antibodies to other cellular constituents can be demonstrated. A raised antibody titre is diagnostic of infection (Pitt et al. 1985) and the immunoglobulins specific for LPS are protective (Pollack and Young 1979). In CF patients also, circulating antibodies in high titre are demonstrable to a number of products of P.aeruginosa.

Both ELISA and radioimmunoassay have been used to measure the titres of serum antibody to the extracellular products of P.aeruginosa. Antibody towards exotoxin A was described by Klinger et al. (1978) and the titre was inversely correlated with the clinical scores of the CF patients. Later Jagger et al. (1982) showed that antibody levels for elastase, alkaline protease and exotoxin A were higher in those patients persistently colonised with the organism. Döring and Høiby (1983) studied the levels of serum antibody towards protease

antigens over a ten year period and demonstrated continuously increasing titres with time.

These data suggest that exotoxin A and proteases are produced, in vivo, during pulmonary infection and those patients with the most severe complications consistently have high serum antibody titres to these antigens, with the exception of elastase. This may indicate the relative importance of individual extracellular products to the establishment of colonisation with P.aeruginosa in the lung.

The production of antibody specific for mucoid exopolysaccharide has also been shown to be associated with long term colonisation with P.aeruginosa (Pier and Elcock 1984) but appears to be non protective. Moreover Moss et al. (1986) showed that the presence of high serum titres to P.aeruginosa especially towards LPS and exotoxin A was associated with the formation of immune complexes and subsequent poor prognosis. Antibody produced towards LPS of a specific serotype has also been shown to inhibit phagocytosis of P.aeruginosa, in vitro, (Shyrock et al. 1985) but it was type specific.

It seems therefore that antibodies to a variety of products of P.aeruginosa do not protect against subsequent colonisation but nevertheless increase in titre as infection progresses. Høiby (1977) proposed that the level of serum precipitins, which had earlier been shown by Burns and May (1968) to be formed in chronic lung infection could be used as an indicator of severity of infection.

In this study I have reported on the high incidence of O-defective forms of P.aeruginosa in CF patients and speculated that these forms may not be recognised efficiently by antibody. A natural

progression was to examine the relative immunogenicity of strains with complete O-antigens compared with those with defective O-antigens in an experimental animal and in CF patients colonised with these strains.

In rabbits the agglutinin response to both forms of antigen was disappointing and the titres obtained were lower than would be normally expected from the routine production of hyperimmune sera. However, immunoblotting with purified LPS showed that there was no antibody response to LPS core components even in rabbits primed with an O-defective strain. On the other hand, antibody to complete LPS was evident in all of the sera regardless of the vaccine strain. This may be interpreted as suggesting that although no wild-type regions of LPS were demonstrable in the O-defective strains, enough high molecular weight material was present to induce an antibody response. It was not confirmed that these antibodies were type specific but a similar finding was reported by Sadoff et al. (1985). These workers produced monoclonal antibodies against 14 of the IATS P.aeruginosa serotypes and found by immunoblotting that the major reaction was given by high molecular weight components. The low molecular weight core region was unreactive with antibody. This was consistent with an antibody directed towards O-side chains rather than core LPS determinants.

Immunoblotting of patient's serum and strains of P.aeruginosa revealed that the low molecular weight core components of P.aeruginosa were poorly recognised by antibody in the serum. The immunodominant region of the LPS was the O-specific side-chains and the core region was only seroreactive in those patients who had

harboured LPS-defective variants for long periods of time. This suggested that other epitopes on the cell surface may be more accessible and induce an antibody response more readily than core components.

In this series of patients antibody to type-specific high molecular weight LPS components was present in the majority of sera tested. By immunoblotting it was possible to show that patients who were persistently colonised with P.aeruginosa had antibody towards O-side chains and some core components of the LPS while those recently colonised recognised only the higher molecular weight epitopes.

The poor immunogenicity of core components was also reported for Salmonella minnesota. Animals primed with core mutants of S.minnesota or E.coli did not give rise to high antibody titres (McCabe 1972) but more recent experiments with passive protection tests revealed that serum from animals immunised with LPS mutants neutralised the toxic effects of endotoxin (Johns, Skehill and McCabe 1983). Some of this effect was due to the formation of antibody towards the active part of the endotoxin i.e. lipid A.

In retrospect it might have been of interest to measure antibody to lipid A in the experimental animals and CF patients. In the latter they are colonised by large numbers of core mutants but do not suffer from the classic toxic effects of circulating endotoxin seen in septicaemia such as shock, rigour and hypotension. It may be that antibody is not formed to the core oligosaccharide in the short term but there is a response to the lipid A components, which although does not reduce colonisation protects against the toxicity of the endotoxin. This may well be worthy of further investigation in CF.

LPS mutants of salmonella have been shown to have altered rates of uptake by murine macrophages. The rate of uptake is inversely proportional to the virulence of the strain and the O-antigenic side chains are thought to promote virulence by preventing complement components binding to the cell membrane (Liang-Takasaki et al. 1983; Grossman and Leive 1984). An O-side chain deficient mutant of P.aeruginosa was opsonised and phagocytosed at a faster rate than the parent strain which had complete polysaccharide sidechains (Engels et al. 1985). Therefore, in the presence of complement LPS-defective strains should be eliminated efficiently. There are no reports known to the author that document the levels of complement in bronchial secretions in CF. If sufficient complement is available in CF sputum then opsonisation of O-defective P.aeruginosa by alveolar macrophages should occur and perhaps their continued presence suggests a lack of local complement production. Furthermore the increased protease content of bacterial or macrophage origin may diminish the levels of this protein.

Most studies of complement activity in CF have been confined to serum. Both classical and alternative complement pathways are functionally normal and levels of each component are either normal or elevated (Speert 1985). Activation of complement in CF serum by both P.aeruginosa and S.aureus is normal (Buescher and Winkelstein 1978) therefore it seems unlikely that persistent infection is due to an abnormality in serum complement composition or function.

Antibodies to type specific antigens of P.aeruginosa are protective and have good opsonic properties but the levels of these antibodies have not been measured in bronchial secretions. Certainly in

systemic infection a rise in antibody to LPS occurs rapidly (Pollack and Young 1979) and opsonins play a vital role in the phagocytic process. The phagocytic capacity of CF pulmonary leucocytes appears to be normal (Speert 1985).

Efficient phagocytosis of many bacterial species including some strains of P.aeruginosa requires serum opsonins. An opsonic defect has been observed in CF serum (Biggar, Holmes and Good 1971; Fick et al. 1981) and has been shown to be due to a decreased affinity of CF IgG for pulmonary macrophages. There appears to be impairment of the attachment of specific anti-pseudomonas antibody to the macrophage complement (Fc) receptor (Fick et al. 1981). There may also be an alteration in the proportions of the IgG subclasses in the bronchial secretions with an increase in IgG2 which despite its affinity for the LPS of P.aeruginosa binds poorly to macrophages (Fick et al. 1986).

The bactericidal activity of CF serum for autologous strains of P.aeruginosa was shown by Thomassen and Demko (1981) to be reduced due to the presence of a blocking factor. Patients whose serum contained this blocking factor were poorer clinically than those in whom it was not present. Penketh et al. (1983^b) also found this factor in the serum of six of 61 CF patients but were unable to show a correlation between clinical status and the presence of the factor. Schiller and Millard (1983) found evidence of a substance, possibly an antibody, in CF sputum which interfered with the bactericidal activity of CF serum for both autologous and heterologous strains of P.aeruginosa. This has not been confirmed by other workers.

The level of non-specific antibody in CF serum has been shown to

increase with the onset of infection (Döring and Høiby 1983; Hancock, Mouat and Speert 1984) and high titres correlate with a poor prognosis (Wood et al. 1976; Wallwork et al. 1974; Jagger et al. 1982). However I was unable to identify the infected state by serological tests. Antibody titres measured by ELISA were high in most cases and although these were greatest when colonisation had been persistent for many years there was not a significantly increased mortality rate in these patients when compared with those recently infected. Döring and Høiby (1983) also found that serum antibody titres to P.aeruginosa increased with time and suggested this indicated the continuous production of antigens by the organism. The high ELISA titres to whole bacterial cells were not directly comparable with the qualitative immunoblots of purified LPS which suggested that much of the antibody in CF serum was directed towards outermembrane proteins. ELISA titres were therefore not indicative of anti-LPS antibody alone. The outermembrane proteins of LPS defective strains are highly conserved and CF serum contained high titres of cross-reacting antibody directed towards these proteins. Studies of the antigenic properties of the outermembrane proteins of P.aeruginosa in CF have shown that high antibody titres occur in the sera of chronically infected patients (Lam et al. 1983; Hancock et al. 1984). By immunoblotting, Hancock et al. (1984) identified the surface exposed protein F as an antigen common to strains of P.aeruginosa and this reacted with antibodies in CF sera. They also found high titres to protein H2 and core LPS but concluded that antibodies to these structures were not protective. It is worthy of consideration that the LPS defects described here

resulted in the exposure of conserved outer membrane proteins which were not reactive in whole cell ELISA tests. Indeed, experiments with monoclonal antibodies against some members of the enterobacteriaceae have demonstrated the ability of O-antigen to shield outer membrane proteins from detection even when reduced amounts of repeating side chains are present (Van der Ley et al. 1986). Therefore the stoichiometric configuration of defective LPS in antigenic variants may shield 'common' protein epitopes on the cell surface and hence make them less accessible to antibody.

The production of mucoid exopolysaccharide 'alginate' by P.aeruginosa is strongly associated with CF. Pier, Matthews and Eardley (1983) showed that purified alginate from P.aeruginosa was immunogenic in animals and that CF patients who harboured mucoid or nonmucoid P.aeruginosa had significantly elevated serum titres towards MEP.

The patients in this study had high levels of antibody towards mucoid isolates and even if nonmucoid and mucoid phenotypes were cultured from the same sample, the highest titre was directed towards the mucoid isolate. However, an ELISA with purified MEP and sera from 29 of the CF patients showed that only five had antibody towards MEP and only in low titre. In contrast high titres towards whole cells of the mucoid strain from which MEP had been prepared were given by most of the patients. Interestingly, two of the five patients had no history of colonisation with P.aeruginosa which contradicts Bryan et al. (1983) who found that only those persistently colonised had antibody to MEP. Furthermore I was unable to demonstrate any immunogenic activity of purified pseudomonas alginate in preliminary experiments with mice.

Woods and Bryan (1985) used a model of chronic pulmonary infection in rats with P.aeruginosa and found that bacterial clearance was associated with a rise in antibody titres to MEP. Immunisation with MEP did not protect animals from infection but antibody to MEP was associated with immune complex formation in the lung and other organs of the animals. The presence of circulating immune complexes has been well documented in CF (McFarlane et al. 1975; Pitcher-Wilmott et al. 1982; Wisniewski et al. 1985) and there is some evidence that deposition of complexes in the lung may mediate tissue damage.

The results of Woods and Bryan (1985) suggest a mechanism in which the production of MEP is associated with deterioration of lung function by the stimulation of antibody production. In contrast other groups imply that MEP acts passively, for example, in the inhibition of phagocytosis (Schwarzmann and Boring 1971; Ruhen et al. 1980).

The proteases produced by P.aeruginosa have been shown to cleave human immunoglobulins, especially IgA, in vitro (Döring et al. 1983). Bronchial secretions of CF patients contain fragments of IgG and IgA (Fick et al. 1984), despite the presence of antibody in high titre to pseudomonas proteases. Degradation of antibody by proteases may be a mechanism by which the organism initiates colonisation of the lung (Döring et al. 1985) but there is little evidence to confirm whether the proteolytic activity of sputum is derived from the organism or from lysis of granulocytes (Döring et al. 1985). Secretory IgA is probably defective in CF, as free J-chain fragments have been observed in CF sputum (McFarlane 1984). This was thought to be an abnormality in either the synthesis or assembly of the dimer SIgA but

presumably could be further evidence of the proteolytic activity of sputum.

The sol phase experiments performed here, although limited by the number of patients, gave useful information as to the integrity of the local immune response towards the organisms present in the sample. The immunoglobulins appeared to be structurally intact and in high titre in some patients. Those patients with the poorest lung function had the lowest titres of sputum antibody in contrast to the study of Schiøtz et al. (1979) who demonstrated high sputum levels of both IgA and IgG in chronically infected patients.

The antibody titre in serum was higher than the corresponding sputum titre which is in agreement with Schiøtz et al. (1979) and Wood, Pennington and Reynolds (1983). These workers suggested that sol phase antibodies were derived from serum exudate through the inflamed mucous membrane. As IgA was the major immunoglobulin found in the sol phase of the sputa examined here this supports the view that bronchial immunoglobulins are produced locally and are independent of the systemic response.

Many attempts have been made to produce a vaccine to prevent colonisation with P.aeruginosa in CF. Ideally, a pseudomonas vaccine for use in CF should have wide cross reactivity between serotypes and have little or no toxic activity due to endotoxin components. The hyperimmune antibody should be capable of minimising adherence of the organism and also have opsonic properties. Cross-reactive, vaccines based on proteins have been found to be non protective in animal studies (Sawada et al. 1984) but LPS vaccines stimulate the production of protective antibody (Pollack

and Young 1979).

The PEV vaccine developed by Burroughs Wellcome is composed in the main of LPS of the IATS serotype strains of P.aeruginosa. A prospective controlled trial of this vaccine was carried out by Langford and Hiller (1984) over three years. The vaccine failed to reduce the rate of colonisation with P.aeruginosa in CF patients. Indeed, patients who received the vaccine experienced more rapid clinical deterioration than those who remained uncolonised and more mucoid isolates of P.aeruginosa were obtained from the vaccinated children than the control group.

Pennington et al. (1975) also had little success in the trial of a heptavalent purified Pseudomonas vaccine in CF. Despite an increase in serum titres there was no improvement in clinical status and many toxic reactions were recorded. Later, Wood et al. (1983) used this vaccine (Pseudogen (R), Parke, Davis and Co.,) intranasally in patients colonised with P.aeruginosa. The vaccine was tolerated well but the low antibody titres in sputum before immunisation did not increase after vaccination, but six of the seven patients showed a slight increase in serum titres. There was no improvement in the clinical condition of patients and the organism persisted even when serum titres were raised.

The emergence of MEP producing strains of P.aeruginosa is so commonly associated with CF that the potential of MEP as a vaccine to prevent colonisation has been investigated (Baltimore 1985). However, as deposition of immune complexes containing antigens to MEP were found in the lungs of immunised animals (Woods and Bryan 1985) vaccines based on MEP may be detrimental to the patients' clinical condition.

Much of the data on pseudomonas vaccines suggest that a high systemic titre of antibody to P.aeruginosa does not benefit patients already colonised. Perhaps, careful monitoring of patients before they become colonised and the use of a vaccine containing high molecular weight LPS components to a few selected serotypes may be beneficial. In this study only three serotypes were common in CF and a multicomponent vaccine such as PEV may be therefore unnecessary.

In conclusion it was not possible to demonstrate evasion of host responses. Despite the alterations in antigenic profile of many of the strains the majority of patients had high antibody levels towards the strains but remained colonised for many years. There was some indication that the local antibody response to strains was poor with comparison to the systemic response which appeared to contain the infection and prevent extension into the bloodstream. There is a paucity of literature on the function of antibodies in sputum especially in the initial stages of infection. Further studies on sputum are therefore indicated given the ease of obtaining adequate specimens particularly in the young patient. Careful monitoring of local immune mechanisms may reveal ways of stimulating protective antibody in bronchial secretions which would be beneficial to the patient if it reduced colonisation with P.aeruginosa.

GENERAL DISCUSSION

Infection/colonisation of the lungs of CF patients with P.aeruginosa has received considerable attention in the last decade but management and treatment of these infections remains a major problem. The biological basis of the predilection and pathogenicity of P.aeruginosa for CF patients is unknown although numerous bacterial products have been identified and implicated in the pathological process. It is clear however that P.aeruginosa in CF is not a single entity but a heterogeneous bacterial population which exhibits many characteristics not found in strains from other infections. This degree of heterogeneity can only have arisen from mutations of several gene functions and apart from the mucoid locus described by Govan and co-workers, the genetic basis of other phenotypes such as serum sensitivity, LPS defects, B-lactam antibiotic hypersensitivity etc. is, as yet, unknown.

This study confirmed that alterations of the outer membrane of P.aeruginosa occurred during the course of colonisation and at least two major defects in synthesis of the immunodominant epitope, LPS, were found. There is little doubt that LPS defective phenotypes other than PA and NT are present in CF as evidenced by the variety of LPS profiles visualised by SDS-PAGE. Furthermore non LPS epitopes present on strains with unaltered outer membranes were expressed in variants upon the loss of LPS constituents. The clinical significance of these serologically variant forms was not revealed by this study but overall the results of the longitudinal survey indicated that patients who were in the terminal stages of infection harboured these strains.

The relative proportions and fluctuation of various serological

states of P.aeruginosa in CF warrant further investigation as some sub-populations of LPS defective strains may be of greater clinical significance than others. The identification of other classes of LPS defects of strains would facilitate such a study but the impression gained so far is that numerous and complex phenotypes of surface antigens will be revealed. The reported loss of virulence associated with LPS defects, serum sensitivity and hypersensitivity to antibiotics is difficult to reconcile with the continued proliferation of these variants in CF patients who, in general, have adequate antibody responses and receive appropriate antipseudomonal therapy. Whilst the serum sensitivity of strains serves as a reasonable explanation for the lack of systemic dissemination from the lungs, the survival of LPS defective and hypersensitive strains is paradoxical. It was not possible to demonstrate an evolutionary advantage, if one exists, of these altered forms, but as the frequency of the various phenotypes differed from sample to sample some form of external pressure may influence the survival of one form over another.

A possible scenario, which is almost entirely speculative, could be that LPS defective strains are phagocytosed in great numbers by macrophages following complement activation by the exposed lipid A component of LPS. These strains survive phagocytosis and cause lysis of the macrophage by the release of proteases and other toxic metabolites. The mechanism by which P.aeruginosa survives in the macrophages is unknown but there is increasing evidence that granulocyte derived proteases released on lysis of the cell contribute significantly to necrosis of lung tissue.

The hypothesis of antigenic variation in strains of P.aeruginosa leading to evasion of antibody response of the CF host was not proven. Certainly, LPS defective forms did not engender the levels of total antibody demonstrable towards typical forms but this may have simply been qualitative differences influenced by the test methods. The function of type specific antibody and anti-core LPS antibody needs further investigation before it can be concluded that the antibody response is adequate towards all forms of P.aeruginosa in CF.

An important ramification of this study was the application of methods used routinely for the identification of subtypes of the species and their failure for P.aeruginosa from CF patients. Serotyping and phage typing are traditionally reliable methods for elucidating the epidemiology of pseudomonas in hospital-acquired infection. As the majority of strains in CF do not express specific O-antigens and at least half of them are insensitive to phage, alternative methods of strain identification are required. Many of the proposals for segregation of patients currently advocated by the Danish CF workers are based on the evidence obtained by sero- and phage typing. If cross infection does occur it must be demonstrated unequivocally by methods of strain differentiation that are apparently not adversely affected by alterations in surface structure. Pyocin typing based on the production of pyocins rather than the sensitivity of strains to pyocins is therefore to be recommended as a primary system for the identification of strains. New methods could then be designed to discriminate further between strains producing the same pyocins.

The detection of antibody in bronchial secretions was encouraging and further work in this area can only be beneficial. Indeed, given the generally high level of serum antibody titres in this study and other published reports, the use of serum antibody determinations to distinguish colonised from infected patients may rightly be questioned. In comparison, the sol phase of sputum provides an easily obtainable specimen, the antibody levels of which appear to be highly relevant to the condition.

The variety of P.aeruginosa isolated from CF patients makes it highly desirable and even necessary that laboratories carry out a more detailed analysis of isolates from routine specimens. Many of the phenotypes can be easily and rapidly identified and although reporting of them may not be of immediate importance to the clinician, it will enable the bacteriological history of patients to be more accurately documented. If, as we suspect, that patients who harbour predominantly atypical forms are most like to suffer exacerbations, then documentation of the bacteriology of individual patients will be justified. What is clear however is that a simple laboratory description of mucoid or nonmucoid pseudomonas from CF is insufficient. Similarly the estimation of antibody titres towards undefined phenotypes, as is currently being advocated, can provide little new information, as the response of the patient is influenced not only by his clinical condition but undoubtedly by the variety of strains he harbours.

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