THE SURFACE OF PSEUDOMONAS AERUGINOSA IN CYSTIC FIBROSIS LUNG INFECTION

DOREEN M. G. COCHRANE

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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by DOREEN MG COCHRANE

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SUMMARY

Chronic experimental lung infection in rats was induced by intratracheal inoculation of agar beads containing Pseudomonas aeruginosa. Bacteria were recovered directly without subculture from the lungs of rats at 14 days post-infection and the outer membrane (OM) antigens were studied. The results indicated that bacteria grew under iron-restricted conditions as revealed by the expression of several iron-regulated membrane proteins (IRMPs) which could also be observed when the isolate was grown under iron-depleted conditions in laboratory media. The antibody response to P. aeruginosa OM protein antigens was investigated by immunoblotting with serum and lung fluid from infected rats. These fluids contained antibodies to all the major OM proteins, including the IRMPs, and protein H1. Results obtained using immunoblotting and enzyme-linked immunosorbent assay indicated that lipopolysaccharide (LPS) was the major antigen recognised by antibodies in sera from infected rats.

The animal model was used to follow the development of the immune response to P. aeruginosa protein and LPS antigens. Immunoblotting was used to investigate the antigens recognised by antibodies in sequential serum samples. An antibody response to the IRMPs and OM proteins D, E, G and H1 and also to rough LPS was detected as early as 4 days post-infection. Results obtained using immunoblotting and crossed immunoelectrophoresis techniques indicated that there was a progressive increase in the number of P. aeruginosa antigens recognised by antibodies in these sera.

Both iron and magnesium depletion influenced protein H1 production. Antibodies in sera from patients with infections due to P. aeruginosa reacted with this antigen.

Results obtained using quantitative gas-liquid chromatographic analysis indicated that growth phase and magnesium and iron depletion also affected the amount of LPS fatty acids, produced by P. aeruginosa. The silver stained SDS-polyacrylamide gels of proteinase K digested whole cell lysates of P. aeruginosa indicated that the 0-antigen and core LPS were both affected by growth phase and specific nutrient depletion.

Key words: Pseudomonas aeruginosa, lung infection, iron, outer membrane proteins, lipopolysaccharide.

To my mother

and family

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ABBREVIATIONS

A	ampere
AMPS	ammonium persulphate
BDH	British Drug Houses
Bis	N, N' methylene bisacrylamide
c	centi
°C	degrees centigrade
CDM	chemically defined media
CIE	crossed immunoelectrophoresis
CIEWIG	crossed immunoelectrophroresis with an intermediate gel
CF	cystic fibrosis
c.f.u.	colony forming unit
CM	cytoplasmic membrane
CV	co-efficient of variation
EDTA	ethylene diamine tetraacetic-acid
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding fragment of immunoglobulin
Fc	crystallisable fragment of immunoglobulin
Fe- CDM	iron-depleted CDM
Fe- TSB	iron-depleted TSB
Fe+ CDM	iron-plentiful CDM
Fe+ TSB	iron-plentiful TSB
Fe- Mg- CDM	iron- and magnesium-depleted CDM
Fe- TSA	iron-depleted TSA
Fla	flagella
GLC	gas liquid chromatography
IATS	International Antigenic Typing Scheme
IRMP	iron-regulated membrane protein
K	kilodalton
kg	kilogram
L	litre
LPS	lipopolysaccharide

M	molar concentration
m	metre
ml	millimetre
µ	micro
min	minute
Mg- CDM	magnesium-depleted CDM
MOPS	morpholinopropane sulphonic acid
NA	nutrient agar
NC	nitrocellulose
NCTC	National Collection of Type Cultures
OD	optical density
OD470nm	optical density at 470nm
OM	outer membrane
PIA	Pseudomonas Isolation Agar
PBS	phosphate buffered saline
PG	peptidoglycan
PMN	polymorphonuclear leucocyte
Rf	retardation factor
Sarkosyl	N-lauryl sodium sarcosine
SDS	sodium dodecyl sulphate
s.e.m.	standard error of the mean
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N tetramethylethylene diamine
TBS	tris buffered saline
TSA	tryptone soy agar
TSB	tryptone soy broth
V	volts
v/v	volume per volume
w/v	weight per volume

1. INTRODUCTION

1.1 COMPOSITION AND STRUCTURE OF THE GRAM-NEGATIVE CELL ENVELOPE

The cell envelope of Gram-negative bacteria is a complex structure that confers shape and rigidity on the cell. This structure regulates the organisms interaction with its environment. The composition of the cell envelope is determined by the environment in which the cell is growing (Brown and Williams 1985a; Brown, 1977, 1975; Ellwood and Tempest, 1972; Holme, 1972). Since the environment is constantly changing so in turn does the cell envelope; this ability to adapt to its surroundings confers major survival advantages on the cell (Brown and Williams, 1985a; Lugtenberg and van Alphen, 1983). This flexibility must be borne in mind when considering published static "structure" of the envelope. The envelope serves to mediate the uptake of nutrients and to protect the cytoplasm from harmful agents. It is now realised that the cell envelope is important in other physiological functions of the cell including resistance to host defence factors such a lysozyme and various leucocyte proteins (Nikaido and Vaara, 1985) and also to detergent action of bile salts and digestive enzymes in the gut for example (Nikaido and Nakae, 1979). The envelope also acts as a strong permeability barrier to many antibiotics. Whether an antibiotic acts at a site within the envelope, or traverses the envelope to reach a target within the cell, changes in the cell envelope will alter the sensitivity of the bacterium to antimicrobial agents (Brown and Williams, 1985b; Brown, 1977; 1975). The pathogencity of bacteria also depends to a large extent on the characteristics of the cell surface (Brown and Williams 1985a; Smith, 1977).

The structure of the Gram-negative envelope and its role in infection has been extensively reviewed (Hammond *et al*, 1984; Beveridge, 1981; Nikaido and Nakae, 1979; Brown 1977; Costerton *et al*, 1974). It may be divided structurally into three layers which support different functions; an outer membrane (OM), a peptidogylcan (PG) layer and an inner cytoplasmic membrane (CM). Many Gram-negative bacteria

also have regularly arranged protein sub-units (S-layers) (Sleytr, 1978) or organised exopolysaccharide layers (Sutherland, 1977) at their external surfaces.

The PG layer confers shape and rigidity on the cell and enables the cell to withstand osmotic pressure. Peptidoglycan consists of a backbone of N-acetylmuramic acid and N-acetylglucosamine cross-linked by peptide chains (Hammond *et al*, 1984). Hobot *et al* (1984) proposed that PG is more highly cross-linked in the part underlying the OM and less so towards the CM and that the PG molecule is highly hydrated so that it forms a gel filling the space between the outer and cytoplasmic membranes. Periplasmic proteins would be freely diffusible within this gel. These periplasmic proteins may be divided into three classes: those with a catabolic function which convert nutrients into a form which can be transported, binding proteins with an affinity for certain nutrients, and degradable enzymes that inactivate harmful substances including antibiotics (Hammond *et al*, 1984; Costerton *et al*, 1974). The periplasmic gel also contains oligosaccharides involved in the regulation of osmolarity (Lugtenberg and van Alphen, 1983).

The CM is a lipid bilayer composed of phospholipids and containing embedded proteins. This membrane contains the enzyme systems of the electron transport chain and oxidative phosphorylation, systems for active transport of solutes and excretion of waste products as well as the synthetic apparatus necessary for the production of exterior layers (Hammond *et al*, 1984; Inouye, 1979). It is joined to the OM at distinct zones of adhesion which are though to be sites for the export of OM components (Bayer, 1979; Smit and Nikaido, 1978).

The OM lies outside the cytoplasmic membrane to which it is convalently attached via lipoprotein (Lugtenberg and van Alphen, 1983; Braun, 1975) (Fig. 1.1.)

1.1.1 Composition of the outer membrane

The outer membrane is a highly specialised bilayer made up of phospholipids, proteins and lipopolysaccharide (LPS) (Lugtenberg and van Alphen, 1983; Nikaido and Nakae, 1979; Inouye, 1979). The major function of the OM membrane as a

FIGURE 1.1

SCHEMATIC DIAGRAM OF THE CELL ENVELOPE OF GRAM-NEGATIVE BACTERIA.

KEY: CM, CYTOPLASMIC MEMBRANE; OM, OUTER MEMBRANE; PG, PEPTIDOGLYCAN; LPS, LIPOPOLYSACCHARIDE; CPS, CAPSULAR POLYSACCHARIDE; PL, PHOSPHOLIPID; POR, PORIN PROTEINS; P, PROTEINS; LP, BRAUN LIPOPROTEIN ; PAL, PEPTIDOGLYCAN-ASSOCIATED LIPOPROTEIN; A, OmpA PROTEIN; PPS PERIPLASMIC SPACE (PERIPLASM); MDO, MEMBRANE-DERIVED OLIGOSACCHARIDES

(Provided by Dr P Lambert)

barrier to antibiotics, detergents and other toxic chemicals is necessarily accompanied by permeability properties which allow entry of nutrients from the environment. The OM constituents serve as receptors for a number of bacteriophages and colicins and facilitate cell-cell interactions during conjugation (Nakae, 1986; Osborn and Wu, 1980). Components of the cell envelope and especially the OM mediate the host-microbe interaction in infection (Brown and Williams, 1985a; Smith, 1977). The individual components of the Gram-negative OM will therefore be considered. A general outline will be presented and where evidence exists details of the P. aeruginosa OM will be included. The phenotypic variability of membrane components will be emphasised.

1.1.1.1 Phospholipids

Phospholipids are a major component of the OM and account for more than 90% of the lipid containing structures in the Enterobacteriaceae (Cronan, 1979). The ratio of saturated: unsaturated fatty acids in these lipids is responsible for maintaining the fluidity of the membrane (Cronan and Gelmann, 1975). Major phospholipids which have been reported in Gram-negative bacteria are phosphatidylethanolamine (PE) phosphatidylglycerol (PG) and phophatidyldiglycerol (DPG). The phospholipids reported to be present in the cell wall of P. aeruginosa NCTC 6750 are DPG, PE and phosphatidylcholine with lesser amounts of lysylphosphatidylglycerol, and PG and phosphatidylserine. The relative proportions of these phospholipids was affected by the limiting nutrients and by the presence of other cations (Kenward *et al*, 1979). The phospholipid composition of the cell wall of Pseudomonas spp. also showed considerable variation with growth rate, nutrient limitation and temperature (Kropinski *et al*, 1987; Gilbert and Brown, 1978; Minnikin *et al*, 1974). For magnesium-depleted cells of P. aeruginosa from chemostat cultures the levels of diphosphatidylglycerol declined with increasing growth rate. This decline was accompanied by an increase in the level of fatty acids

and neutral lipids (Gilbert and Brown, 1978).

1.1.1.2 LPS

LPS is located exclusively in the outer leaflet of the OM of the Gram-negative bacteria (Mühlradt and Golecki, 1975). It is an amphipathic molecule consisting of three regions each with a distinct composition and biological function. Integrated in the OM bilayer is the hydrophobic lipid A region, consisting of fatty acids linked to glucosamine. Extending outwards from the lipid A is the core region consisting of a short carbohydrate chain and from the core, in smooth strains, a longer carbohydrate polymer, the O-side chain (Hammond *et al*, 1984; Wesphal *et al*, 1983; Wicken and Knox, 1980). LPS can act as a receptor for bacteriophage (Braun and Hantke, 1981) and plays an important role in cell wall integrity and function (Henning, 1975). It also plays an important role in the pathogencity of Gram-negative bacteria. The LPS 0-side chains represent one of the main surface antigens of Gram-negative bacteria and are referred to as the 0-antigen (Luderitz *et al*, 1966; Morgan and Partridge, 1941). Injection of LPS-containing bacteria or of purified LPS into experimental animals causes a wide spectrum of so-called endotoxic reactions. These reactions are generally non-specific and acute, and include such phenomena as fever, changes in white cell counts, shock and death after high doses (Schlessinger, 1977; Kass and Wolff, 1973). The lipid A portion is responsible for many of the endotoxic properties of LPS (Wicken and Knox, 1980). The pathophysiological effects of P. aeruginosa LPS are listed in Table 1.1.

Lipid A regions of LPS from different species of Gram-negative bacteria share a general structure based on a phosphorylated β1-6 glucosamine disaccharide to which fatty acids are attached by ester and amide bonds (Westphal *et al*, 1983). Unlike phospholipids which have only two fatty acids connected to the backbone structure, LPS molecules have 6 or 7 fatty acids linked to the glucosamine disaccharide backbone (Nikaido and Vaara, 1985). Lipid A is unique among bacterial fatty acids in containing hydroxy fatty acids (Hammond *et al*, 1984). The absence

TABLE 1.1

PATHOPHYSIOLOGICAL EFFECTS OF P. AERUGINOSA LIPOPOLYSACCHARIDE

From Kropinski *et al* (1985)

from lipid A of 3-hydroxytetradecanoic acid (3OH-$C_{14:0}$) and the presence of 2-hydroxydodecanoic acid (2OH-$C_{12:0}$), 3-hydroxydecanoic acid (3OH-$C_{10:0}$) and 3-hydroxydodecanoic acid (3OH-$C_{12:0}$) are characteristic of P. aeruginosa (Wilkinson and Galbraith, 1975). Small amounts of straight chain fatty acids also occur in lipid A from P. aeruginosa and other enteric bacteria; unsaturated fatty acids are absent from lipid A (Nikaido and Vaara, 1985; Hammond *et al*, 1984). LPS from P. aeruginosa appears to be a less potent endotoxin than the LPS from other Gram-negative bacteria (Sadoff, 1974) and this may be due to the absence of 3OH-$C_{14:0}$. The lipid A component appears to assume a highly ordered conformation on the cell surface, with the fatty acid chains being tightly packed in a dense hexagonal lattice and the diglucosamine backbone forms domains in a parallel arrangement in the membrane. The high state of order of lipid A may be an important factor in the structural role and permeation barrier functions of LPS in the OM (Labischinski *et al*, 1985).

The core is a complex oligosaccharide consisting of an inner and an outer region. The inner core region contains heptose and the unique sugar 3-deoxy-D-manno-2-octulosonate (KDO). KDO links the core polysaccharide to lipid A via an acid labile bond (Wicken and Knox, 1980). The outer core region of P. aeruginosa differs significantly from that of the other enteric species. It contains glucose, rhamnose, heptose, galactosamine and possibly alanine, KDO and high concentrations of phosphate (Kropinski *et al*, 1985; Wilkinson, 1983). The heptose constituent of P. aeruginosa contains equal amounts of L-glycero-D-manno-heptose and D-glycero-D-manno-heptose (Hammond *et al*, 1984). The core region of P. aeruginosa strain PA01 has been estimated to contain 10 phosphate groups (Kropinski *et al*, 1985).

The 0-side chain consists of repeating oligosaccharide sub-units. The number of repeating units can show considerable variation even in a culture of one strain (Hitchcock and Brown, 1983; Jann *et al*, 1975) conferring great variability on

the molecular make up of the bacterial cell surface (Palva and Mäkelä, 1980; Goldman and Leive, 1980; Chester and Meadow, 1975). LPS isolated from a smooth strain of P. aeruginosa PA0 1 and resolved by SDS-PAGE revealed >50 species of repeating units (Kropinski *et al*, 1985). The side chains of P. aeruginosa contain unbranched tri- or tetra-saccharides and are rich in rhamnose and the n-acetylated aminosugars including hexosamine, fucosamine and quinovosamine (Kropinski *et al*, 1985; Wilkinson, 1983). The chemical structure of the 0-antigen subunit is extremely variable and it is this property which forms the basis of immunological typing of of P. aeruginosa strains (0-serotyping) (Bergan, 1975). Studies carried out mainly be Dmitriev and co-workers, have elucidated the chemical composition of a number of different serotypes of P. aeruginosa (see Kropinski *et al*, 1985 for refs). Some important differences were found: Homma serotypes 0:1, 0:2, 0:3 and 0:6 were found to contain uronic acids which give the side chain an electrophoretic charge which is important in electrophoretic separation, some serotypes were also found to lack cis-vicinyl hydroxyls in the side chains which may be important in their chemical reactivity (Kropinski *et al*, 1985).

The smooth to rough morphology mutation coincides with the disappearance of 0-antigen leaving a rough LPS consisting only of core oligosaccharide bound to lipid A (Westphal *et al*, 1983). Rough mutants of P. aeruginosa have been isolated (Kropinski *et al*, 1979; Koval and Meadow, 1975).

P. aeruginosa core LPS is more highly phosphorylated than that of other Gram-negative bacteria (Wilkinson and Galbraith, 1975). Phosphate groups in the KDO-lipid A region of LPS have a high affinity for divalent cations (Mg^{2+} and Ca^{2+}). The LPS of P. aeruginosa is also unusual in that the overall capping frequency (ie the percentage core LPS components covered with 0-antigen) is estimated to be much lower than that of Salmonella typhimurium or Escherichia coli (Kropinski *et al*, 1985) so that a greater percentage of the core determinants are exposed at the cell surface. Figure 1.2 shows a diagrammatic representation of P. aeruginosa LPS based on an IATS serotype 5 (Homma type 2) strain.

FIGURE 1.2

PROPOSED STRUCTURE OF P. AERUGINOSA LPS BASED ON A SEROTYPE 5 STRAIN (ADAPTED FROM KROPINSKI *ET AL* 1985)

Major fatty acid composition of P. aeruginosa LPS. The hydroxy fatty acids may be amide- or ester-linked to glucosamine. The non-hydroxy fatty acids may be only ester-linked to the 3-OH fatty acids

	Fatty acid	Systematic name
hydroxy fatty acids	$2OH\text{-}C_{12:0}$	2-hydroxydodecanoate
	$3OH\text{-}C_{10:0}$	3-hydroxydecanoate
	$3OH\text{-}C_{12:0}$	3-hydroxydodecanoate
Non-hydroxy fatty acids	$C_{12:0}$	lauric acid
	$C_{16:0}$	palmitic acid

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Adapted from Kropinski et al (1985)

-3)L-Rha(α 1-4)L-GalNAcA(α 1-3)D-QuiNAc(α 1-)

There has been very few studies which have considered the phenotypic variability of P. aeruginosa LPS. Gilbert and Brown (1978) reported reductions in the KDO content of P. aeruginosa LPS with increasing growth rate, also magnesium-limited cultures contained more KDO than glucose-limited cells from chemostat cultures. Day and Marceau-Day (1982) also investigated the effect of growth rate and magnesium-depletion on the KDO and heptose content of P. aeruginosa. Their results indicated that these components varied independently of one another and with growth rate and nutrient conditions.

1.1.1.4 Outer membrane proteins

The OM is poor in enzymic activity. The enzymes which do occur are involved in repair and modification of OM components (Lugtenberg and van Alphen, 1983). Identification of protein components has depended upon techniques of separation using gel electrophoresis (Schnaitman, 1974). A comparison of the CM and OM fractions reveals relatively few heavily staining bands in the OM of Gram-negative bacteria (Lugtenberg *et al*, 1975). The OM of E. coli which has been well characterised contain three major classes of protein: the pore proteins (porins), OmpA protein and lipoprotein. These proteins facilitate the transport and diffusion of nutrients and any other small hydrophilic molecules across the OM from the external environment, stabilise the structure and anchor the OM to the PG layer (Lugtenberg and van Alphen, 1983; Nikaido and Nakae, 1979). The OM also contains some other "minor" proteins. Many of these minor proteins are inducible or derepressible proteins and under appropriate environmental conditions reach levels in the OM comparable with those of the major proteins (Lugtenberg *et al*, 1983; DiRienzo *et al*, 1978).

For the OM to act as an efficient barrier, the interstices between the protein molecules and LPS lipids should remain tightly sealed. There is now evidence for a strong interaction between outer membrane proteins and lipids in E.coli and

Salmonella spp. (Nikaido and Vaara, 1985).

Porin proteins produce relatively non-specific pores or channels that allow the passage of relatively small hydrophilic molecules across the OM (Nakae, 1986; Nikaido and Vaara, 1985; Nikaido and Nakae, 1979). They are characterised by their tight but non-covalent association with the PG layer so that they remain attached to the PG when cells are extracted with SDS at non-denaturing temperatures (Osborn and Wu, 1980). Porins must span the OM since they also serve as receptors for bacteriophages and bacteriocins (Lugtenberg and van Alphen, 1983; Osborn and Wu, 1980). Diffusion of a molecule through these aqueous channels not only depends on its size and hydrophilicity, but also its charge, most pores being cation selective. A second class of pore-forming proteins with more specific functions exists; the Lam B protein of E. coli, which is involved in uptake of oligosaccharides of the maltose series, is an example (Osborn and Wu, 1980).

Lipoprotein is the most abundant protein in the OM of many Gram-negative species. Lipoprotein is an unusual polypeptide that is lipid substituted at the N-terminus. About one third of the copies of this protein are convalently bound to the PG layer through the $-NH_2$ group at its C-terminal lysine to carboxy groups of diaminopimetic acid residues whereas the rest occurs in association with the OM (Nikaido and Vaara, 1985; Braun, 1975). Lipoprotein may act as an anchor linking the OM to the PG layer, however its exact function is unknown (Inouye, 1979). Lipoproteins have been found in all the Enterobacteriaceae studied and in P. aeruginosa (Lugtenberg and van Alphen, 1983).

A number of proteins have been identified and characterised in the OM of P. aeruginosa (Hancock and Nikaido, 1978; Mizuno and Kageyama, 1978). The nomenclature assigned by Mizuno and Kageyama (1979a; 1979b; 1978) has been generally adopted. Hancock and Carey (1979) have established SDS-PAGE conditions under which major OM proteins of P. aeruginosa strain PA0 1 separate into 8 bands, designated D1, D2, E, F, G, H1, H2 and I in order of decreasing mobility in the gel. Figure 1.3 shows a diagrammatic representation of P. aeruginosa

proteins separated according to the method of Hancock and Carey (1979); the function of these proteins is also indicated. Among these proteins D1, D2, F, G and H1 are all heat modifiable, since their mobility in SDS-PAGE was determined by the solubilisation temperature. Proteins F, H2 and I are non-covalently bound to the underlying PG layer (peptidogylcan associated proteins) and proteins D1, D2 and E are in some degree induced when P. aeruginosa is grown with glucose as carbon source.

Proteins D1, D2, G and H1 all appear to belong to the same class of heat-modifiable proteins since heating of these proteins between 70-100 °C in SDS caused a decrease in their mobility on SDS-PAGE (ie an increase in their apparent molecular weights). The effect of heat-modification was reversed by addition of LPS to the heat modified forms of D1, G and H1 (Hancock and Carey, 1979). Heat modifiability arises as a result of structural rearrangements within the protein molecule under denaturing conditions and leads to an apparent increase in the molecular weight of that protein (Lugtenberg and van Alphen, 1983).

Protein F has been identified as the major protein of P. aeruginosa. It is also a heat modifiable protein; the nature of its heat modification is different to that of the proteins D1, D2, G and H1. Unlike these latter proteins long periods of boiling in SDS are necessary to affect the change of protein F to its heat modified form (Hancock and Carey, 1979). Protein F is rich in β sheet structure (Mizuno and Kageyama, 1979b) which may explain the mechanism by which its heat modification occurs (Nakamura and Mizushima, 1976). Protein F has been found to be non-covalently bound to the underlying PG (Hancock *et al*, 1981; Mizuno and Kageyama, 1979b). However, this protein must span the OM since it was labelled when whole cells of PAO 1 were reacted with ^{125}I-labelled peroxidase (Lambert and Booth, 1982). Also, monoclonal antibodies to protein F were shown, using immunofluorescence techniques, to interact with surface components of different serotypes of P. aeruginosa but not with a porin-deficient mutant (Mutharia and Hancock, 1983).

Proteins H1 and H2 share similar molecular weights and were not resolved in the earlier studies using SDS-PAGE, so these proteins were designated protein H (Mizuno and Kageyama, 1979b). Analysis of protein H from the OM of P. aeruginosa indicated that it contained covalently linked fatty acids. A 21K peptidoglycan associated protein in the OM of E. coli K-12 prepared under the same conditions had a similar fatty acid and amino acid composition (Mizuno, 1979). The P. aeruginosa protein studied by Mizuno is most likely protein H2 as it is a peptidoglycan associated protein (Hancock *et al*, 1981), unlike protein H1. Monoclonal antibody to protein H2 did not bind to intact smooth P. aeruginosa cells suggesting that this protein may not be surface exposed under the growth conditions used. Also, when whole cells of P. aeruginosa strain PAO 1 were reacted with ^{125}I–labelled peroxidase this protein was not labelled (Lambert and Booth, 1982). It was noted that monoclonal antibodies to protein H2 bound to rough LPS-deficient mutants. This was though to be due to the unmasking of antigenic determinants by loss of 0-side chains of LPS or to rearrangements of OM components (Mutharia and Hancock, 1983). Protein H1 is induced in the OM of P. aeruginosa grown in magnesium-deficient media and becomes a major OM component (Anwar *et al*, 1983b; Nicas and Hancock, 1983; 1980). A linear relationship has been found between Mg^{2+} levels in the cell envelope and the OM of protein H1 present. Protein H1 is though to replace Mg^{2+} at polycationic binding sites in the LPS of P. aeruginosa grown in magnesium-depleted media (Nicas and Hancock, 1980).

Protein I in the OM of P. aeruginosa is also a lipoprotein and is analogous to the Brauns lipoprotein of E. coli (Mizuno and Kageyama, 1979a). It is rich in α helical structure (Mizuno and Kageyama, 1979b) and exists in both free and to a lesser extent bound form in the OM (Hancock *et al*, 1981; Mizuno and Kageyama, 1979a).

Proteins D1 and D2 are of similar molecular weight (Hancock and Carey, 1979) and were not identified as separate polypeptides in earlier studies (Mizuno and Kageyama, 1979b). Both these proteins are strongly induced in the OM of cells

FIGURE 1.3

SCHEMATIC DIAGRAM OF THE CELL EVELOPE OF P.AERUGINOSA. (For key see Fig 1.1)
D1, D2, F, H1, H2, I AND P ARE OM PROTEINS.

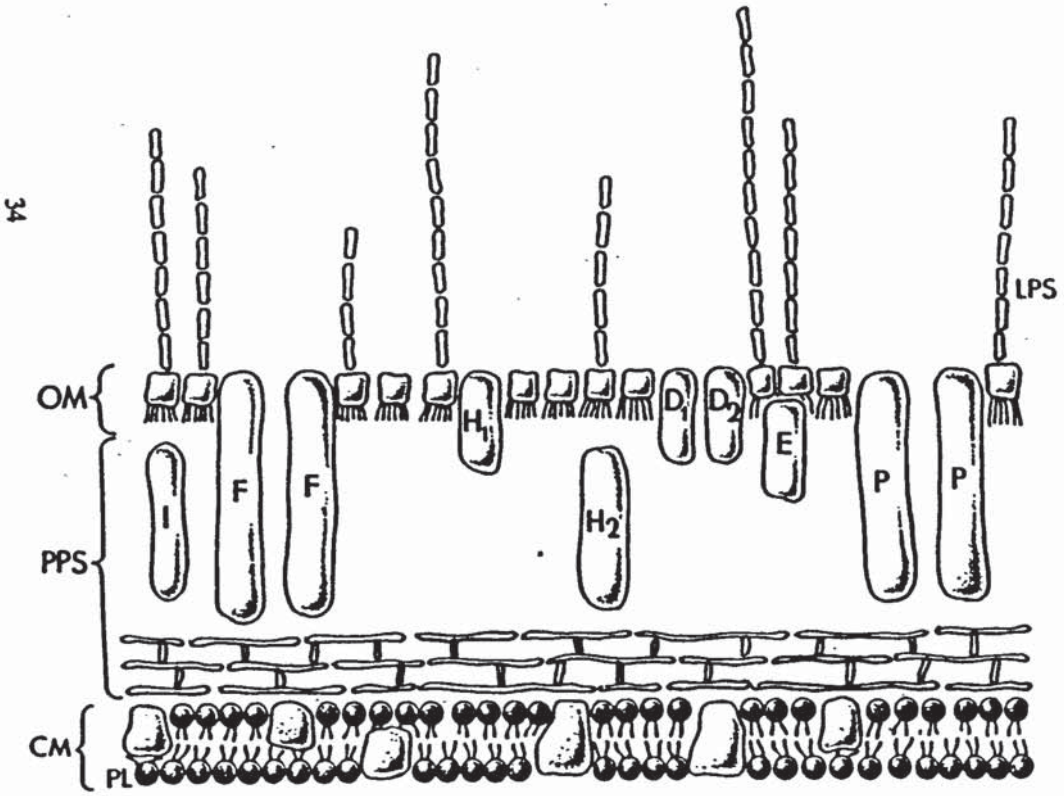
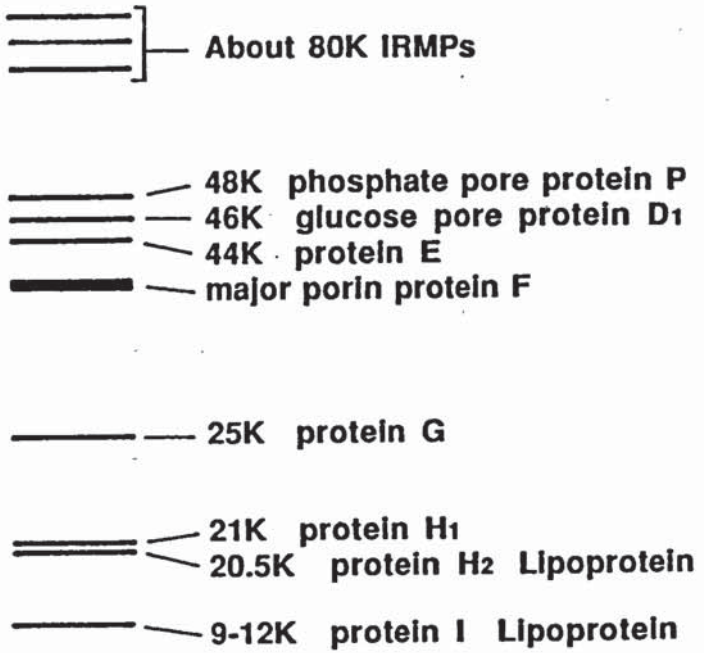


FIGURE 1.4

PATTERN OF OM PROTEINS OF P. AERUGINOSA PRODUCED BY ELECTROPHORESIS ON 14% ACRYLAMIDE GELS AFTER DENATURING AT 100 °C IN PURE SDS AND MERCAPTOETHANOL. THE MOLECULAR WEIGHT AND FUNCTION OF THE PROTEINS ARE SHOWN, WHERE KNOWN.



grown on media containing glucose as the sole carbon source (Hancock and Carey, 1979, 1980). Protein D1 functions as a pore (Hancock and Carey, 1980). Protein E is also slightly induced after growth of the organisms on glucose, although it is a constitutive protein. P. aeruginosa also produces as 48K protein, designated protein P, upon phosphate starvation (Hancock *et al*, 1982). This protein forms a very strong anionic specific channel when reconstituted in lipid bilayers and is proposed to be a porin involved in a phosphate uptake system (Hancock *et al*, 1982). The function of OM protein G has not been described.

Iron limitation of P. aeruginosa induces production of a number of high molecular weight OM proteins (Brown *et al*, 1984; Sokol and Woods, 1983; Ohkawa *et al*, 1980; Meyer *et al*, 1979; Mizuno and Kageyama, 1978) which are assumed to be involved in the uptake of iron because of their appearance in iron-deprived conditions and their similarity in molecular weight (70-90K) to the iron-binding proteins of the enteric bacteria. However, the genes encoding for these proteins have not be mapped as they have for E. coli (Griffiths, 1987b; Griffiths, 1983).

Ohkawa *et al* (1980) demonstrated production of 4 high molecular weight iron-regulated membrane proteins (IRMPs) in the OM of PAO 1 grown in iron-deficient media; however, nine mutant strains which were resistant to pyocin S2 all lacked one of the IRMPs, designated Fe-b protein, indicating a possible role for this protein as a receptor for pyocin S2. This may suggest that the Fe-b protein will be surface exposed in the OM of strains expressing this protein.

In addition to the high molecular weight IRMPs in the OM of P. aeruginosa a 14K protein which is proposed to be involved in the uptake of ferripyochelin has been reported by other workers (Sokol and Woods, 1986; Sokol, 1984; Sokol and Woods, 1983). The experimental protocol adopted by Sokol and Woods (1983) involves preparation of OMs from P. aeruginosa strain PAO 1 grown in iron-deficient media. Following incubation with ^{55}[Fe]-ferripyochelin the OM samples are

denatured and subjected to SDS-PAGE. The ferripyochelin binding protein is then detected by autoradiography. This method, appears to assume that bonds between the ferripyochelin and the protein involved in its uptake are not disrupted by this procedure, and this has not been confirmed experimentally (for fuller discussion see section 3.1.5). As other workers have not detected this protein it may suggest that the result obtained by Sokol and co-workers may be an artefact arising from their experimental procedure. An alternative suggestion may be provided based on the work of Owen *et al* (1980). These investigators used crossed immunoelectrophoresis to analyse the iron-containing antigens present in the OM vesicles of E. coli grown in the presence of excess ^{59}Fe. Seven discrete antigens were detected by autoradiography. The immunoprecipitate corresponding to Braun's lipoprotein contained tightly bound iron, therefore Owen *et al* (1980) suggested there may be an *in vivo* association of Braun's lipoprotein with other iron-containing components possibly an iron-siderophore receptor protein.

1.1.1.5 Exopolysaccharides

Many Gram-negative bacteria produce extracellular polysaccharides (exopolysaccharides) present in various forms around the cell. These are often categorised according to their degree of firmness of association with the cell and may be described as rigid, flexible, integral or peripheral exopolysaccharides (Costerton *et al*, 1981). The glycocalyx (defined as any polysaccharide containing component outside the cell wall) of P. aeruginosa may be shed largely into the medium and forms a thick, flexible, peripheral, glycocalyx (Costerton *et al*, 1981; Sutherland, 1977). The importance of these exopolysaccharides as an almost universal component of the bacterial cell was established by direct electron microscopic observation of bacterial cells in nature and disease, in which polyanion-specific stains were used to detect these polysaccharides (Costerton *et al*, 1981).

Most strains of P. aeruginosa secrete a high molecular weight polysaccharide slime distinct from the polysaccharides of LPS and this purified slime was found to

be two or three times more toxic than LPS for mice (Sensakovic and Bartell, 1974). Mucoid strains of P. aeruginosa from CF lung infection elaborate an extensive exopolysaccharide component distinct from this slime layer at their surface (Doggett *et al*, 1964; Doggett, 1969). Exopolysaccharide of mucoid P. aeruginosa is an acetylate co-polymer of β-D-mannuronic acid and α-L-guluronic acid, which closely resembles the alginates produced by marine algae, and is similar to the polymer of Azotobacter vinelandii (Sutherland, 1977; Evans and Linker, 1973).

Acid hydrolysis shows that the polysaccharide contains some homogenous sequences of either poly-mannuronic acid or poly-guluronic acid interspersed with sequences containing both polymers (Evans and Linker, 1973). Analysis of the alginate obtained from different P. aeruginosa strains indicated differences in their mannuronic:guluronic acid ratios. The acetyl content of these alginates was found to be proportional to the mannuronic acid content (Fyfe and Govan, 1983). Alginate biosynthesis was studied using a number of mucoid mutants derived from the non-mucoid PAO 381 strain. When the composition of the polymers from these strains, grown for the same period of time under identical growth conditions was analysed, all samples had a high mannuronic acid content ranging from 75% to 95%. Their viscosity and degree of acetylation also varied significantly and a linear relationship was observed between % acetylation and viscosity over the range 2.3 to 8.6% acetate content (Fyfe and Govan, 1983 ref 75).

Annastassiou *et al* (1987) analysed the alginate produced by a non-mucoid revertant variant derived by serial passage of a heavily mucoid P. aeruginosa strain. The slime from the non-mucoid isolate contained 16% uronic acids as compared to the mucoid parent strain which contained 65% dry weight uronic acids. Furthermore, extracts from six clinical macroscopic non-mucoid P. aeruginosa isolates were found to contain 8 to 14% uronic acids. Alginate production would appear to be shared by all P. aeruginosa strains.

Biosynthesis of pseudomonas alginate is controlled by chromosomal genes and involves at least three regions of the chromosome (Ohman, 1986; Fyfe and

Govan, 1983; McGeorge *et al*, 1986). Change to the mucoid form may be coded for by a phage (Martin, 1973). Alginate synthesis is very expensive in energy terms (Jarman and Pace, 1984) which suggests that a clear survival advantage is gained by the organism from alginate synthesis in those environments where the mucoid state predominates. The role of P. aeruginosa alginate in the aetiology of chronic P. aeruginosa lung infection will be considered further in section 1.3.

1.1.1.6 Flagella and fimbriae

Flagella and fimbriae are protein structures anchored in the OM. Flagella are composed of three distinct substructures, the filament, the hook and the basal structure and are responsible for the mobility of bacteria allowing them to exhibit chemotactic behaviour (Silverman and Simon, 1977). Each flagellin is made up of a number of repeating polypeptide units called flagellin (Silverman and Simon, 1977). An initial report by Montie and Stover (1983) identified flagellin of P. aeruginosa as a 53K species. In a more recent study Allison *et al* (1985) studied the electrophoretic mobility of purified flagellins from a large number of laboratory and clinical strains. Their results indicated that the majority of strains had flagellins of 53K (b-type flagellins). A more heterogenous group of a-type flagellins with molecular weights in the range of 52K-45K were obtained from other isolates (a-type flagellins).

Many Gram-negative bacteria possess surface fimbriae which are revealed by electron microscopy as thin non-sinusoidal filaments radiating from the surface of the cell. Fimbriae play an important role in the attachment of bacteria to surfaces. Fimbriation is a reversible trait and is most likely determined by chromosomal genes (Hammond *et al*, 1984). Most strains of P. aeruginosa produce chromosomally encoded fimbriae, which in most cases are polar. They are expressed in relatively small numbers (2-12 per pole (Bradley, 1972). The fimbriae of P. aeruginosa K strain serve as receptors for bacteriophage and are built up of sub-units of 17.8K (Hammond *et al*, 1984). Fimbriae have been implicated in the adherence of P. aeruginosa to human buccal epithelial cells and damaged corneal epithelial cells

(Woods *et al*, 1980; Reichert *et al*, 1983).

1.1.2 The role of divalent cations in the Gram-negative cell envelope

Divalent cations are known to play several important roles in the bacterial cell envelope. They are essential in maintaining the stability of the CM (Lederberg, 1956), regulating the activity of several enzyme systems, especially those enzymes which are involved in the synthesis of the cell wall and as structural components of the cell wall (Costerton *et al*, 1974; Asbell and Eagon, 1966). Eagon (1969) reported that magnesium and calcium are major elements and that iron is a minor element in P. aeruginosa; zinc, lead, copper, manganese and strontium were present as trace elements. The cation composition of P. aeruginosa shows marked variation depending on the nature of the medium (Kenward *et al* 1979) and on the presence of other cations (Kenward *et al*, 1979; Boggis *et al*, 1979a).

Ca^{2+} and Mg^{2+} form stabilising cross-links between highly anionic groups in the LPS-protein-phospholipid molecules in the OM (Costerton *et al*, 1974). The LPS of P. aeruginosa is highly phosphorylated and phosphate groups in the KDO-lipid A region have a high affinity for divalent cations (Wilkinson, 1983b; Wilkinson and Galbraith, 1975). P.aeruginosa is characteristically lysed by EDTA as a result of removal of the Mg^{2+} and/or Ca^{2+} cross-links (Boggis *et al*, 1979; Brown and Melling 1969a; 1969b). Under conditions of magnesium-depletion P. aeruginosa may replace Mg^{2+} ions with protein H1, since a linear relationship was demonstrated between levels of Mg^{2+} and protein H1 in the membrane (Nicas and Hancock, 1980). However, induction of protein H1 in the OM of magnesium-depleted P. aeruginosa was repressed in media supplemented with Ca^{2+} and Mn^{2+} indicating that cells prefer to replace Mg^{2+} with these alternative metal ions rather than protein H1 (Anwar *et al*, 1983a, 1983b; Nicas and Hancock, 1980).

1.2 THE INFLUENCE OF THE *IN VIVO* ENVIRONMENT ON THE SURFACE PROPERTIES OF GRAM-NEGATIVE BACTERIA

There is much evidence to indicate that the surface composition and biological properties of the bacterial surface are largely determined by the growth environment (Brown and Williams, 1985a). The influence of osmolarity, Eh, pH and temperature have long been recognised. Other factors which are less well recognised are the effects of specific nutrient limitation (Brown and Williams, 1985a; Dalhoff, 1985; Ellwood and Tempest, 1972; Holme, 1972), the rate of growth (Brown and Williams 1985a; Taylor, 1984) and whether the culture is replicating in suspension or on a surface (Costerton, 1984; Costerton *et al*, 1983; Costerton *et al*, 1981): it is these factors which will be emphasised here. The structural flexibility of the cell envelope is important in understanding the host-bacterium interaction in infection since phenotypically induced alterations in the surface components of a bacterium will affect its virulence and immunogenicity, and also its interaction with host defence mechanisms and sensitivity to antibiotic therapy (Brown and Williams, 1985a; 1985b; Costerton *et al*, 1979; Smith, 1977). The surface properties of bacteria *in vivo* are in turn likely to be influenced directly by host defence mechanisms and the presence of antibiotics at subgrowth inhibitory concentrations (Brown and Williams, 1985a; Dalhoff, 1985).

Direct electron microscopic examination has revealed that the adherent surface associated microcolony mode of growth is an important feature in the growth of many pathogenic bacteria *in vivo* especially in chronic infections (Costerton, 1984; Costerton *et al*, 1983; Costerton *et al*, 1981). The microcolony mode of growth is perhaps best exemplified by P. aeruginosa growing in the lungs of CF patients with chronic infection (Lam *et al*, 1980). Since the microcolony mode of growth and its role in CF lung infection will be considered further in section 1.3 it will not be reviewed here but rather the effects of specific nutrient limitation and growth rate on

the surface properties of bacteria will be illustrated. The general plasticity of the bacterial envelope *in vitro* will be outlined initially with emphasis on features which are important to the host-bacterium interaction in infection, together with a brief review of the surface properties of bacteria grown *in vivo* in chamber implants. This will be followed by a review of our current knowledge concerning nutrient deprivation and growth rate *in vivo*. Relatively little information exists concerning the effect of nutrient limitation and growth rate on the CM or the production of bacterial exoproducts but where such evidence exists it will be included.

1.2.1 General plasticity of the Gram-negative cell wall *in vitro* and *in vivo*

A large body of evidence has accumulated to showing that bacteria grown *in vitro* adapt readily to their growth environment (Brown and Williams, 1985a; Ellwood and Tempest, 1972; Holme, 1972, see also section 1.1). This plasticity of the bacterial cell wall confers a considerable survival advantage on the cell.

Limitation of growth medium phosphate, sulphate or iron all resulted in the induction of new OM proteins in E. coli (Lugtenberg and van Alphen, 1983). The ratio of OmpF/OmpC (the two major porin proteins) is dependent on the composition of the growth medium, although the sum of the amounts appears constant (Lugtenberg and van Alphen, 1983). These proteins enable the organism to scavenge its environment selectively for depleted nutrients. In addition to induction of new OM proteins iron limitation also imposes changes in the metabolism of the pathogen. The activity of ribosomes isolated from iron-depleted cells of E. coli was greater when compared with that of iron-plentiful cells (Iorio and Plocke, 1981).

Phenotypically induced alterations in the bacterial cell surface also affect the susceptibility of the bacterium to antimicrobial agents. Induction of protein H1 in the OM of P. aeruginosa grown in magnesium-depleted media has been correlated with increased resistance of the cells to polymyxin and gentamicin (Nicas and Hancock, 1980). Under conditions of iron-limitation in chemostats at different growth rates P.

aeruginosa (wild-type and its mucoid variant) showed altered penicillin binding proteins compared to cells grown in complex media and with each other (Turnowsky *et al*, 1983). In a recent study the bactericidal activity of two β-lactam antibiotics on E. coli was compared for generation times ranging from 0.7 to 12 hours. All killing rates were a constant function of the bacterial generation time; slowly growing bacteria became progressively more phenotypically tolerant to β-lactam antibiotics as the generation time was extended (Tuomanen *et al*, 1986).

Growth rate and specific nutrient limitation also affect the phospholipid, (Minnikin *et al*, 1974) fatty acid, (Cozens and Brown, 1978)) LPS (Day and Marceau-Day, 1982; Cozens and Brown, 1978) and cation content (Boggis *et al*, 1979a) of the Gram-negative cell. Expression of pili is also influenced by growth rate. The production of fimbrial adhesions K99 and F41 by enterotoxigenic E. coli was found to be correlated with both specific growth rate (μ) and pH in chemostat grown cells (van Verseveld *et al*, 1985). A significant production of fimbriae was only detected at μ values higher than 0.2 $hour^{-1}$ corresponding to a doubling time of 29 hours. Phenotypically induced alterations in the fatty acid and neutral lipid content of P. aeruginosa was associated with changes in the susceptibility of the cells to disinfectants (Cozens and Brown, 1978). Alterations in the cation content of magnesium-depleted batch and chemostat cells was associated with increased resistance to EDTA, polymyxin and cold shock (Kenward *et al*, 1979). Slow-growing magnesium-limited P. aeruginosa were much more resistant to killing in *in vitro* whole blood assays than were fast-growing magnesium-limited or slow-growing carbon-limited cells (Finch and Brown, 1978). Pseudomonas cepacia was much more sensitive to complement-mediated serum killing when carbon-depleted than when oxygen- or iron-depleted; these changes were attributed to alterations in the surface components of the bacterium (Anwar *et al*, 1983c).

Production of P. aeruginosa extracellular enzymes, including protease and elastase, were all reduced in iron-plentiful as compared to iron-depleted media and production of these enzymes varied in a complex manner with specific growth rate

(Ombaka *et al*, 1983; Bjorn *et al*, 1979). Low concentrations of iron *in vitro* enhanced the production of exotoxin A in Pseudomonas aeruginosa (Bjorn *et al*, 1978) and of the phage-mediated diptheria toxin in Corynebacterium diptheriae (Pappenheimer, 1977). Production of extracellular polysaccharides by mucoid P. aeruginosa was studied in batch cultures depleted of phosphorus, iron, carbon, magnesium and sulphur. Phosphate-, nitrogen- and iron-depleted cells produced more exopolysaccharide than cells grown under the other nutrient depletions (Ombaka *et al*, 1983).

An approach which as been useful in the study of the surface properties of bacteria grown *in vivo* has been the use of chamber implants (Day *et al*, 1980; Penn *et al*, 1976). Artifacts can be introduced in studies using chamber implants since they exclude cellular antimicrobial defences although permitting soluble humoral factors to penetrate (Brown and Williams, 1985a). Attention must also be drawn to the *in vitro* culture conditions for the inocula used in these studies. Little is known about the availability of nutrients, but it is known that iron (Griffiths, 1983) and glucose (Smith, 1977) (in chambers) may be growth-limiting. Despite some of the limitations associated with this technique some important results have been obtained.

Strains of E.coli harbouring the Col V plasmid were grown *in vivo* in chamber implants and the sensitivity of these cells to serum-killing was investigated. Differences in the serum sensitivity of organisms of the same strain to the bactericidal action of human serum were found in some cases between organisms of the same strain grown *in vivo* or *in vitro* in nutrient broth. Comparison of the protein composition of the bacterial cell envelopes revealed differences between *in vivo* and *in vitro* grown cells (Finn *et al*, 1982).

Gonococci grown *in vivo* in chamber implants resisted the killing action of human serum (Penn *et al*, 1976). This phenotypically acquired resistance to serum was lost after only a few generations in laboratory media. Serum susceptibility and induced serum resistance in strains (of gonococci) grown in chambers differed in their sensitivity to pyocins indicating differences in LPS structures or masking of

some determinants (Winstanley *et al*, 1984). It is not known if these changes arise from phenotypic variation in the bacterium in response to the *in vivo* environment or from selection of resistant strains.

Electron microscopic examination of laboratory grown and animal-passaged isolates of the Gram-negative anaerobe Bacteroides fragilis revealed significantly more capsule around the later. This observation correlated with increased resistance of the organism to phagocytosis (Simon *et al*, 1982).

1.2.2 Nutritional deprivation *in vivo*

1.2.2.1 Iron

In the 1940's Schade and Caroline (1944; 1946) first discovered that specific iron-binding proteins present in blood and also in the whites of eggs inhibited the growth of certain bacteria. The active component in egg white was later shown the be ovotransferrin (Alderton *et al*, 1946). The component in the blood was found to be a similar iron-binding protein named transferrin to indicate its importance in iron transport (Schade and Caroline, 1946). The ability of iron-binding compounds to abolish the antibacterial effects of body fluids *in vitro* is now well recognised; this is a property which is specific to iron (Weinberg, 1984; Griffiths, 1983; Finkelstein *et al*, 1983; Bullen, 1981).

The solubility of iron is extremely low; at physiological conditions ferric iron tends to oxidise, hydrolyse and polymerise forming essentially insoluble ferric hydroxide and oxyhydroxide polymers (May and Williams, 1980). The solubility constant for ferric hydroxide has been estimated to be about 10^{-36} so that free Fe^{3+} in excess of 2.5×10^{-15} M could be precipitated as insoluble ferric hydroxides (Bullen *et al*, 1978). The toxic effects of iron are avoided *in vivo* since most of the body's iron is located intracellularly in ferritin or haemosiderrin. Extracellular iron in body fluids is attached to the iron-binding proteins transferrin and lactoferrin. Transferrin is found mainly in the serum whilst lactoferrin is found in many exocrine secretions including tears, saliva, bronchial mucus, and nasal exudate in addition to milk

(Morgan, 1981; Aisen and Listowsky, 1980). Human polymorphonuclear leucocytes (PMNs) also contain lactoferrin (Bullen and Armstrong, 1979). PMN lactoferrin is released on degranulation of cells at an area of sepsis. After combining with iron in the infected region the metal saturated protein is ingested by macrophages (van Snick *et al*, 1974).

Transferrins consist of a single polypeptide chain and contain two iron-binding sites. They exhibit maximal iron-binding activity at pH values above 6.5 (Bezkorovainy, 1987; Morgan, 1981). The two iron-binding sites appear to be identical functionally (Bezkorovainy, 1987). Binding of iron by transferrins is accompanied by binding of an anion on a 1:1 molar basis. Physiologically this ion is carbonate; binding of iron does not occur in the absence of carbonate (or other anion in non-physiological conditions) (Schlabach and Bates, 1975).

An important property of lactoferrin is its ability to bind iron as the pH declines below 4.0, this will allow it to maintain a low-iron environment within the acidic phagolysosome which is necessary for the bactericidal function of other components (Ambrusco and Johnston, 1981). Lactoferrin can also be an effective scavenger of iron at sites of infection where the pH has been lowered by release of acid from tissues and/or from metabolically stimulated leucocytes (Weinberg, 1978). Lactoferrin is also highly unsaturated normally which would allow it to function as an iron-binding protein rather than an iron-transport agent. The affinity constant of lactoferrin for iron is 10^{52}, compared to transferrin at 10^{36} (Griffiths, 1987a; 1987b). So although the body contains much iron, the amount of free iron in equilibrium with the iron-binding proteins is of the order 10^{-18} M, which is too low to support bacterial growth (Cox, 1985; Morgan, 1981; Bullen *et al*, 1978).

Although virtually all the iron in vertebrates is bound to protein some iron is also thought to be complexed to low molecular weight ligands, such as citrate or phosphate, and it has been suggested that these low molecular weight molecules may mediate the transfer of iron both inside and outside the cells. Physiological concentrations of citrate, for example, do catalyse Fe^{+} exchange between transferrin

molecules (Aisen and Liebman, 1968).

Serum iron concentrations decrease rapidly immediately after infection and during inflammation and fever (Cartwright *et al*, 1946). This change, which is referred to as the iron-specific "nutritional immunity", is thought to be attributable primarily to an increase in storage of iron in the liver and to a lesser extent to decreased absorption from the gut (Weinberg, 1984). This decrease can be reproduced experimentally by injecting small amounts of bacterial endotoxin (Baker and Wilson, 1965) which acts by stimulating release from PMNs of a factor which seems identical to leucocyte pyrogen (Merriman *et al*, 1977). The subsequent changes which lead to reduction of serum iron are not however clear. van Snick *et al* (1974) have reported evidence for an accelerated removal of iron from the serum transferrin pool during inflammation and that the macrophages of the reticuloendothelial system may play a central role in this alteration. Macrophages must quickly initiate synthesis of the iron-binding protein ferritin leading to inhibition of iron release and so preventing its normal recycling to transferrin. Accumulation of lactoferrin at sites of inflammation with its ability to bind iron in the more acidic conditions which often prevails at such sites may also be important (Morgan 1981; Aisen and Listowsky, 1980). Evidence has also been presented to suggest that, in mice at least, hypoferremia is not due to an accelerated removal of iron from serum transferrin but to an altered processing of iron within the reticuloendothelial system which limits the supply of this element to the extracellular pool (Letendre and Holbein, 1984).

The hypoferremia of infection is thought to have an important role in modulating the inflammatory response. Neutrophils produce superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). In the presence of Fe^{3+} the O_2^- is converted to O_2 and Fe^{2+} (Haber and Weiss, 1934). This then leads to formation of Fe^{3+} and an hydroxyl radical (OH^-). Apart from its role in bacterial killing (Ambrusco and Johnston, 1981) the hydroxyl radical may also contribute to acute tissue damage during inflammation (Sacks *et al*, 1978).

Levels of transferrin and lactoferrin also undergo considerable variation in many disease states. Hunter *et al* (1984a) reported that 28 out of 47 adults with acute myeloid leukaemia had low levels of transferrin and the levels of transferrin saturation were above 50%. Whilst alterations in the saturation level of transferrin may not affect the course of infection it was found that sera containing highly saturated transferrin had far less ability to inhibit a strain of P. aeruginosa than did normal sera; the higher the level of saturation the less the inhibition. Low levels of transferrin and/or failure to rise with nutritional therapy is a poor prognostic indication in patients with cancer and other serious diseases (Hunter *et al*, 1984b).

Gutteberg *et al* (1984) reported that the concentration of lactoferrin was significantly increased in all but one of 11 children suffering from meningococcal septicaemia who had been ill for less than 18 hours. Lactoferrin was thought to be derived from polymorphs. Wolach *et al* (1984) reported plasma lactoferrin levels of 1.5 ± 1.8 mgL^{-1} for plasma lactoferrin in normal male controls and found that patients with more than a 30% burn wound had levels of 10-40 mg L^{-1}. There was also a positive correlation between the extent of a burn wound and the level of lactoferrin on the first day after injury.

1.2.2.2 Deprivation of nutrients other than iron

Whilst there is little information concerning the deprivation of nutrients other than iron in the body there is some evidence to suggest that some other trace metal ions, including magnesium and zinc, and also phosphate may be limiting.

An important factor which may predispose for hypomagnesemia is treatment with aminoglycoside antibiotics which cause renal nephrotoxicity and may result in increased urinary magnesium excretion (Cronin, 1979). It has been suggested that magnesium depletion is probably common in patients with CF (Green *et al*, 1985). Sympotmatic hypomagnesemia was reported in 12 patients who had decreased magnesium serum values. The patients had all been receiving aminoglycoside antibiotics (Green *et al*, 1985). Broughton *et al* (1968) also reported decreased

levels of magnesium in sera from patients with burn wounds. The levels of zinc in the serum may also fall in response to fever and inflammation (Pekarek and Engelhardt, 1981).

Weinberg (1974) reported that patients infected with Gram-negative organisms reduce the levels of phosphate in their plasma to levels that are suboptimal for microbial growth (<1mM). P. aeruginosa produces pyocyanin, a bright blue-green pigment, *in vitro* in medium which has low phosphate concentrations and relatively high iron concentrations (Cox, 1985). The fact that P. aeruginosa produces a blue pigment during human infection has been recognised since the organism was first isolated from blue pus by Gessard in 1882 and suggests that the levels of phosphate are low at the site of infection. P. aeruginosa also produces a heat stable hemolysin and alkaline phosphatase together with phospholipase C in low phosphate, but not in high phosphate, media (Liu, 1979). These observations lead Liu to propose that these products may function together as a phosphate scavenging system. Continued production of these virulence factors by isolates from clinical infection is taken to indicate that phosphate may be limiting *in vivo*. Vasil *et al* (1985) examined the incidence of production of phospholipase C by clinical isolates of P. aeruginosa from various clinical infections using a synthetic substrate assay. Urinary tract isolates produced more phospholipase C than other strains tested and some lung isolates also produced very high levels of phospholipase C.

1.2.2.3 Response of Gram-negative bacteria to iron deprivation

Iron is essential for many bacterial enzymes including those involved in the electron transport system, the flavoproteins and of oxygen metabolism (see Griffiths, 1987a). In E. coli the process of DNA replication is known to be dependent on iron. Iron-containing ribonucleotide reductases catalysed the transfer of ribonucleotides to the deoxyribo-nucleotides by substituting a hydroxyl group at position 2' on the molecules in hydrogen; this is an obligatory step in DNA synthesis. Therefore, iron appears to be an essential element for all forms of life (Griffiths, 1987a) except the

lactobacilli which seem to use vitamin B12 co-enzyme as a factor for the reduction of ribonucleoside triphosphates (Archibald, 1983). Pathogenic bacteria must therefore be capable of acquiring iron if they are to multiply in the iron-restricted environment in the body. Griffiths (1987b) has outlined 4 mechanisms by which bacteria might be expected to assimilate protein bound iron or aquire it from liberated haem in the body. These are:

1. through proteolytic cleavage of the iron-binding glycoprotein leading to release of iron from the iron-binding site.
2. by reduction of the Fe^{3+} complex to Fe^{2+} and subsequent release of Fe^{2+}.
3. through direct interaction between receptors on the bacterial cell surface and the Fe^{3+} complex.
4. by producing low molecular weight iron-chelating compounds, known as siderophores, that are able to remove iron from the Fe^{3+}-glycoprotein complex and deliver it to the bacterial cell (Neilands, 1981; 1984).

The mechanism for iron assimilation by bacteria which is best understood at present is that which depends on the production of siderphores. Whilst evidence exists that some species utilise one of the other three mechanisms these have been less well elucidated at present (Griffiths, 1987b).

Many Gram-negative bacteria growing in iron-deprived conditions *in vitro* secrete low molecular weight chelators which are part of what is termed the "high affinity" iron transport systems (Neilands, 1981). A "low affinity" iron-uptake system is also thought to operate when iron is freely available but little is known about the mechanisms involved.

The high affinity iron transport systems produced by bacteria of the genera, Escherichia, Klebsiella, Salmonella and Shigella have been studied most. When grown under conditions of iron-deprivation *in vitro* E. coli, K. pneumoniae, S. typhimurium and some species of Shigella secrete the iron chelator enterobactin (enterochelin). This compound, which is a cyclic triester of 2, 3-dihydroxy-N-benzoyl serine, is synthesised under conditions of iron-deprivation and removes iron

from the iron-binding proteins and promotes bacterial growth (Neilands, 1984; Griffiths, 1983).

The most outstanding feature of siderophores is their very high affinity for ferric iron; enterobactin has an affinity constant for iron of approximately 10^{52} at neutral pH (Harris *et al*, 1979). Organisms which secrete this siderophore are therefore capable of competing efficiently with host glycoproteins for iron. The enterobactin iron-uptake system has been extensively characterised *in vitro* using the laboratory strain E. coli K12 but it is known that pathogenic strains of E. coli also synthesise this siderophore. It has been demonstrated that enterobactin was produced *in vivo* in experimental infection (Griffiths and Humphreys, 1980). The peritoneal washings from guinea pigs infected with E. coli contained enterochelin and its degradable products which provided evidence that the bacteria grow *in vivo* under iron-restricted conditions.

Whilst the main endogenous system used by E. coli, Salmonella, and Klebsiella for accumulating iron appears to be enterobactin mediated iron-uptake these organisms also utilise a variety of hydroxamate type siderophores which are not synthesised by the organisms themselves but are produced by other micro-organisms (Neilands, 1984; 1981; Konisky, 1979). Citrate is another compound which may supply iron to E. coli since some strains are known to induce a citrate mediated iron-uptake system when grown *in vitro* in an iron-limited media containing citrate (Hancock *et al*, 1976). Many strains of E. coli which are capable of causing general extraintestinal infection in man carry the Colicin V (Col V) plasmid and synthesise a plasmid encoded hydroxamate type siderophore called aerobactin in addition to enterobactin (Crosa, 1984; Braun, 1981).

An essential part of high-affinity iron-uptake systems based on siderophores is the production of outer membrane protein receptors for the iron-siderophore complexes and cellular enzymes which are involved in the release of iron from the iron chelators (Neilands, 1981). Specific receptors are required for the iron-siderophore complexes since their molecular weight exceeds the exclusion limit of the

small water filled porin channels which are present in the OM of Gram-negative bacteria (Nikaido and Nakae, 1979). In E. coli the proteins involved in iron-uptake have been extensively characterised genetically and these proteins are designated according to their molecular weights as the 83K, 81K, 78K and 74K proteins (Griffiths, 1983; Braun *et al*, 1976). The 81K (Fep A) protein is the receptor for Fe^{3+} -enterochelin and is the product of the fep gene. The 78K protein is the receptor for ferrichrome and is the product of the ton A gene. An outer membrane protein specified by the Col V plasmid regulated by iron and involved in aerobactin mediated iron transport has also been identified (Bindereif *et al*, 1982).

Pseudomonas species are known to produce several siderophores when grown *in vitro* in iron-depleted media, these are pyoverdin, pyochelin ferribactin and ferrioxamines (Neilands, 1984; 1981; Cox 1980; Cox and Graham, 1979; Liu and Shokrani, 1978). Of these pyochelin and pyoverdin are of the most interest as regards infection. Pyochelin (Fig 1.5) is a salicyl acid-substituted cysteinyl peptide compound and is therefore a phenolic type siderophore (Cox *et al*, 1981). When pyochelin was added to iron-deficient cultures it promoted growth of P. aeruginosa and also reversed growth inhibition by the iron chelator EDDA (Cox and Graham, 1979). The uptake of iron from ferripyochelin by P. aeruginosa involves two stages, an energy-independent step, presumably involving binding to the cell surfaces, followed by an energy-dependent process (Cox, 1980). Pyoverdin (Fig 1.4) the yellow green fluorescent pigment produced by P. aeruginosa when it is grown in iron-limiting conditions has a binding constant for iron of 10^{32}. (Wendenbaum *et al*, 1983). Pyoverdin has also been shown to promote the growth of P. aeruginosa in the presence of transferrin (Cox and Graham, 1979). Pyoverdin negative strains have been isolated from human infections; in contrast pyochelin negative strains have not been found and this siderophore has a dramatic effect on the virulence of P. aeruginosa in mice (Cox, 1982). However recent work suggests that the synthesis of pyoverdin rather than pyochelin may be more important for growth of P. aeruginosa in serum.

1

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FIGURE 1.5

STRUCTURAL FORMULAE OF TWO SIDEROPHORES FROM P. AERUGINOSA (1) PYOVERDIN, (2) PYOCHELIN (FROM GRIFFITHS, 1987b).

In *in vitro* studies with pyochelin and/or pyoverdin deficient mutants pyoverdin was found to be by far the most active siderophore for promoting iron-uptake by P. aeruginosa in serum containing transferrin (Ankenbauer *et al*, 1985). The pH conditions also have a dramatic effect on the activity of P. aeruginosa siderophores. In dialysis type experiments pyochelin and/or pyoverdin converted unavailable iron bound to transferrin into siderophore bound iron or iron utilisable by P. aeruginosa only at acid pH of 5.0 and 6.0 and not at pH of 7.4 (Sryiosachati and Cox, 1986). When tested individually pyoverdin was found to be a more effective siderophore than pyochelin in mobilising iron from transferrin at pH values between 5.0 and 6.0 (Sryiosachati and Cox, 1986). Care must therefore be taken in interpreting the results of earlier *in vitro* assays carried out using glucose containing media as catabolism of glucose may have altered the pH conditions throughout the period of the assay and altered siderophore mediated iron-uptake. The effect of pH on activity of siderophores may have important implications in infection. The pH at sites of local tissue necrosis and inflammation may be slightly acidic favouring siderophore mediated iron-uptake by bacteria (Cox, 1985).

Iron limitation in P. aeruginosa also induces production of several high molecular weight OM proteins which are thought to be involved in uptake of iron (see section 1.1).

1.2.3 OM composition of Gram-negative bacteria grown *in situ* in infection

Since surface structures play a central role in the host-bacterium interaction in infection it is essential to study the surface components of bacteria recovered directly and without subculture from *in vivo*. Expression of IRMPs in the OM of pathogenic bacteria grown *in situ* in infection would provide biochemical evidence that bacteria replicate *in vivo* in an iron-restricted environment. Griffiths *et al*, (1983) found that E. coli strain 0111 recovered directly and without subculture from the peritoneal

cavities of infected guinea-pigs expressed IRMPs of molecular weights 74K and 81K and these proteins were present in amounts equal to, or even greater than, the other major OM proteins. The same pattern of IRMPs were induced following growth of the isolate in laboratory media with added transferrin. A pathogenic strain E. coli 018 K1 H7, which carries the Col V plasmid, induced not only the IRMPs seen *in vitro* but also an extra OM protein of 68K when grown *in vivo* (Griffiths *et al*, 1985). It is not known if this protein is directly related to bacterial iron metabolism; its expression was not related to the presence of the Col V plasmid. When sera from some normal healthy adults were examined for the presence of naturally occurring antibodies to the IRMPs of E. coli 0111 and 018 some individuals had antibodies which reacted strongly with these proteins including the ferric-enterochelin receptor protein (81K). Scortino and Finkelstein (1983) also reported that Vibrio cholerae expressed iron-regulated membrane proteins when grown *in vivo* in the intestines of infant rabbits.

Understanding of the surface changes which occur in pathogenic bacteria as they grow *in vivo* has particular relevance to the development of preventive and therapeutic measures against bacterial infections. Clearly it was essential to consider the surface structures of pathogenic bacteria in naturally occurring clinical infections. Brown *et al* (1984) provided the first direct biochemical evidence that bacteria grow in iron-restricted conditions in the human host. Three IRMPs were expressed in the OM of mucoid P. aeruginosa recovered directly and without subculture from the sputum of a CF patient. These high molecular weight proteins were also expressed following growth of the same isolate *in vitro* in iron-depleted chemically defined media. Protein H1 was also induced in the OM of cells recovered from *in vivo*. Immunoblotting with the patient's own serum revealed the presence of antibodies to all the major OM proteins including protein H1 and the IRMPs. Since protein H1 is induced by magnesium-deprivation (Anwar *et al*, 1983a; Nicas and Hancock, 1980) it would suggest that the CF lung may provide a magnesium-deficient environment for growth of bacteria. The patient had been receiving antibiotic therapy with

tobramycin and pipericillin. It was possible that there was competition between the aminoglycoside tobramycin and magnesium for anionic cell-surface sites normally occupied by divalent cations.

Anwar *et al* (1985) also provided evidence that bacteria replicated under iron-restricted conditions in burn wound infections. Serum from a patient with burn wounds contained antibodies which reacted with the IRMPs expressed by the patients own strain of P. aeruginosa grown *in vitro* in iron-depleted media. The patient's serum had been collected two days after onset of clinical symptoms of wound infection and thus represented the acute-phase antibody response.

In a study of 12 enteric strains (E. coli, P. mirabilis, and K. pneumoniae) recovered by differential centrifugation from the urine of patients with urinary tract infections, Lam *et al* (1984) reported that five of the strains exhibited two or more IRMPs. It was also noted that fresh isolates possessed fewer porin type proteins than the same strains cultured *in vitro* in tryptic soy broth. Similar results were reported by Shand *et al* (1985) for P. mirabilis and K. pneumoniae isolated directly from the urine of patients with urinary tract infections. Three IRMPs were expressed in the OM of *in vivo*-grown K. pneumoniae whilst growth of the same strain in iron-depleted laboratory media resulted in the induction of six IRMPs (Shand *et al*, 1985). Immunoblotting revealed the presence of antibodies in the patient's serum to all the IRMPs induced by growth *in vitro* in iron-depleted media. Recently, Black *et al* (1986) demonstrated that four strains of Neisseria meningitidis isolated from patients with meningococcal disease expressed IRMPs when grown *in vitro* in iron-limited media. Convalescent serum from each of the patients contained antibodies to two or more of these IRMPs.

The results obtained from the few investigations in which bacteria have been recovered directly from infection clearly indicate that pathogenic bacteria express IRMPs when growing *in vivo*. Furthermore, immunoblotting with serum from infected patients has indicated that these proteins are antigenic and are seen by the patient's immune system even in the acute stages of the infection. It will be important

to establish if these proteins are surface exposed and therefore are available to react with these antibodies. The siderophore receptors of some pathogenic stains of E. coli have been shown to be surface exposed and to react with fairly large proteins in solution (Griffiths *et al*, 1983). Kadurugamuwa *et al* (1985a; 1985b) found that subinhibitory concentrations of several cephalosporins lead to reduction in enterobactin production by K. pneumoniae growing in iron-depleted conditions and also to reduction in capsule formation. Reduction in capsule formation resulted in exposure on the bacterial cell surface of a number of OM proteins, including the IRMPs, which were otherwise occluded (Kadurugamuwa *et al*, 1985b).

1.2.4 Growth rate of bacteria *in vivo*

The growth rat of bacteria *in vivo* in infection is dependent on a number of factors related not only to the organism but also to the site of infection and the status of the host's defence mechanisms. Approaches to the study of *in vivo* doubling times of bacteria include the use of radioactively labelled cells (Eudy and Burrous, 1973) as well as temperature-sensitive mutants (Morris Hooke *et al*, 1985; Sordelli *et al*, 1985). These techniques are however open to criticism; labelled cells may, for example, be cleared more quickly by the host's defence mechanisms than the remainder of the bacterial population.

In some animal models of infection division rates approaching those achieved *in vivo* have been demonstrated. P. aeruginosa has been shown to achieve initial division rates of 30 mins in the lung and 20 mins in the peritoneal cavity (Morris Hooke *et al*, 1985; Sordelli *et al*, 1985), they suggest that differences in growth rates of the organism in the two different sites may be due to differences in pH and nutrient availability. The status of the host's defence mechanisms also influence the replication rate; the doubling times in the lungs of mice rendered granulocytopenic was reduced to 16 mins (Sordelli *et al*, 1985). The iron-restrictive nature of the *in vivo* environment may contribute to the slower growth rate of some bacteria *in vivo*. For example, certain strains of E. coli had doubling times of 28 mins when grown *in*

vitro in iron-depleted media but in the presence of transferrin or lactoferrin the doubling time was reduced to 38 mins (Bullen *et al*, 1968). In experimental peritoneal infection sublethal doses of E. coli typically grew for a few hours and then declined. However, if animals were injected with iron-containing compounds the same sublethal dose multiplied much more quickly and killed the animals (Bullen *et al*, 1968). The size of the inoculum used in these studies clearly is going to affect the interaction with the host's defence mechanisms and possibly the apparent doubling time of bacteria. Clearance must therefore be considered.

In many experimental infections the bacterial doubling time increases as the infection proceeds and there is much evidence to indicate that bacteria multiply slowly in the later stages of many chronic experimental infections (Eudy and Burrous, 1973; Brock, 1971; Meynell and Subbiah, 1963). Eudy and Burrous (1973) studied the doubling time of E. coli and P. mirabilis in experimental kidney infections and reported doubling times of 2.3 and 0.9 hours respectively in the first 7 hours after infection. The doubling times increased in subsequent stages of the infection and in both cases were as slow as 20 hours in the 24 to 48 hour period. The results of these animal model studies suggest that bacteria grow slowly in many chronic infections. Whilst deprivation of nutrients may contribute to the slow growth rate *in vivo* other factorincluding the host's immune response will also be important.

1.3 THE HOST-BACTERIUM INTERACTION IN CYSTIC FIBROSIS

1.3.1 Lung infection in CF

Fibrocystic disease of the pancreas, now known simply as cystic fibrosis or CF, has been recognised as a disease entity only since the late 1930's (Andersen, 1938). CF is the most common genetically inherited disorder and is inherited as an autosomal recessive trait (Danks *et al*, 1965; Merritt *et al*, 1962). Progress in the understanding of genetic aspects of CF have been quite remarkable during the past three years. Several laboratories have attempted to identify the CF gene by the use of genetic techniques, in particular the use of linkage analysis. Using this approach the CF gene has been located on chromosome 7q31 (Wainwright *et al*, 1985; Knowlton *et al*, 1985). Work is ongoing to identify the gene. It has been suggested that several genes may be involved and one of these may be located on chromosome 1 (Dorin *et al*, 1987). CF is the most frequent cause of chronic suppurative lung disease in children and young adults of Northern European descent. The incidence in this population is 1 in 2000 live births; it is less frequent in other racial groups (Brunecky, 1982; Kulczycki and Shauf, 1974; Merritt *et al*, 1962). In the United Kingdom some 450 babies with CF are born annually (Goodchild and Dodge, 1985). The clinical features of the disease result from generalised disorder of the exocrine glands including malabsorption, chronic bronchopulmonary infections, and high sweat electrolytes (di Sant' Agnese and Davis, 1976; Wood *et al*, 1976).

Soon after birth the bronchioles of CF patients may become obstructed with thick tenacious secretions (Zuelzer and Newton, 1949). The sticky nature of this mucus may lead to stagnation in the lower bronchioles and these static secretions become infected. Infection stimulates further mucus secretion and so a viscous circle is set up. Mucopurulent secretions, which are difficult to clear by coughing, gradually fill the bronchioles and bronchi. Infection gradually destroys the bronchial epithelium and extends into the peribronchial tissues. This leads to peribronchial

fibrosis as well as to weakness of the bronchial walls, with increased dilatation and bronchiectasis which becomes irregularly scattered throughout the lungs (Goodchild and Dodge, 1985; Wood *et al*, 1976; Andersen, 1962). Recurrent and chronic lung infection remains the major cause of morbidity and mortality for these patients (Goodchild and Dodge, 1985).

Thirty years ago life expectancy for patients with CF was very short but in Britain 50% of children with CF are now surviving to the age of 20 years in reasonable health. Early diagnosis, more effective comprehensive treatment and recognition of milder cases have all contributed to this improved prognosis (Weller, 1986; Goodchild and Dodge, 1985). Treatment of the lung infection is mainly aimed at control of the infection with aggresive antibiotic therapy and removal of thick bronchial secretions by chest physiotherapy (Weller, 1986; Davis and di Sant' Agnese, 1984; Høiby *et al*, 1982).

Serious lung disease is restricted to a limited number of pathogens; these are P. aeruginosa, Staphylococcus aureus and Haemophilus influenzae, however Proteus spp, Klebsiella spp, E. coli, viruses, mycoplasma and fungi can all cause respiratory infection in these patients (Høiby, 1982; Mearns, 1980; May *et al*, 1972). Some recent reports have drawn attention to an increasing incidence of P. cepacia infection in CF; one of these has documented an increase in the prevalence of P. cepacia from 10% in 1971 to 18% by 1981 in a population of approximately 500 patients (Isles *et al* 1984).

S. aureus is the pathogen most frequently isolated from the lungs of patients after initial diagnosis (Mearns, 1980) and unless specifically treated persists in the broncial secretions of these patients (Marks, 1981). Since the introduction of effective anti-staphylococcal drugs in the 1950's the incidence of S. aureus infection has declined whilst that of P. aeruginosa has increased so that P. aeruginosa is now the organism most frequently isolated from the respiratory tract of CF patients (Høiby, 1982; Mearns, 1980).

Attempts have been made to identify the selective nature of sputum from CF patients which accounts for colonisation by only a few bacterial species. Sputum from CF patients were analysed and found to have significant elevations of Na^+, K^+, Ca^{2+} and Mg^{2+} (Kilbourn, 1984; 1980; 1978). Increased concentrations of the calcium-modulating proteins calmodulin and calcitonin have been detected in CF sputum (Kilbourn, 1984) and also in lung tissue from CF patients (Wolf *et al*, 1986). It was suggested that the increase Ca^{2+} concentration makes the sputum more susceptible to colonisation by S. aureus. However, after repeated infections the Ca^{2+} ion concentration appeared to be reduced and the lung became more susceptible to infection by other micro-organisms (Kilbourn, 1978). These alterations in the Ca^{2+} concentration may be related to the levels of the calcium-modulating proteins.

1.3.2 P. aeruginosa in CF lung infection

Lung infections in CF patients caused by S. aureus and H. influenzae may be successfully treated, and in most cases these pathogens may be eradicated by antibiotic therapy (Høiby *et al*, 1982). By contrast once P. aeruginosa is established in the lungs of these patients it is rarely, if ever, eliminated despite aggressive antibiotic therapy (Weller, 1986; Høiby et al, 1982). P. aeruginosa may be isolated from the respiratory tract of over 70% of CF patients at any given time (Pier, 1985), and poor clinical prognosis in CF patients is largely associated with infections caused by this organism (Høiby *et al*, 1977). Therefore the role of P. aeruginosa in CF lung infection shall be the focus of this section.

A striking feature of P. aeruginosa isolates from CF lung infection is the high incidence of mucoid alginate producing strains (Doggett, 1969; Doggett *et al*, 1964; Iacocca *et al*, 1963). Although mucoid strains account for only 0.8-2.1% of clincial isolates in other human infections up to 80% of all P. aeruginosa isolates from CF lung infection are mucoid (Doggett *et al*, 1964). Whilst non-mucoid strains of P. aeruginosa are often isolated initially following colonisation of the patients lungs these are subsequently replaced by mucoid strains (Høiby, 1974). The non-mucoid

strains isolated from the lungs of patients soon after initial colonisation can be allocated to a specific 0-serotype on the basis of agglutination reactions with standard typing sera (Penketh *et al*, 1983) unlike the mucoid strains which frequently tend to be nontypable (Zierdt, 1978). The mucoid alginate may mask the LPS components important in the serotyping reactions. Pyocin typing, phage typing and antibiogram patterns have all be useful in studying these mucoid and non-mucoid isolates from CF lung infection. The mucoid isolates are frequently unstable *in vitro* and split off non-mucoid variants; results of typing studies indicate that the non-mucoid variants belong to the same sero group, pyocin type and phage type and have the same biochemical properties as the mucoid parent strain (Høiby, 1982; Govan, 1975; Williams and Govan, 1973; Zierdt and Schmidt, 1964).

The mucoid alginate produced by these isolates plays an important role in the aetiology of chronic P. aeruginosa lung infection in CF (Govan and Harris, 1986; Costerton *et al*, 1979). Due to the low incidence of mucoid P. aeruginosa isolates from other clinical infection features of the CF lung environment may play an important role in selection of mucoid P. aeruginosa strains. Factors which select for mucoid P. aeruginosa *in vitro* and which may be relevant to the *in vivo* evironment will therefore be reviewed.

Surfactants. Govan (1975) demonstrated an increased stability of mucoid P. aeruginosa in culture media containing various surfactants including dipalmitoyl lecithin which is present in the lungs. Since mucoid P. aeruginosa prevails in chronic pneumoniae associated with CF lung infection, the surfactant in such lungs has to be different from that of normal lungs. Whether this is the case is not yet known.

Cations. Since the concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} are all elevated in CF sputum this prompted the study of the effect of cations on the stability of mucoid P. aeruginosa. Boyce and Miller (1980; 1982) found that calcium and magnesium

concentrations had no effect on either the total viable count or the frequency of non-mucoid revertants in batch cultures of mucoid P. aeruginosa. The concentrations of cations used in these experiments were similar to those present in the sputum from CF patients. These workers did notice some differences in the morphology of the alginate produced by organisms recovered from cation-depleted media and grown on nutrient agar (Boyce and Miller, 1982).

By contrast, the concentrations of iron in the growth medium had a profound effect on the selection of non-mucoid mutants in unshaken batch cultures of mucoid organisms. In medium containing high concentrations of iron non-mucoid derivatives accumulated to a greater than 100-fold higher frequency than mucoid forms. Rates of accumulation of non-mucoid derivatives were lower in media containing low concentrations of iron (Boyce and Miller, 1982). Jones *et al* (1977) in an earlier study had assessed the stability of a wild-type strain and its mucoid variant in chemostat cultures limited by sulphate, magnesium and iron at a fixed dilution rate (D 0.12 h^{-1}). After 2 days a 10% inoculum of the mucoid strain was added to the wild-type and 10% of the wild-type strain was added to the mucoid culture and their stability was followed for 10 days (about 34 generations). Only in the iron-limited culture did the mucoid variant tend to replace the wild-type strain.

This work was extended in a later study in which the stability of the same mucoid strain was assessed in iron, carbon, nitrogen, phosphorus, magnesium and sulphur limited chemostat cultures over a range of dilution rates (Ombaka *et al*, 1983). The mucoid form was more stable under iron-limitation than under all the other nutrient limitations studied. Growth rate also affected the stability of the mucoid strain. Of the nutrient limitations tested phosphate, nitrogen and iron limitation resulted in the greatest production of extracellular polysaccharide. This is relevant to the *in vivo* infection since Brown *et al* (1984) provided direct biochemical evidence to indicate that P. aeruginosa grew under iron-restricted conditions in the CF lung. In addition the growth rate of bacteria in chronic

infections tends to be slow (see section 1.2.4 for refs).

Antibiotics. The stability of mucoid strains *in vitro* may also be increased by the inclusion of low levels of carbenicillin in the medium (Govan and Fyfe, 1978; Govan, 1975). Although differences in drug sensitivity between mucoid and non-mucoid P. aeruginosa are not always observed (Markowitz *et al*, 1980; Anderson, 1974; May and Ingold, 1973) it has been suggested that some mucoid isolates have a small but significant increase in resistance to carbenicillin, gentamicin and tobramycin (Govan and Fyfe, 1978; Govan, 1976). The apparent association of bacterial alginate with increased resistance was confirmed by demonstration of a direct relationship between increased resistance and transfer of the genetic determinant for alginate production into previously non-mucoid strains by bacterial conjugation. Mucoid variants demonstrated increased resistance only on a culture medium which allowed alginate production (Govan, 1976).

The significance of this slightly increased resistance is in the advantage it would give to the mucoid variant in the presence of low drug levels in the lung. Pharmacologic studies indicate that the antibiotics show poor penetration into the lung and commonly do not reach the minimum inhibitory concentration (Levy, 1986; Marks et al, 1971).

Oxygen. Krieg *et al* (1986) have recently reported that selection of the mucoid and non-mucoid phenotypes of P. aeruginosa *in vitro* may be accomplished by varying the levels of air available to the culture. A high reversion rate was demonstrated in batch cultures of mucoid strains of P. aeruginosa, which were not aerated. These reversion of the non-mucoid to the mucoid phenotype could be controlled if air was supplied to the culture at a minimum rate of at least 0.5 $Lmin^{-1}$. The mucoid phenotype was also maintained for extended periods of time by the addition of air. However, increasing the supply of air to the culture altered the number of viable cells present and is also likely to have altered the growth rate of the organism. The

significance of these results is therefore unclear.

Emergency of mucoid strains *in vivo* in the CF lung may be the result from the selective pressure resulting from probably more than one factor. The selective advantage of the mucoid strains to resist host defence mechanisms will also be important.

1.3.3 The microcolony mode of growth

In its natural and pathogenic environments P. aeruginosa demonstrates two complementary modes of growth: the adherent microcolony and the mobile swarmer cell. The mobile swarmer cell provides the movement necessary to colonise a new environment or cause initial colonisation of a compromised tissue or organ. Following contact with the tissue two factors will tend to cause the swarmer cells to change to the microcolony mode of growth; these are, the tendency to attach to a favourable surface and the selective pressure of intact host defence factors (Costerton, 1984; Costerton *et al*, 1983; 1981). Since many P. aeruginosa isolates from CF lung infection produce copious amounts of alginate it was not surprising that electron microscopic examination revealed that P. aeruginosa exists within glycocalyx enclosed microcolonies in the CF lung (Lam *et al*, 1980). The fibrous nature of the anionic glycocalyx consitutes an ion-exchange resin and may act to futher concentrate nutrients around the adherent cells. (Costerton *et al*, 1981; Costerton *et al*, 1979). However, it has also been suggested that the alginate may function as barrier to the uptake of antibiotics and essential nutrients including iron (Govan and Harris, 1986). Cells present within microcolonies are notoriously refractory to antibiotic therapy (Costerton and Marrie, 1983).

Evidence obtained from animal model experiments indicate that the microcolony mode of growth also plays an essential role in overcoming host defence mechanisms. In the animal model of chronic lung infection developed by Cash *et al*

(1979) beads of polysaccharide matrix containing P. aeruginosa are inoculated into the lungs of normal rats. These "artificial" microcolonies withstand the clearance and defence mechanisms which kill a corresponding number of unprotected cells within hours (Costerton, 1984). P. aeruginosa infection in CF is chronic and non-disseminating; the microcolony mode of growth may be essential to understanding how P. aeruginosa persists in the lungs of these patients for many years (Costerton, 1984). The role of bacterial alginate and the microcolony mode of growth in overcoming host defences is considered further in section 1.3.4.

1.3.4 Interaction of P. aeruginosa with host defence mechanisms in CF

Host defence against bacterial infection involves the complex interaction between non-specific and specific mechanisms. Non-specific mechanisms are mediated by fever, inflammation and restriction of iron availability and by antimicrobial factors such as complement and lysozyme. Specific immune defences rely on the co-ordinated response of antibodies, phagocytic cells, lymphocytes and complement. These defences interfere with bacterial growth, promote the uptake and killing of bacteria by phagocytes and inhibit the toxic properties of bacterial components and exoproducts (Mims, 1982, Young and Armstrong, 1972).

When considering the host defence mechanisms against a bacterial species then the virulence factors of that bacterial species must also be considered (Neu, 1983; Peterson, 1980). Virulence factors can be divided into two general groups: cell surface components and extracellular products. Surface components of P. aeruginosa important in pathogenesis include LPS, proteins, pili and alginate or other surface polysaccharides (Vasil, 1986; Aduan and Reynolds, 1979). Extracellular factors which contribute to virulence of P. aeruginosa in lung infections include proteases, exotoxin A, hemolysins, exotoxin S, lipase and pyocyanin (Woods and Sokol, 1986; Pitt, 1986; Liu, 1979).

Non-specific defence mechanisms in the CF lung. Most microbial agents gain access to the human host via the mucosal portals of the intestinal or respiratory tracts (Smith, 1977). Mucus, present on the surface of the respiratory tract prevents attachment of bacteria to the epithelium, and is cleared by exhalation or by the mucocilary transport system. A major component of human mucosal secretions is a glycoprotein known as mucin (McNabb and Tomasi, 1981). Human tracheobroncial mucins have receptors for surface adhesons of both mucoid and non-mucoid strains of P. aeruginosa (Ramphal *et al*, 1987; Ramphal and Pier, 1985). Mucus protects against microbial attachment through specific chemical interactions with glycoproteins, glycolipids, and other surface moieties of the epithelium which contain receptors for bacterial adhesins. Johanson *et al* (1979) showed marked increased adherence of P. aeruginosa to buccal epithelial cells from CF patients compared to cells from control persons in *in vitro* assays. Adherence of non-mucoid strains appeared to be pilus-mediated. Adherence correlated inversely with the amount of cell surface fibronectin and directly with salivary proteases (Woods *et al*, 1980).

Alterations in the physical properties of respiratory secretions from CF patients, which may occur secondary to infection (Wood *et al*, 1976), may intefere with mucocilary clearance. The increased Ca^{2+} concentrations, which are characteristic of the CF lung environment (Gibson *et al*, 1971; Wood *et al*, 1976), alters the rheologic properties of alginate and favours microcolony formation (Govan and Harris, 1986). The microcolony mode of growth may also reduce clearance of P. aeruginosa by the normally highly efficient mucociliary transport system (Costerton *et al*, 1981). Pseudomonas alginate also appears to enhance extracellular lipase activity in a time- and concentration-dependent manner *in vitro* (Wingender and Winkler, 1984) and so may provide the bacterium with a further possible mechanism for causing tissue damage in the lung. Proteases produced by P. aeruginosa promote mucin release from tracheal epithelial cells (Klinger *et al*, 1984) and so may further exacerbate an underlying mechanism of distress in the lung.

Extracellular factors produced by P. aeruginosa may also interfere with cilary activity. Wilson *et al* (1985) reported that a factor present in supernatants from P. aeruginosa cultures interfered with the cilary activity of human epithelial cells *in vitro*. More recently, Hingley *et al* (1986) reported that P. aeruginosa elastase and alkaline protease disrupted the function and structure of demembranated cilia extracted from porcine trachea.

Another factor which plays an important role in host defence mechanisms in the lung is surfactant (Colacicco *et al*, 1973) which has strong antibacterial activity. It has been suggested that the glycocalyx increases the resistance of cells to surfactants in a manner analogous to LPS from other Gram-negative bacteria (Costerton *et al*, 1981). LPS from P. aeruginosa and other Gram-negative bacteria forms complexes with lung surfactant and so interferes with its activity (Brogden *et al*, 1986).

P. aeruginosa produces two hemolysins, one of which is the heat labile phospholipase C and the other is a heat-stable glycolipid (Johnson and Boese-Marazzo, 1980). Phospholipase C hydrolyses certain phospholipids into diacyl glycerol and phosphoryl choline and it acts preferentially on phospholipids containing quarternary ammonium groups, for example, phosphatidylcholine (Liu, 1979). Phosphatidylcholine, which constitutes about 75% of lung surfactant, is critical to lung physiology and may function as a non-immune opsonic factor for alveolar macrophages (Reynolds, 1985). Vasil *et al* (1985) reported that P. aeruginosa isolates from CF lung infection produced high levels of phospholipase C when tested *in vitro*. It has been suggested that P. aeruginosa produces phospholipase as a means of acquiring phosphate (Liu, 1979). Phospholipase C is, therefore, likely to have a special significance in respiratory infections, by interfering with the activity of phospholipids.

The tracheobronchial secretions also contain locally produced proteins including lysozyme and lactoferrin which have non-specific antibacterial activity

(McNabb and Tomasi, 1981). Lysozyme is capable of lysing certain bacterial cells or converting them to spheroplasts. The bacteriostatic activity of lactoferrin, an iron-binding protein, has been attributed to its ability to interfere with iron uptake by bacteria (McNabb and Tomasi, 1981; Bullen, 1981; Weinberg, 1978). However, there is evidence to indicate that the mechanism by which lactoferrin alters bacterial growth is more complex than simple nutritional deprivation. Several studies have reported that purified lactoferrin inhibited the growth of E. coli 0111 (Stuart *et al*, 1984; Bullen et al, 1972). Stuart *et al* (1984) reported that the effect of purified apo-lactoferrin on the growth of E. coli 0111 was a kinetic delay effect; however, the outcome of these growth experiments may have been related to the subinhibitory concentration of lactoferrin used. It was suggested that lactoferrin may bind to the microbial cell membrane for activation of its iron-binding capacity. Smith *et al* (1977) reported that concentrations of lactoferrin, in saliva from CF patients, were 4-fold higher than for normal persons. Iron-limitation may be an important factor contributing to the selection of mucoid variants of P. aeruginosa in the CF lung (see section 1.3.2).

Complement. Complement components are present in bronchial secretions in low concentrations compared with those in serum but they increase during inflammation and are key factors in lung defence (Piedra and Ogra, 1986). The complement system comprises at least 12 different proteins capable of causing lysis and cell death of Gram-negative bacteria (Taylor, 1983; Peterson, 1980). Proteins of the classical pathway have been designated C1 through C9. In addition to lysis of bacteria some components cause local inflammation which focuses some of the body's defence mechanisms on the site of infection; some act as chemotaxins (C3a and C5a) and some act as opsonins (C3b) which enhance the ingestion of invading bacteria by phagocytes. This latter function is one of the primary roles of the complement system since most pulmonary pathogens are resistant to direct killing by complement. Complement may also be bactericidal when associated with specific antibody on

surfaces of certain bacterial species. The terminal proteins of the complement system (C6, C7, C8 and C9) are involved in this biological activity.

The complement system may also be activated by an alternative pathway mechanism without antibody (Gotze and Muller-Eberhard, 1976). Three separate proteins, factor B, factor D and properdin, associate in an inflammed respiratory tract with activation of C3 to its opsonically active form C3b. The polysaccharide portion of LPS from Gram-negative bacteria is responsible for activation of complement by the alternative pathway, whereas lipid A activates complement via the classical pathway in the absence of specific antibodies (Morrison and Kline, 1977).

P. aeruginosa strains are generally effectively opsonised with C3b by the classical pathway and, to a lesser extent, by the alternative pathway (Perterson, 1980). Lack of an 0-antigen side chain was associated with increased sensitivity of P. aeruginosa to opsonisation and phagocytosis (Engels *et al*, 1985). The majority of P. aeruginosa strains isolated from CF lung infection contain reduced amounts of 0-antigen side chains (Ojeniyi *et al*, 1985; Hancock *et al*, 1983) and are sensitive to killing by serum in *in vitro* assays (Pitt *et al*, 1986; Hancock *et al*, 1983). The presence of alginate surrounding cells within the CF lung may explain how these serum sensitive strains persist in the lung (Govan and Harris, 1986). Phenotypically induced alterations in surface components of the bacterial cell may also alter the sensitivity of the bacterium to complement mediated killing (Finch and Brown, 1978; Anwar *et al*, 1983c). P. aeruginosa elastase has a destructive effect on complement components, of the protein components of the complex only C4 and C7 are not degraded by elastase (Schultz and Miller, 1974). Elastase thus depresses the inflammatory response, inhibits chemotaxis of neutrophils and lowers their phagocytic activity (Kharazami *et al*, 1984b).

Phagocytic cells. Alveolar macrophages are the primary cells involved in surveillance of the bronchial and alveolar surface and maintaining the sterility of the

lower airways (Green and Kass, 1964). As well as their role in phagocytosis stimulated macrophages secrete a variety of factors including components of the complement system, superoxide anion, and also elastase, collagenase and neutral protease enzymes (Hunninghake *et al*, 1979). Alveolar macrophages also regulate the specific adaptive immune response of the host by processing antigens and by physical association with lymphocytes (Quie, 1986). During inflammation macrophages located in the interstitium of the lung parenchyma and a ready reserve of PMNs in the marginated systemic pool may be recruited into the lung; other phagocytes, including blood monocytes and eosinophils, may be recruited into lung but the role of these latter phagocytic cells in defence against bacterial infection is not as prominent (Reynolds, 1985). Analysis of lung lavage fluid from patients with chronic lung infection indicated the cell count to reflect mainly PMNs, a normal percentage of lymphocytes and a reduced percentage of alveolar macrophages (Reynolds, 1985).

The specific adaptive antibody response to microbial surface antigens provides the host with opsonic antibodies for phagocytosis by phagocytic cells. Unstimulated alveolar macrophages appear to have receptors for the Fc component of IgG and C3b (Hunninghake *et al*, 1979). When microbes are efficiently opsonised and engulfed phagolysosomes develop; the phagocytic cell undergoes an oxidative burst, concentrating highly lethal microbicidal factors into the phagolysosomes. Phagocytes also contain non-oxygen requiring factors including myeloperoxidase and lysozyme which are bactericidal. In addition, cationic proteins are bactericidal for certain Gram-negative species. Other hydrolytic enzymes from azurophilic granules may be involved in bactericidal mechanisms as well as digestion and degradation of organisms that have been killed by oxidative or other mechanism within phagocytic cells (Root and Cohen, 1981).

Thomassen *et al* (1981) compared the functional activity of alveolar macrophages obtained from the lungs of CF patients with those from normal healthy

individuals. The CF alveolar macrophages functioned normally in the presence of normal serum but phagocytosis by alveolar macrophages from both CF and normal patients was inhibited by a heat-stable factor in CF serum. Studies by Fick *et al* (1984; 1985) have shown that antibody to P. aeruginosa LPS isolated by affinity chromatography from serum and broncoalveolar lavage fluid from CF patients showed poor activity in mediating opsonic uptake and killing in conjunction with alveolar macrophages. Antibody was shown to be fragmented into Fab, $F(ab')_2$ and Fc pieces which indicates that there was proteolytic activity in the lung. Analysis of the proteolytic components present in sputum from CF patients indicate that it was due to elastase released from PMNs (Suter *et al*, 1984; Döring *et al*, 1983), rather than P. aeruginosa protease or elastase.

Many of the extracellular products and surface components of P. aeruginosa interfere with phagocytosis. Mucoid exopolysaccharide inhibits *in vitro* opsonophagocytosis of P. aeruginosa mediated by antibody to LPS (Baltimore and Mtichell, 1980). Schwarzmann and Boring (1971) also reported that P. aeruginosa slime had an antiphagocytic effect on rabbit polymorphonuclear leucocytes. Studies with *in vitro* grown microcolonies of P. aeruginosa indicated that the sheer size of the microcolony may be sufficient to interfere with phagocytic killing by macrophages (Costerton *et al*, 1979). It is important to note that studies of the phagocytic activity of alveolar macrophages from CF patients have been carried out *in vitro* with no attempt to mimic conditions which occur in the CF lung.

Phagocytic activity of macrophages is also impaired after exposure to low doses of P. aeruginosa exotoxin A (Pollack and Anderson, 1978), probably due to the activity of this enzyme on protein synthesis. Protease produced by P. aeruginosa also inactivates PMNs by inhibition of chemotaxis and chemiluminescence (Kharazami *et al*, 1984a; 1984b). It was noted that elastase derived from PMNs inactivated exotoxin A *in vitro* (Döring *et al*, 1985). It is not clear if this enzyme will be active *in vivo* in the inflammed lung.

Humoral response. The lung is now recognised as an important immunologic organ which contributes to host immunity (Bienenstock, 1984). In the upper respiratory tract the major local mucosal immune response is that found in the secretory IgA class. Secretory IgA is synthesised by plasma cells distributed in the lamina propria of the bronchial tract (Tomasi, 1976). It contains an IgA dimer covalently bound to a secretory component. The secretory component stabilises the polymer and renders it less susceptible to proteolytic attack (McNabb and Tomasi, 1981; Tomasi, 1976). When secretory IgA binds to Gram-negative bacteria carbohydrate moieties of the secretory component become exposed, liability to phagocytosis is reduced and interaction with mucus is increased (Edebo *et al*, 1985). The major function of secretory IgA is not as an opsonising antibody but rather its functional role *in vivo* appears to be in interference with attachment to the mucosal membrane (Edebo *et al*, 1985; Stendhal, 1983). The mechanism of its anti-adhesive effect is not well understood. It is possible that specific antibody may agglutinate bacteria or interact with receptors on bacteria or the epithelial cell. Secretory IgA also inhibits antigen uptake by mucosa and neutralises toxic exoproducts of bacteria (van Furth, 1981; McNabb and Tomasi, 1981). Secretory IgA has no bactericidal activity except when complement and lysozyme are present (van Furth, 1981).

IgG is the predominant antibody found in the peripheral airways. IgG may also be found in large amounts in secretions of the upper respiratory tract and may be associated with transudation from serum, particularly during respiratory infection (Bienenstock, 1984). In contrast to secretory IgA, binding of IgG to the surface of bacteria renders them more hydrophobic favouring phagocytic interaction (Stendhal, 1983). IgG alone promotes phagocytosis of bacteria by neutrophils and macrophages without the participation of complement but the opsonic support of phagocytosis is enhanced by complement activation. IgG reacts with soluble antigens to cause precipitation and with insoluble antigens to cause agglutination (Mims, 1982; Roitt, 1972). IgM class antibodies have similar activity to IgG; however, IgM antibodies

are not normally present in the lung (Kazmierowski *et al*, 1977).

Immunofluorescence studies of P. aeruginosa present in the sputum from CF patients indicated that bacteria were coated with IgA and to a lesser extent IgG and IgM antibodies (Hann and Holsclaw, 1976). However, Schiøtz *et al* (1979) reported that anti-pseudomonal antibodies present in the sputum sol phase from CF patients with chronic lung infection were predominantly of the IgG class. There is no discrepancy in these results since one study was concerned with the antibodies present on the surface of P. aeruginosa in the lung and the latter study considered the unbound antibodies present in sputum.

It is well recognised that sera obtained from CF patients with chronic P. aeruginosa lung infection contains elevated titres of IgG, but not IgA or IgM, antibodies to P. aeruginosa antigens (Brett *et al*, 1986; Baltimore *et al*, 1986). These antibodies are directed to P. aeruginosa toxins and cellular antigens.

Results obtained with animal model experiments indicate that P. aeruginosa proteases and exotoxin A contribute to lung pathology in chronic pulmonary infection (Cash *et al*, 1983). The activity of these enzymes may only be important in the early stages of the infection prior to induction of an immune response to these antigens (Döring *et al*, 1985). Sera from CF patients contains high levels of antibodies to P. aeruginosa alkaline protease and elastase (Klinger *et al*, 1978). Whilst the titres of antibody in sputum from CF patients is lower than in serum, free proteases were not detected in sputum samples suggesting that the levels of antibodies were sufficient to neutralise these enzymes (Döring *et al*, 1983).

The hemolysins produced by P. aeruginosa are relatively small molecules. Due to their low molecular weight these products may evade the hosts immune response and so contribute to P. aeruginosa virulence in chronic infection (Döring, 1987).

The antibody response in sera from CF patients to P. aeruginosa cell components has been extensively studied using crossed-immunoelctrophoresis

technqiues. Using this technique up to 64 antigens of P. aeruginosa can be detected (Høiby and Axelsen, 1973). The presence of two or more precipitins in sera from CF patients was taken as an indication of P. aeruginosa lung colonisation (Høiby, 1977). During chronic infection a high and increasing number of P. aeruginosa antigens were detected; up to 61 precipitins were formed with sera from a patient with chronic P. aeruginosa lung infection (Döring and Høiby, 1983; Høiby *et al*, 1977). A high and increasing number of precipitins has been correlated with poor clincial prognosis in these patients (Høiby *et al*, 1977). It has also been reported that an increasing antibody response correlated with the emergence of stable mucoid strains in these patients lungs (Høiby, 1974). However, a major difficulty associated with this technique relates to the problem in identifying antigens involved in precipitin formation.

1.3.5 Role of immune complexes in tissue damage

The observation of high and increasing titres of antibody in serum and sputum from CF patients with chronic lung infection lead to the suggestion that immune complexes may play a central role in tissue damage in these patients (Høiby *et al*, 1986; Moss and Lewiston, 1980, Høiby and Axelsen, 1973). McFarlane *et al* (1975) found deposits of immunoglobulins (mainly IgG and IgM) and complement components (C1q, C3 and C4) in the respiratory and gastrointestinal tracts of CF patients at post-mortem examination. Many investigators have subsequently found increased levels of immune complexes in serum or sputum from CF patients (see Høiby *et al*, 1986 for refs). These immune complexes are thought to be formed locally in the lung but may spill over into the serum and be detected as circulating immune complexes.

Incidence and levels of IgG containing immune complexes appear to correlate strongly with decreases in pulmonary function in CF patients (Dasgupta *et al*, 1987; Moss *et al*, 1980) and also with increased serum IgG titres to P. aeruginosa

(Dasgupta *et al*, 1987). In one study involving 49 patients colonised with P. aeruginosa, the relevance of circulating immune complexes and serum antibodies to discrete antigens of P. aeruginosa was investigated. The 14 patients who died had a significantly higher incidence of immune complexes than the 35 survivors. Patients who died also had much higher levels of IgG antibodies to P. aeruginosa LPS and exotoxin A than did the survivors (Moss *et al*, 1986). P. aeruginosa LPS and also alkaline protease and elastase have been identified in immune complexes from CF patients serum or sputum (Döring *et al*, 1984; Berdischewsky *et al*, 1980).

The biological properties of immune complexes are determined largely by their ability to activate complement and to interact with other host cells. Since IgG, IgM and aggregated IgA may activate complement by the alternative or classical pathways all these classes of antibody may be involved in immune complex formation. Activation of complement results in generation of chemotaxins, release of histamine from basophils, serotonin from thrombocytes and protease from neutrophils and these factors all contribute to tissue damage (Høiby and Schiøtz, 1982). However, tissue damage is thought to occur primarily by release of lysosomal enzymes and oxygen radicals from stimulated PMNs during successful phagocytosis, "frustrated" phagocytosis and after cell death (Høiby *et al*, 1986). Frustrated phagocytosis may occur owing for instance to large immune complexes formed by the exopolysaccharide matrix of microcolonies and antibodies directed against it (Høiby *et al*, 1986), or other antigens which it contains. Woods and Bryan (1985) reported that rats immunised with alginate from mucoid P. aeruginosa and which were subsequently unable to clear a challenge of P. aeruginosa from their lungs, developed deposits of immune complexes in their lung tissues. These workers were unable to identify mucoid exopolysaccharide in these antigen-antibody complexes. Electron microscopic examination has revealed the presence of large deposits of immune complexes at the surface of P. aeruginosa microcolonies present in the lungs of chronically infected rats which had been immunised with LPS prior to infection with P. aeruginosa. These immune complexes contained deposits of IgG,

IgA and IgM (Dr JW Costerton, personal communication). Further work is clearly required to elucidate all the antigens involved in immune complex formation.

1.4 AIMS

The aim of this project was to study the effect of the lung environment on the surface antigens of P. aeruginosa. Mucoid P. aeruginosa grown *in vivo* in the lungs of a CF patient has been shown to grow under iron-restricted conditions (Brown *et al*, 1984). The aim of this study was to extend these observations to further investigate the effect of lung environment on the surface protein and LPS antigens expressed by P. aeruginosa. Chronic experimental P. aeruginosa lung infection in rats was induced using the model developed by Cash *et al* (1979). Mucoid P. aeruginosa was recovered directly without subculture from the lungs of rats 14 days after infection. SDS-PAGE and immunoblotting techniques were used to study the protein and LPS antigens present in OMs prepared from these *in vivo*-grown cells and also from the same isolate grown *in vitro* in iron-plentiful and iron-depleted media. The antibody response in the lungs and sera from infected rats would be compared. It was necessary to adapt the SDS-PAGE system to allow optimal electrophoretic resolution of LPS in proteinase K digests of P. aeruginosa OMs and whole cell lysates.

It has been reported that several of the major OM proteins are highly conserved in different serotypes of P. aeruginosa (Mutharia *et al*, 1982), and so may be potential candidates for vaccine development. It was proposed to study the cross-reaction of antibodies in sera from infected rats with OM antigens of P. aeruginosa strains of different serotypes. The OM antigens would be prepared from cells grown *in vitro* under nutrient conditions which mimicked those *in vivo*.

A sequential study of the antibody response to P. aeruginosa during chronic lung infection would allow the antigens which elicit an antibody response to be identified. Immunoblotting and crossed immunoeletrophoresis techniques were used to investigate which antigens were recognised by antibodies in body fluids from infected rats and at which stage in the infection these antibodies were produced.

Results of these animal model studies indicated that LPS was a major antigen recognised by antibodies in sera from infected rats. The effect of growth phase and specific nutrient limitation on the LPS produced by P. aeruginosa was investigated using qualitative and quantitiative techniques.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacteria

The laboratory organisms used in this study were Pseudomonas aeruginosa NCTC 6750 and M7, a mucoid variant of P. aeruginosa NCTC 6750, isolated in the laboratory by the method described by Govan and Fyfe (1978). Mucoid P. aeruginosa strains PAO 579 and PA 492a were both kindly provided by Dr John Govan (Department of Bacteriology, Medical School, Edinburgh). The mucoid mutant strain PAO 579 was isolated from a serotype 5 strain PAO 381 (Govan *et al*, 1983). Strain PA 492a was isolated from the sputum of a CF patient (Govan *et al*, 1983; Govan & Fyfe, 1978).

Sputum from which P. aeruginosa strains were isolated was kindly provided by Dr Judith Williams (Birmingham Children's Hospital, Birmingham). Other clinical isolates used in this study were obtained from our laboratory culture collection. Clinical isolates of P. aeruginosa used are listed in Table 1. Serotyping was kindly carried out by Dr Malcolm Noy (Microbiology Department, Selly Oak Hospital, Birmingham). Clinical strains were identified as P. aeruginosa by Gram-stain, positive oxidase reaction, growth on Pseudomonas Isolation Agar (PIA) (Difco, West Molesey, Surrey) and on the basis of results of other biochemical tests performed using API 20E strips (API Laboratories Ltd, Basingstoke, Hants).

Enterobacteriaceae. The strains Escherichia coli ECJM, Klebsiella pneumoniae KPEB, Proteus mirabilis PMFB and Serratia marcescens SM were obtained from our laboratory culture collection.

Non-mucoid P. aeruginosa and Enterobacteriaceae strains were maintained on nutrient agar slopes at 4 °C. Mucoid P. aeruginosa were maintained in 3% sterile skimmed milk contained in Bijoux bottles and stored at 4 °C for routine use, or at –70 °C. Strains were subcultured at approximately three monthly intervals.

TABLE 2.1

P. AERUGINOSA STRAINS: SOURCE, SEROTYPE AND COLONIAL MORPHOLOGY

Sputum isolates		
	colony type	serotype#
PANW	rough	NT†
PAAT	rough	NT
PAAA	mucoid	NT
PAJM	mucoid	NT

Serotype strains		
	colony type	serotype#
C17	smooth	0:1
10662	smooth	0:2
CI8	smooth	0:3
CI9	smooth	0:4
0072	smooth	0:5d
PAGS	smooth	0:6
CI10	smooth	0:7
CI11	smooth	0:8
CI12	smooth	0:9
CI16	smooth	0:10
CI4	smooth	0:11
CI14	smooth	0:12
CI13	smooth	0:13
9841	smooth	0:14
9766	smooth	0:15
CI14	smooth	0:17
17TS	smooth	0:17

Others		
	Colony type	Source of isolate
PAE1	smooth	Otitis externa
PAFF	smooth	urinary tract infection

International Antigenic Typing Scheme (IATS)
† NT = nontypable

2.1.2 Chemicals

Chemicals and reagents used and not specified in the text were Analar grade (BDH Chemicals Ltd, Poole, Dorset). All solutions were prepared in double distilled water.

2.1.3 Media

2.1.3.1 Chemically defined media

The chemically defined medium designed by Noy (1982) for growth of P. aeruginosa was used with the modification that the concentration of NaCl was increased from 0.5 mM to 5 mM so the medium was isosmotic with body fluids (Shand, 1985). The chemically defined medium is described in Table 2.2.

2.1.3.2 Complex media

Nutrient agar was obtained from Oxoid (London SE1) and PIA was obtained from Difco (West Molesey, Surrey). Both were prepared and sterilised according to the manufacturers' instructions.

Removal of iron from tryptone soy broth: Two ion-exchange columns were filled with Chelex 100 ion-exchange resin (Bio-Rad Laboratories Ltd, Herts) to a column height of 20 cm after settling had occurred. The resin had been conditioned to pH 7.4 according to the manufacturer's instructions (Bio-Rad Technical Bulletin No 2020). Triple strength tryptone soy broth (TSB) (Lab M, London Analytical and Bacteriology Media Ltd, Salford, Lancs) was passed three times down the first column and then twice down the second column at a flow rate of 2 ml min^{-1}. After treatment essential metal ions were replaced by addition of a micronutrient solution of essential metal ions to a final concentration of $CaCl_2$, 5 x 10^{-7} M; HBO_3, 5 x 10^{-7} M; $CoCl_2$, 5 x 10^{-8} M; $CuSO_4$, 1 x 10^{-8} M; $ZnSO_4$, 1 x 10^{-8} M; $MnSO_4$, 1 x 10^{-7} M and $(NH_4)_6 Mo_7 O_{24}$, 5 x 10^{-19}M in the single strength medium (Fe-TSB). For iron-plentiful TSB (Fe + TSB), $FeSO_4$ was added to a final concentration of 0.02 mM.

TABLE 2.2

CHEMICALLY DEFINED MEDIA CDM_{10}

Nutrient	Molar concentration
NaCl	5.0 mM
KCl	0.62 mM
$K_2HPO_4.3H_2O$	3.2 mM
$(NH_4)_2\ SO_4$	40.0 mM
$MgSO_4.7H_2O$	0.4 mM
Glucose	40.0 mM
MOPS *a*	50.0 mM
$Fe\ SO_4.7H_2O$ *b*	0.062 mM

a MOPS (Morpholinopropane sulphonic acid) buffered to pH 7.8 with NaOH

b $FeSO_4$ solution was acidified with conc. H_2SO_4 to prevent precipitation during autoclaving

Iron-depleted CDM (Fe- CDM) contained no added iron salts.

Magnesium-depleted CDM (Mg- CDM) contained 0.02 mM added magnesium.

Specially purified agar (Fisher Scientific Co, New Jersey, USA) was added to Fe- TSB to a final concentration of 2% w/v, autoclaved at 121 °C for 15 mins and agar plates were poured (Fe-TSA).

2.1.4 Blood, Serum and Sputum Samples

2.1.4.1 Patient's serum and sputum

Patient's serum was separated from blood obtained by venipuncture during the course of clinical investigation. A small portion of the blood was allowed to clot at room temperature for 2 hours, then centrifuged at 2,000 x g for 5 mins and the supernatant serum collected and stored at -20 °C.

Sputum samples were obtained from a CF patient who was hospitalised due to acute exacerbation of pulmonary infection. Samples were pooled, freeze-dried and stored at -20 °C prior to use.

2.1.4.2 Raising of hyperimmune antisera

P. aeruginosa PAO 579 or PA 492a were each grown to early stationary phase in Fe+ TSB and Fe- TSB. Cells were harvested by centrifugation at 8,000 x g for 10 mins at 20 °C and resuspended in sterile pyrogen free 0.85% saline (Travenol Laboratories Ltd, Thetford, Norfolk). Cells were fixed by exposure to formalin (final concentration 4% v/v) for two hours and washed twice with sterile pyrogen free saline.

Male Sprague-Dawley rats were immunised by intraperitoneal injection of a 0.1 ml volume of formalin-fixed bacterial suspension (approx 10^9 P. aeruginosa ml^{-1}) at two and three day intervals for two weeks. Two days after the last injection the rats were bled by cardiac puncture. Blood was allowed to clot for two hours at room temperature and serum separated by centrifugation at 2,000 x g for 10 mins. Supernatant serum was stored in 1 ml aliquots at -20 °C.

Pre-immune blood was also collected from control rats not immunised with

P. aeruginosa and serum separated as above. This pre-immune serum was used to check for pre-existing antibodies to P. aeruginosa.

2.1.5 Equipment

Manufacturers' addresses are only cited once. Equipment and apparatus used in this study and not specified in the text was supplied by:-

Automatic pipettes - Gilson Pipetman, P-200 and P-1000; Anachem, Luton Beds.
- Digital multichannel pipette; Flow Laboraotries Ltd, Irvine, Scotland.

Balances - macro, Oertling HC22; Oertling Ltd, Orrpington, Kent.
- micro, Sartorius 1702; Sartorius Instruments Ltd, Belmont, Surrey.

Blender - Kenwood Model A524 blender; Fisons Scientific Apparatus, Loughborough, Leics.
- Waring Blender; Eberbach Co, Ann Arbar Michigan.

Blood collecting tubes - 10 ml stoppered plain polypropylene tubes; Sterilin Ltd, Teddington, Middlesex.

Centrifuges - Beckman J2-21 high speed centrifuge; Beckman RIIC Ltd, High Wycombe, Bucks
- Eppendorf centrifuge 5412,
- MSE Super Minor bench centrifuge; Measuring and Scientific Equipment, Crawley, Surrey.

ELISA plate washer - model 120; Flow Laboratories Ltd.

ELISA plate reader - Titertek Multiscan model 310C; Flow Laboratories Ltd.

Electron microscope - Hitachi H-600; Hitachi, Japan

Freeze dryer - Edwards Modylo freeze dryer; Edwards High Vacuum Ltd, Crawley, Surrey.

French press - Amicon Corp, Gloucester, Glos.

Gas liquid chromatography equipment - Chromatography series 204 attached to PM 8222 dual-pen recorder; Philips/Pye-Unicam, Cambridge, Cams.

Gel electrophoresis apparatus - large – made in house by Aston Services.
mini – Model 360 mini vertical slab gel and Model II - mini-Protean 125 BR vertical slab gel; Bio-Rad Laboratories Ltd, Watford, Herts.

Gel dryer - Bio-Rad model 224 gel slab drier; Bio-Rad Laboratories Ltd.

Immunoblotting tank - Trans Blot Cell; Bio-Rad Laboratories Ltd.

Immunoblotting power pack - model 250/2.5; Bio-Rad Laboratories Ltd.

Incubators - Gallenkamp orbital shaking incubator, Gallenkamp London.

Laser densitometer -Ultroscan Laser Densitometer LKB2202; LKB Instruments Ltd, Croydon, Surrrey, run by an Apple II Europlus computer (Gelscan software).

Membrane Filters - Gelman Acrodisc, Gelman Sciences, Brackmills, Northampton.

Microscope Wild Model M20 binocular phase contrast microscope; Micro-Instruments Ltd, Oxford.

pH meter - PT1-15; Fisons Scientific Apparatus.
Photography Equipment - Nikon camera FM; Nippon Kogaku KK, Tokyo, Japan with Ilford Pan F film or Kodak technical pan film 2415.
Power pack electrophoresis - Bio-rad model 500/200; CIE - Bio-Rad model 3000/300; Bio-Rad Laboratories Ltd.
Rotary evaporator - Buchi Rotavapor-R; Fisons Scientific apparatus.
Sealable tubes - Screw top culture tubes with teflon lined caps; Sterilin, Teddington, Middlesex.
Sonicator - Daw soniprobe; Dawe Instruments Ltd, Acton, London.
Spectrophotometers - Unicam SP600; Pye-Unicam Instruments, Cambridge.

- Cecil CE 292 Digital; Cecil Instruments, Cambridge.
- Plastic cuvettes for spectrophotometry, Brand, Gallenkamp.
- Scanning uv spectrophotometer; Unicam 8000; Pye-Unicam Instruments.
- Quartz cuvettes for uv spectrophotometer; Helma, Westcliff-on-Sea, Essex.

Syringes - Gillette Surgical, Isleworth, Middx.
Water bath - Grant Instruments Ltd, Barrington, Cambs.
Whirlimix - Fisons Scientific Apparatus.

2.1.6 Antibodies and conjugates used in immunological assays

Congugates used in patients studies were horseradish peroxidase conjugated anti-human IgG, IgA and IgM. These were supplied initially by Miles, Rehovot Israel and then by Miles Scientific, Croydon, Surrey. For animal studies rabbit anti-rat IgM and goat anti-rat IgA antibodies were used. Also horseradish peroxidase congugated anti-rat IgG, anit-rabbit IgG and anti-goat IgG were used. These were supplied by Miles Scientific, Croydon, Surrey.

2.1.7 Preparation of glassware

All glassware was rinsed in tap water, fully immersed in Extran 5% v/v (BDH Chemcials, Atherstone, Warkwickshire) and allowed to stand overnight at room temperature. It was then rinsed once in distilled water, once in 1% v/v HCl, six times in single distilled water and three times in double distilled water before drying at 60 °C. Glassware was sterilised by dry heat at 160 °C for 3 hours.

2.2 EXPERIMENTAL METHODS

2.2.1 Measurement of bacterial concentration

2.2.1.1 Viable counts

This method was used to enumerate P. aeruginosa in inocula and also in the lungs of experimentally infected rats at the time of sacrifice. Ten-fold serial dilutions were made in sterile phosphate buffered saline (PBS) (pH 7.4). Volumes of 100 µl were plated in duplicate on well-dried nutrient agar, PIA and Fe- TSA plates. Plates were incubated at 37 °C for 30 hours and colonies were enumerated using a colony counter.

2.2.1.2 Measurement of optical density

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

$$OD \propto \log \frac{Io}{I}$$

where Io = intensity of the incident light, and I= intensity of the emergent light provided that the light path is constant. The relationship obeys the Beer-Lambert law up to an OD of about 0.3 (Kenward, 1975). Above this absorbance the OD increases less than proportionally due to secondary scattering of light (Meynell and Meynell, 1970). If the suspensions were diluted to an absorbance less than 0.3 proportionality was restored (Kenward, 1975). Measurements of absorbance were made at a wavelength of 470 nm to minimise absorption by metabolic products of the bacteria such as pyocyanin. An aliquot of the growth medium was retained for use as a blank

and as a diluent for optical density measurements. An optical density of 1.0 at 470 nm indicates a concentration of approximately 10^9 cells P. aeruginosa ml^{-1}.

2.2.2 Growth measurements

Growth of bacteria in CDM and complex media was followed by measuring changes in optical density of the culture with time. Flasks containing media, pre-warmed to 37 °C, were inoculated with cells from an overnight culture, grown under the corresponding nutrient depletion, to give an OD 470 nm of approximately 0.01. Each flask was agitated at 180 rpm at 37 °C on a shaking orbital. Samples for estimation of OD were removed ascepticially at appropriate intervals, dilutions being made in fresh growth media when necessary. Undiluted samples were returned to the flask to prevent undue reduction in volume, diluted samples were discarded.

2.2.3 Dry weight determinations

Glass sample pots were allowed to equilibriate overnight on the freeze-dryer and their individual weights recorded. The bacterial cells were fixed in 1% v/v formalin for two hours. Cells were harvested by centrifugation at 10,000 x g for 10 mins and the pellet washed twice with distilled water and resuspended to an estimated optical density of 40 at 470 nm. Three 2 ml aliquots were placed in separate sample pots and freeze-dried to constant weight.

2.2.4 Preparation of samples for electron microscopy

2.2.4.1 Negative staining and shadowing

Copper grids (200 mesh)(Fisher Scientific Co; New Jersey, USA) were coated with Formvar (0.25% ethylene dichloride, Fischer Scientific Co). The bacterial suspension was diluted when necessary with PBS (pH 7.4) to around 10^8 cells ml^{-1}. Four drops of the sample were mixed with 1 drop of 1% (w/v) phosphotungstic acid (pH 7.0). A drop of the suspension was placed on the support

side of the Formvar grid with a Pasteur pipette. After 30 seconds excess liquid was removed by touching the drop with filter paper.

For carbon shadowing a drop of diluted culture was placed on the Formvar grid and excess liquid removed after 30 seconds as above. The grid was mounted on the table in a vacuum evaporator. The carbon was deposited by angle shadowing for 6 seconds under vacuum. Negatively stained or carbon shadowed preparations were observed using an Hitachi H-600 electron microscope.

2.2.5 Rat model studies

2.2.5.1 Preparation of inocula

Chronic pulmonary infection of male rats was established by intratracheal inoculation of mucoid P. aeruginosa in an agar bead slurry using the method developed by Cash *et al* (1979). A 1 ml suspension of early stationary phase cultures was added to 1 ml of a 2% w/v agar solution (specially purified) (Fisher Scientific Co, New Jersey, USA) and mixed; 3 ml of peanut oil was then added. The mixture was stirred vigorously for 10 mins on ice to allow beads to form, after which the suspension was centrifuged at 5,000 x g for 15 mins. The supernatant was removed by aspiration and the pellet was resuspended in sterile PBS (pH 7.4). The beads were allowed to settle and the supernatant removed. This washing procedure was repeated three more times. In a separate study (Reid *et al*, 1985) beads prepared using this procedure, were found to be well formed and to range in size from 20-150 μm (mean 48 μm).

2.2.5.2 Infection procedure

Fourty male Sprague-Dawley rats (150-200 g) were individuallly anaesthetised using anhydrous ether. The trachea was exposed and a volume of the agar bead suspension, contining approximately 10^5 viable bacteria was introduced into the lower left lobe of the rats using a 16 gauge needle with a blunted tip (Fig 2.1).

FIGURE 2.1

RAT MODEL OF CHRONIC LUNG INFECTION.

The rats were placed in cages and they recovered full activity within 1 hour. Animals received food and water *ad libitum*. The animals were sacrificed 14 days post-infection by intramuscular injection of 1 ml sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Mississauga, Canada). Blood was collected by cardiac puncture. The lungs of 10 rats were removed and rinsed with sterile PBS ((pH 7.4) and placed in separate tubes containing 30 ml sterile PBS. The number of viable P. aeruginosa was determined after homogenising the lungs. The lungs from the remaining rats were collected, pooled and stored in PBS at -20 °C. Serum was separated from blood as described in section 2.1.4.

The infection was repeated. One group of rats were infected with PA 492a grown in Fe+ TSB (Group A) or Fe- TSB (Group B). Four animals from each group were sacrificed 1, 4, 7, 14, 21 and 28 days post-infection. Blood was collected from each rat and serum separated. The lungs of three rats from each group were collected and the number of viable bacteria ml^{-1} lung homogenate enumerated as before. Lung lavage fluid was collected by infusion of PBS into the lung via the trachea.

2.2.5.3 Recovery of bactria from *in vivo*

Lungs from thirty rats, which had been stored in PBS, were homogenised vigorously for 60 seconds. Lung cell debris was removed by coarse filtration through Whatman No 44 ashless cellulose paper. Material retained by the filter was washed with saline and the washings added to the bulked filtrate. The bacteria were harvested by centrifugation using alternate fast (8,000 x g for 10 mins) and slow (3,000 x g for 10 mins) spins until the supernatant, viewed by light microscopy, contained mostly bacteria with some cell debris. The pellet was washed twice with saline, resuspended in distilled water and OMs prepared.

2.2.5.4 Preparation of lung fluid for immunoblotting

The supernatant fluid obtained during recovery of bacteria from the lungs of infected rats was freeze-dried and resuspended in 100 ml Tris-HCl 10 mM, 0.9% saline pH 7.4 containing 0.3% Tween 20 (TBS/Tween) and stored at -20 °C. The fluid was designated infected rat lung fluid.

Similarly, lungs from hyperimmunised or control rats were homogenised and lung cell debris removed by centrifugation at 3,000 x g for 10 mins. The freeze-dried supernatants were reconstituted in TBS/Tween and designated immunised rat lung fluid and control rat lung fluid respectively.

2.2.6 Outer membrane preparation

2.2.6.1 Sarkosyl extraction

Outer membranes were routinely prepared by the method of Filip *et al* (1973), using Sarkosyl to solubilise the cytoplasmic membrane. Cells were harvested by centrifugation at 10,000 x g for 10 mins at 4 °C and washed twice with 0.85% saline by centrifugation. The pellet obtained was resuspended in 20 ml of distilled water. After sonication for 8 x 30 second intervals in an ice bath with 30 second intervals for cooling Sarkosyl (N-lauryl sarcosine) was added to the broken cell suspension to a final concentration of 2% w/v. The mixture was incubated at room temperature for 45 mins. Unbroken cells were removed by centrifugation at 7,000 x g for 10 mins and OMs collected by centrifugtion at 38,000 x g for 30 mins at 4 °C. The OM fraction was washed with distilled water and finally resuspended in 1 ml distilled water and stored at -20°C.

2.2.6.2 Sucrose density ultracentrifugation

Outer membranes were also isolated using the sucrose density ultracentrifugation method of Hancock and Nikaido (1978) with some modifications.

Four litres of cells (2 x 2L culture volumes) were harvested by centrifugation

at 10,000 x g for 10 mins. This and all subsequent operations were carried out at 4 °C. The reagents and French press were pre-cooled overnight to 4 °C. Cells were washed once with 30 mM Tris-buffer pH 8.0 and resuspended in 20 ml of 20% (w/v) sucrose in 30 mM Tris-buffer pH 8.0 containing 2 mg deoxyribonuclease (bovine pancreas type III; Sigma, Poole, Dorset) and 2 mg ribonuclease (bovine pancreas type 1-AS; Sigma, Poole, Dorset). The cells were then passed three times through a French press at 15,000 lb/in^2 after which 4 mg of egg white lysozyme (Sigma, Poole, Dorset) was added and the mixture was allowed to stand in an ice-bath for 20 mins. The preparation was diluted with 20 ml of 30 mM Tris-pH 8.0. Cell debris was removed by centrifugation at 7,000 x g for 10 mins. The supernatant was diluted in 25 ml of a 20% (w/v) sucrose solution in 30 mM Tris, pH 8.0 and 4 ml were layered unto a sucrose step gradient containing 9 ml each of 70% (w/v) sucrose and 60% (w/v) sucrose in 30 mM Tris, pH 8.0. The gradient was centrifuged at 100,000 x g for 14 hours in an MSE 20° angle rotor. Afterwards three bands were observed, the lower band containing the OM fraction was removed using a Pasteur pipette. These samples were diluted with distilled water and centrifuged at 38,000 x g for 60 mins. The pellet was washed twice more and finally resuspended in 2 ml distilled water and stored at –20 °C.

2.2.7 Extraction of LPS

An extraction procedure based on the hot-phenol method of Westphal and Jann (1965) was used to extract LPS. Four litres of culture (2 x 2L) grown to early stationary phase at 180 rpm in an orbital shaking incubator at 37 °C were harvested by centrifugation at 10,000 x g for 10 mins. The cells were washed twice with saline and resuspended in 20 ml of 30 mM Tris-HCl pH 8.0. Cells were broken using 10 x 30 second cycles of sonication in an ice bath with 30 second intervals for cooling. The suspension was incubated with 200 μg ml^{-1} deoxyribonuclease (bovine pancreas type III; Sigma Chemical Co, Poole, Dorset), 50 μg ml^{-1} ribonuclease (bovine pancreas type I-AS; Sigma Chemical Co, Poole, Dorset) and 200 μg ml^{-1} lysozyme

with shaking for 2 hours at 37 °C. After addition of 5 ml tetrasodium EDTA (0.5 M) and 200 μg ml^{-1} protease (Streptomyces griseus type XIV; Sigma Chemical Co, Poole, Dorset) the preparation was incubated for 18 hours at 37 °C. The protease was inactivated by heating at 80 °C for 20 mins. The mixture was cooled to 70 °C and a pre-heated 90% (w/v) phenol solution containing 50 g phenol was added and the mixture was stirred vigorously for 10 mins. The suspension was cooled in an ice bath and centrifuged at 10,000 x g for 10 mins at 4 °C. The upper aqueous layer was carefully removed taking care not to disturb the protein-aqueous material at the interface. The lower layer was extracted twice more with water and the aqueous layer dialysed against running tap water for 3 days.

To help precipitate the LPS, 25 mM $MgCl_2$ was added to the contents of the dialysis tubing and LPS was collected by centrifugation at 40,000 x g for 4 hours. The pellet was resuspended in distilled water and washed once by centrifugation and freeze-dried. The KDO concentration of a suspension of LPS (10 mg ml^{-1}) in water was determined as described in section 2.2.4.3. The absence of nucleic acids and proteins was confirmed by lack of absorption at 260 nm and 280 nm using a Pye Unicam SP 8000 uv spectrophotometer with distilled water as blank.

2.3 ANALYTICAL PROCEDURES

2.3.1 Fatty acid analysis

Fatty acids were extracted from whole bacterial cells or extracted LPS by mild alkaline hydrolysis using the method of Moss (1978) and were analysed by gas liquid chromatography (GLC).

Glassware used throughout this procedure was immersed in 1M H_2SO_4 for 18 hours, rinsed with double distilled water and finally with specially pure hexane (BDH Chemicals, Poole, Dorset). Suspensions containing P. aeruginosa whole cells

(10 mg ml^{-1} dry wt) or extracted LPS (10 mg ml^{-1} dry wt) in water were prepared and after thorough mixing 500 µl of each sample to be analysed was placed in sealable tubes (Sterilin, Teddington Middlesex) and 2.5 ml of a 5% (w/v) NaOH solution in 50% (v/v) methanol was added. The tubes were sealed and the fatty acids were saponified by heating the mixture to 100 °C for 30 mins. The saponified mixture was allowed to cool and adjusted to pH 2 with concentrated HCl. The fatty acids were derivatised to methyl esters by addition of 2.5 ml boron trifluoride methanol complex (14% boron trifluoride, BDH Chemicals Ltd, Poole, Dorset) and heating to 80 °C for 5 mins. Fatty acid methyl esters were extracted from the mixture with 5 ml chloroform/petroleum ether 60-80 (1:4). The solvent layer containing the fatty acid methyl esters was placed in a round-bottomed flask and evaporated to dryness using a rotary evaporator. The residue was redissolved in 40 µl hexane and evaported at room temperature to a final volume of approximately 10 µl. Control tubes to which bacteria or LPS were not added were also prepared. A loading of 2 µl of the final preparation was used for analysis. GLC conditions were similar to those described by Lambert and Moss (1983).

Column - 3 m by 2 mm I.dia x 5 mm O.dia glass packed with 3% SP-2100 DOH on 100/120 Suppelcoport (Supelco Chromatography Supplies, Suppelchem, Sawbridgwworth, Herts).
Column temperature programme - 150 °C 1 min, increase at 2 °C/minute to 225 °C, hold for 10 minutes.
Gas pressure - hydrogen 14 psi (for flame ionisation detector)
- air 6.5 psi (for flame ionisation detector)
- nitrogen 23.5 psi - 20 ml/minute as carrier gas through column

Fatty acids in samples were identified by comparison with the retention times of fatty acids in a reference standard containing 23 fatty acids (Bacterial fatty acid methyl ester mixture concentration at 10 mg ml^{-1}; Supelco Inc, Supelchem, Sawbridgeworth, Herts). P. aeruginosa did not contain heptadecanoic acid ($C_{17:0}$) fatty acid. This fatty acid was used as an internal standard to quantitate the GLC

analysis. A solution of heptadecanoic anhydride (Sigma Chemical Co, Poole, Dorset) was prepared at a concentration of 1 mg ml^{-1} in hexane (specially purified) and 50 µl of this solution was placed in sealable tubes. When the solvent evaporated samples for analysis were added and after derivatisation GLC analysis was carried out as described above. Knowing that the peak area of heptadecanoic methyl ester internal standard was equivalent to 50 µg $C_{17:0}$ the peak areas of P. aeruginosa fatty acids could be used to estimate the amount of each fatty acid in a given weight of sample.

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

2.3.2.1 Proteins

Whole cell and outer membrane protein preparations were separated by gel electrophoresis using the method of Lugtenberg *et al* (1975) (as modified by Anwar *et al* 1983a). Optimal separation of the major OM proteins of P. aeruginosa and the Enterobacteriaceae used in this study was achieved using 14% acrylamide gels. 10% acrylamide gels, which allow greater separation of high molecular weight proteins at the expense of lower molecular weights, were also used in assigning molecular weights. Polyacrylamide gels were prepared according to the schedules in Table 2.3. Solutions were mixed in a 250 ml beaker and stirred with a magnetic stirrer. Polymerisation was initiated by the addition of NNN'N'-tetramethylethylene diamine (TEMED) and the solutions poured between the plates of the electrophoresis equipment. The stacking gel was prepared in the same way and when the running gel had polymerised the stacking gel was poured on top. A comb was inserted and the stacking gel left to set. On removing the comb the gel was fitted into an electrophoresis tank and the electrode buffer which contained 0.025 M Tris, 0.19 M glycine and 0.1% pure SDS, pH 8.3, was added to the top and bottom of the tank.

Samples for SDS-PAGE analysis were prepared as described in section

2.3.2.3 and were loaded into wells of the stacking gel and electrophoresis carried out at 40 mA (large, in house system) or 10 mA (mini systems). When the tracking dye had moved 10 cm (large, in-house system) or 6 cm (mini systems) from the top of the separating gel electrophoresis was stopped and the gel were stained for protein overnight in a solution of 0.1% Coomassie brilliant blue R-250 (Sigma Chemical Co, Poole, Dorset) in 50% methanol-10% acetic acid, then destained in 5% methanol-10% acetic acid.

Molecular weights of proteins separated by SDS-PAGE were estimated from a calibration curve of $\log_{10}$ molecular weight of protein standards against Rf where

$$Rf = \frac{\text{distance moved by standard protein through the running gel}}{\text{distance moved by bromophenol blue-marker through the running gel}}$$

Standard marker proteins used were β-galactoside (116K) phosphorylase-b (97.4K) bovine albumin (66K), ovalbumin (45K), carbonic anhydrase (30K) trypsinogen (24K), and lysozyme (14.3K) (Sigma Chemical Co, Poole, Dorset) and were separated on 10% and 14% (w/v) acrylamide gels. Coomassie blue stained gels were scanned with an LKB 2202 Ultrascan laser densitometer run by an Apple II Europlus computer (Gelscan software, LKB Instruments Ltd, Croydon, Surrey). Using the scan obtained the calibration curve was plotted.

2.3.2.2 LPS

The gel system of Lugtenberg *et al* (1975) did not resolve P. aeruginosa LPS into discrete bands. The gel system was modified to allow analysis of the LPS from proteinase K digests of P. aeruginosa OMs and whole cell lysates. The schedule for preparation of polyacrylamide gels for LPS analysis is shown in Table 2.4, the system used is based on that of Laemmli (1970) with the modified stacking buffer described by Palva and Mäkelä (1980). Gels were electrophoresed at a constant current of 22 mA (large, in-house system) or 7.5 mA (mini systems) per gel. Electrophoretically resolved LPS was stained with silver nitrate or electrophoretically

transferred to nitrocellulose (NC) paper and reacted with serum.

2.3.2.3 Preparation of samples for SDS-PAGE

Proteins: The protein concentration of OM and whole cell antigen preparations was determined as described in 2.2.4.1 and adjusted to 10 mg ml^{-1} protein. Samples were mixed with an equal volume of sample buffer (Table 2.3). Samples were routinely denatured by heating at 100 °C for 10 mins. To identify OM protein H1 samples were also denatured at 37 °C for 10 mins prior to electrophroesis (under these conditions protein H1 migrates with an apparent molecular weight of 19 K compared to 21 K following denaturing at 100 °C for 10 mins).

Lipopolysaccharide: Bacterial cell proteins were digested with proteinase K(Sigma Chemcial Co, Poole, Dorset) using the method of Hitchcock and Brown (1983) prior to electrophoretic separation. 50 µl of a suspension of bacterial cells (10 mg ml^{-1} dry wt) and 30 µl of sample buffer were heated at 100 °C for 10 mins. When OM preparations were used they were diluted 1:1 with distilled water prior to addition of sample buffer and denaturing. On cooling 25 µg proteinase K solubilised in 10 ml sample buffer, or sample buffer alone, as a control, was added to each tube. After incubation at 60 °C for 90 mins the samples were analysed by SDS-PAGE.

Solutions of extracted LPS from P. aeruginosa or commercially available E. coli serotype 011.B4 LPS (Sigma Chemical Co, Poole, Dorest (1mg ml^{-1}) were also analysed. Samples were mixed with an equal volume of sample buffer and heated at 100 °C for 10 mins before electrophoretic analysis.

2.3.3 Silver stain of LPS

The method used for staining LPS was that of Tsai and Frasch (1982). LPS was fixed in the polyacrylamide gel by immersion overnight in 40% absolute alcohol–5% acetic acid solution. The solution was replaced by fresh fixing solution containing 0.7% periodic acid and oxidation allowed to proceed for one hour.

TABLE 2.3

GELS AND DENATURING BUFFER FOR SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS RESOLUTION OF PROTEINS

Ingredient	Running gel 10%	Running gel 14%	Stacking gel	Denaturing buffer
Stock 1	12.5 ml	18.75 ml		
Stock 2			5 ml	
10% w/v SDS soln	1.5 ml	1.5 ml	0.3 ml	5 ml
1.5M Tris [a] pH 8.8	18.75 ml	18.75 ml		2.5 ml
0.5M Tris pH 6.8			7.5 ml	
Dist. H_2O	26 ml	18.5 ml	16.0 ml	5 ml
TEMED [b]	0.14 ml	0.14 ml	0.08 ml	
10% AMPS [c]	0.2 ml	0.2 ml	0.1 ml	
Glycerol				2.5 ml
2-mercapto-ethanol				0.25 ml
5% bromo-phenol blue				0.2 ml

Stock 1 - 44 g acrylamide + 0.8 g N, N' methylenebisacrylamide (bis) in 100 ml distilled water

Stock 2 - 30 g acrylamide + 0.8g bis in 100 ml distilled water

a - Tris (hydroxymethyl) aminoethane
b - N, N, N', N' tetramethylene diamine
c - ammonium persulphate, prepared daily

TABLE 2.4

GELS FOR SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS RESOLUTION OF PROTEINS AND LPS

Ingredient	Running gel (14%)	Stacking gel
Stock 1	18.9 ml	
Stock 2		3.3 ml
10% SDS soln	0.6 ml	0.2 ml
3.0 M Tris pH 8.8	7.5 ml	
Tris/Na_2HPO_4 [a] buffer pH 7.8		5.0 ml
Dist. H_2O	30.0 ml	10.45 ml
TEMED	0.03 ml	0.15 ml
1.5% AMPS	3.0 ml	1.0 ml

a 3.25g Tris + 3.48 g $NaH_2PO_4.2H_20$ in 100 ml distilled water, adjusted to pH 7.8 with conc. HCl.

The gel was rinsed in 1L volumes of double distilled water at 30 min intervals to remove unreacted periodic acid. Staining reagent was prepared by adding 5 ml of 20% silver nitrate solution to a mixture of 2 ml conc. ammonium hydroxide and 28 ml 0.1M NaOH. A transient brown precipitate formed which disappeared with mixing, the volume of the staining reagent was made up to 150 ml with double distilled water. The gel was stained for 45 mins at 37 °C with shaking. After staining the gel was again washed with 1L volumes of double distilled water at 30 min intervals. The stain was developed with a solution containing 50 mg of citric acid and 0.5 ml 37% formaldehyde L^{-1} double distilled water. LPS in the gel stained brown. Reaction was terminated by replacing the developing solution with 40% absolute alcohol-5% acetic acid solution. Gels were photographed immediately.

2.3.4 Immunoblotting

Bacterial cell components separated in acrylamide gels were transferred to nitrocellulose (NC) paper (pore size 45 μm; Bio-Rad Laboratories Ltd, Watford, Herts) by the method of Towbin *et al* (1979). The gel was sandwiched against NC paper and placed in a transblot cell filled with ice-cooled transfer buffer (125 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) and surrounded with ice. Transfer was carried out at a constant voltage of 80 V for 2 hours followed by 50 V for 18 hours.

After transfer proteins were visualised by staining with 1% w/v amido black in 10% methanol-7% acetic acid or be reaction with antisera. Complete quantitative transfer of protein and LPS was confirmed by staining the gel after transfer with Coomassie blue stain or silver stain respectively.

For visualisation of antibody-antigen reactions the NC paper was incubated with whole or LPS-absorbed serum diluted 1 in 50 in TBS/Tween or with lung fluid for 4 hours at 37 °C. The paper was then washed thoroughly with TBS. Binding of human IgG, IgA or IgM were investigated by subsequently incubating the NC paper

with horseradish-peroxidase goat anti-human IgG, IgA or IgM conjugates respectively diluted 1 in 1000 in TBS/Tween. The NC paper was again washed thoroughly and antigenic sites visualised with 25 μg ml^{-1} solution of 4-chloro 1-naphthol in TBS containing 0.1% H_2O_2.

Binding of IgG in rat lung fluids or dilute sera was investigated using horseradish-peroxidase rabbit anti-rat IgG. For IgA and IgM studies a two step incubation was required. Rinsed NC was incubated for two hours with goat anti-rat IgA or rabbit anti-rat IgM. After rinsing the NC paper was incubated with horseradish-peroxidase rabbit anti-goat IgG or goat anti-rabbit IgG conjugates as appropriate for a further two hours. These antibodies were all diluted 1 in 1000 in TBS/Tween. Antigenic sites were visualised as before.

For immunodetection of LPS antigens the nitrocellulose was baked at 70 °C for 1 hour (Sturm *et al*, 1984) immediately after the initial blocking stage. This procedure improved the resolution of the high molecular weight portions of the O–antigen.

Strip immunoblotting was carried out when the reaction of several antisera or different dilutions of one antiserum with the antigenic determinants in one preparation was to be investigated. A comb with one large well was placed in the stacking gel of the polyacrylamide gel. After transfer of electrophoretically separated antigens to the solid phase the NC paper was divided into 6 mm strips. One strip was stained with amido black, the remaining strips were placed in trays which were divided into individual tracks. The remaining stages of the immunoblotting procedure were carried out in these trays.

The reproducibility of OM antigen transfer to NC paper and the immunoblotting procedure was investigated. The OM antigens of a range of Gram-negative organisms were transfered to NC paper and visualised by amido black staining or by immunoblotting with hyperimmune rabbit serum to P. aeruginosa. One stain and one immunoblot was carried out on three separate occasions. These

procedures were entirely reproducible.

2.3.5 Absorption of antisera with extracted LPS

Sera were absorbed with extracted LPS from the patient's or animal's own isolate to avoid immunological detection of proteins due to co-migrating LPS (Poxton *et al*, 1985). One ml of a solution containing 5 mg extracted LPS was mixed with an equal volume of serum and the mixture was tumbled for 1 hour at 37 °C followed by incubation overnight at 4 °C. The serum was clarified by centrifugation at 4, 000 x g for 15 mins. A further 1 ml of LPS suspension was added and the absorption procedure repeated at least once more. The serum, designated LPS-absorbed serum, was sterilised by filtration (0.45 μm filter) and stored at -20 °C.

Fig 2.2 shows the reaction of hyperimmune and control rat serum before and after LPS-absorption with P. aeruginosa OM proteins (Fig 2.2a) and whole cell proteinase K digests (Fig 2.2b). No qualitative differences were noted in the reaction of IgG, IgA and IgM antibodies with P. aeruginosa OM proteins following LPS-absorption. IgG, IgA and IgM in whole antisera reacted with LPS of the homologous strain. There was no reaction when proteinase K digested whole cells were probed with LPS-absorbed serum using anti-rat IgG, IgA and IgM as the second antibody. Serum from control rats did not react with P. aeruginosa OM proteins or LPS on immunoblots.

2.3.6 Enzyme-linked immunosorbent assay (ELISA)

An ELISA method was developed based on that described by Borowski *et al* (1984) for detection of IgG, IgA and IgM antibodies to P. aeruginosa surface antigens. Early stationary phase cells were harvested by centrifugation at 10,000 x g for 10 mins at 20 °C and the pellet was resuspended in PBS pH 7.4. Preliminary experiments were carried out in which hyperimmune antiserum diluted 10^{-1} - 10^{-6} was titrated in ELISA plate wells coated with a suspension containing 10^5-10^9 P. aeruginosa c.f.u. ml^{-1}. A coating suspension containing 10^8 c.f.u. ml^{-1} was found to

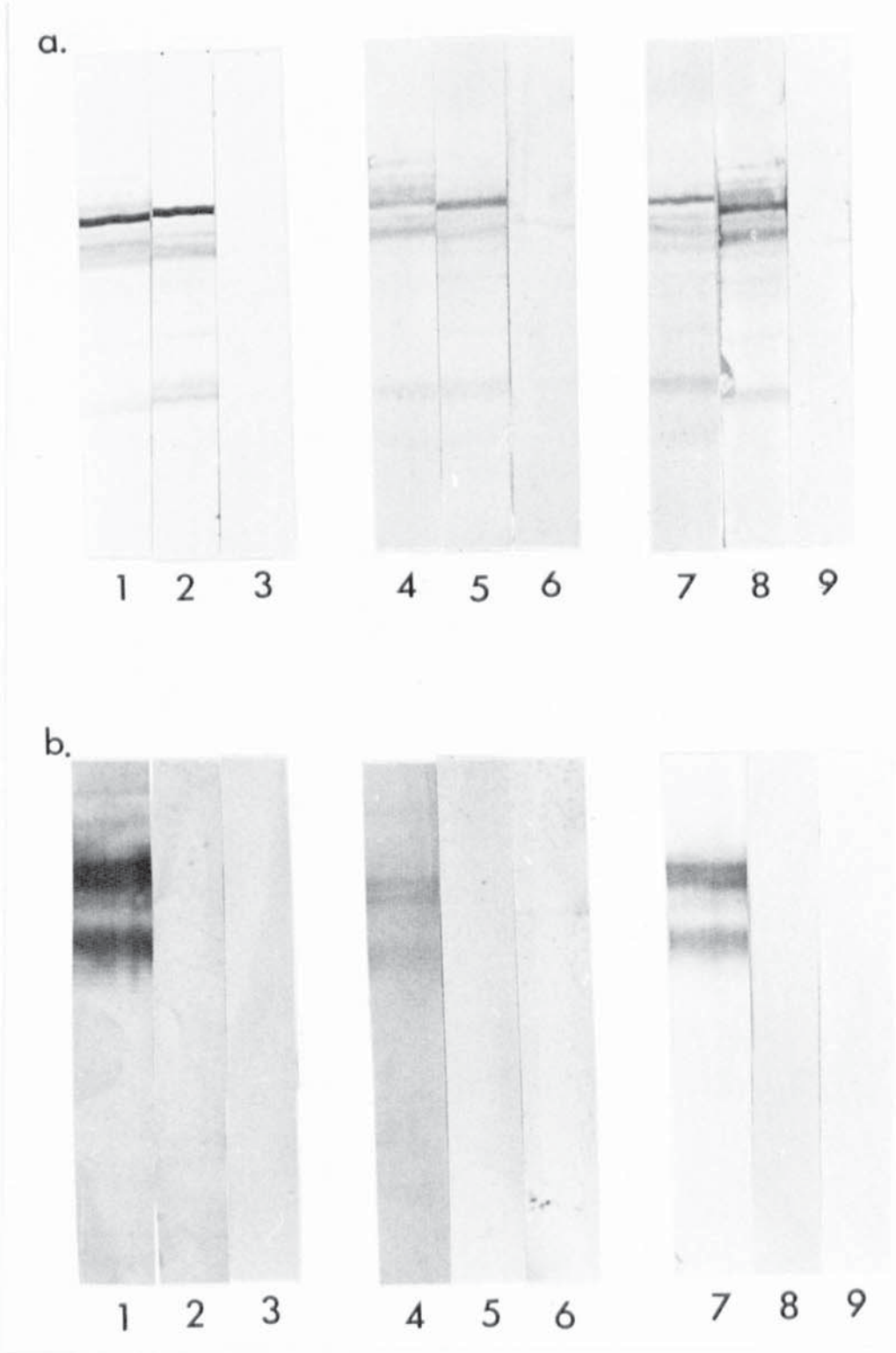


FIGURE 2.2, a and b

IMMUNOBLOT ANALYSIS OF PA0 579 OM ANTIGENS (Fig 2.2a) AND PROTEINASE K DIGESTS OF PA0 579 OMs (Fig 2.2b) AFTER REACTION WITH WHOLE SERUM (LANES 1, 4, 7) AND LPS-ABSORBED SERUM (LANES 2, 5, 9) FROM RATS IMMUNISED WITH THE HOMOLOGOUS STRAIN AND PRE-IMMUNE RAT SERUM (LANES 3, 6, 9). THE SECOND ANTIBODY USED WAS ANTI-RAT IgG (LANES 1-3), ANTI-RAT IgA (LANES 4-6) AND ANTI-RAT IgM (LANES 7-9).

give optimal sensitivity and low background due to non-specific absorbance.

To each well 250 µl of whole cell suspension was added. Plates were coated by shaking at room temperature for 1 hour. Formalin at a final concentration of 1% (v/v) was added to each well and plates were incubated for 14 hours at 4 °C. Plates were washed 4 times with PBS, pH 7.4 containing 0.5% Tween 20. Non-specific protein binding sites were blanked with a solution containing 1% (w/v) bovine serum albumin in RPM1 1640 (Gibco Ltd, Paisley, Scotland) containing 10% (v/v) foetal calf serum (Gibco Ltd) by incubating for 30 mins with shaking at room temperature then 2 hours at 37 °C. The wells were washed as above and to each well was added 50 µl of serially diluted serum in gelatin phosphate buffer (0.07 M sodium phosphate buffer pH 6.5 containing 0.2% gelatin) or plain phosphate gelatin buffer. The plates were incubated for a further 2 hours with shaking at room temperature. The plates were again washed as above then incubated for 2 hours with 100 µl anti-antibody or horseradish-peroxidase conjugates. The optimal dilutions of horseradish-peroxidase linked conjugates and anti-antibody preparations for use in ELISA were pre-determined by checkerboard titrations. The plates were washed as above and 100 µl of a substrate solution added. The substrate solution was prepared by dissolving 10 mg 3,3',5,5';-tetramethylbenzidine (Sigma Chemical Co, Poole, Dorset) in 1 ml of dimethylsulphoxide and adding to 100 ml of 0.1 M sodium acetate/citrate buffer pH 6. Immediately before use 8 µl of H_2O_2 was added and the plates shaken at room temperature for 5 mins. The reaction was stopped by adding 50 µl of 2N H_2SO_4 to each well and a yellow colour developed. Absorption at 450 nm was measured using a Dynatech plate reader. Control wells to which gelatin phosphate alone (blanks) or anti-antibody and horseradish peroxidase conjugates were added consistently produced absorbance values <0.2. The mean absorbance of the blanks was subtracted from the mean absorbance of test wells. Corrected absorbance readings ≥0.1 optical density units at 450 nm were considered positive. Results were expressed as the reciprocal of the serum dilution giving a corrected absorbance of 0.1

optical density units at 450 nm.

The interassay co-efficient of variation (CV) (CV=standard deviation/mean x 100%) were calcuated from the endpoint IgG titres in sera from infected rats carried out in triplicate, repeated on five occasions. The mean interassay CV was 8.8%. The intraassay CV of one ELISA run was also calculated from absorbance values on eight duplicate wells of the same sample. The mean interassay variation for IgG was 1.7%.

The results presented are the mean end-point titres calculated from assays carried out in triplicate on one occasion. All assays were repeated at least once.

2.3.7 Crossed immunoelectrophoresis (CIE)

Crosed immunoelectrophoresis was performed with an intermediate gel (CIEWIG) for all studies using the method of Weeke (1973). The intermediate gel contained either antigen (for crossed-line immunoelectrophoresis) or saline (0.145 M).

A 1% (w/v) agarose suspension (Seakem Agarose ME; FMC, Maine, USA) with medium electroendosmosis was prepared in Tris-barbital buffer pH 8.6. The barbital buffer was prepared by dissolving 4.48 g barbituric acid CIV (Fisher Scientific Co, New Jersey, USA) 8.86 g Tris-base (Sigma, USA) and 0.108 g calcium lactate (Fisher Scientific Co, New Jersey, USA) in 1L of water. The pH of the buffer was adjusted to pH 8.6 with a few drops of 1N NaOH if necessary. The agarose solution was cooled to 48 °C and poured onto a glass plate in an agarose/surface area ratio of 0.18 ml/cm^2. Electrophoresis was carried out on a water-cooled 10 °C flat-bed electrophoresis apparatus with Tris barbital pH 8.6 buffer. The first-dimensional separation was carried out at 10 Vcm^{-1} until a bromophenol blue-labelled human albumin marker had migrated 26 mm. A 6 cm x 1 cm agarose strip was transferred to the hydrophobic side of gel bond (FMC Bioproducts, Rockland, USA) (6 cm x 5 cm). The intermediate gel containing 0.145

M saline or OM (16.7 µl cm^{-2} protein) was poured. A 400 µl aliquot of serum was mixed thoroughly with 1% w/v agarose which had been cooled to 50 °C and immediately poured unto the gel bond taking care to coat the entire remaining surface area. Electrophoresis was carried out perpendicular to the first dimension separation at 2V cm^{-1} for 18 hours.

The gels were pressed, washed and stained as described by Weeke (1973). The gel was sandwiched between sheets of damp blotting paper and pressed using a 1kg weight for 10 mins. The gels were washed in 0.85% saline for 20 mins and pressed again for 10 mins. The gels were dried in a stream of hot air and stained with Coomassie blue R-250. The stain contained Coomassie blue R-250 in 0.5% in ethanol 45%-acetic acid 10%. Excess staining solution was removed by immersing gels in destaining solution containing ethanol 45%-acetic acid 10%.

2.3.8 Quantitative analyses

2.3.8.1 Lowry protein assay

The protein concentration of whole cell and OM preparations were determined using the assay developed by Lowry *et al* (1951). Bovine serum albumin (Sigma Chemical Co, Poole, Dorset) standards (0-200 µg/ml) or samples for determination were made up in 0.35 ml volumes of distilled water. Each sample was mixed with an equal volume of 1M NaOH and heated at 100 °C for 5 mins. On cooling 3 ml of a solution which was prepared by mixing 1 ml of 1% (w/v) $CuSO_4.7H_2O$ with 1 ml of 2% (w/v) NaK tartrate solution and 100 ml of 2% (w/v) sodium carbonate in 0.1M NaOH was added. After leaving for 10 mins at room temperatre 0.3 ml Folin Ciocalteau reagent (Sigma Chemical Co, Poole, Dorset) diluted 1:1.5 with water was added to each tube and the contents mixed by vortexing. After 30 mins the absorbance at 750 nm was recorded. A calibration curve of protein concentration against absorbance at 750 nm of known standards was used to estimate the protein concentration of samples.

2.3.8.2 KDO assay

Extracted LPS: A method based on that of Osborn (1963) was used. Samples of KDO standard (10-100 µg) NH_4^+ salt (Sigma Chemical Co, Poole, Dorset) or 100 µl, 50 µl and 25 µl samples of a suspension of extracted LPS (1mg/ml) were made up to 0.25 ml with 0.05 M H_2SO_4 and hydrolysed by heating in sealable tubes at 100 °C for 30 mins. On cooling, 0.25 ml periodic acid (0.025 M in 0.0625 M H_2SO_4) was added. After warming to 55 °C for 20 mins 0.5 ml of 2% (w/v) sodium arsenite (Sigma Chemical Co, Poole, Dorset) in 0.5 M HCl was pipetted into each tube and the contents mixed thoroughly. After 3 mins 2.0 ml of 0.3% (w/v) thiobarbituric acid in distilled water was added and the tubes sealed and heated at 100 °C for 20 mins. After cooling to room temperature, the absorbance of each solution at 550 nm was measured. By plotting a standard calibration curve of KDO concentration against OD 550 nm the KDO content of samples was calculated.

Whole Cells: Cells were harvested by centrifugation at 10,000 x g for 10 mins and washed once with saline. A pellet of cells (wet weight 50 mg) was suspended in 2.5 ml H_2SO_4 (0.05M) and heated at 100 °C for 30 mins. After cooling cell debris was removed by centrifugation at 17,000 x g for 15 mins. Nine 0.25 ml volumes of the supernatant were placed in separate tubes and 0.25 ml H_2SO_4 (0.5M) alone or spiked with 40 µg KDO or 80 µg KDO were added to three tubes containing samples. The assay was performed as described above for KDO determination of extracted LPS. The spiking procedure was used to determine if interfering substances such as DNA were contributing to the end-point absorbance.

3. RESULTS AND DISCUSSIONS

3.1 EFFECT OF IRON DEPLETION ON GROWTH AND ON SURFACE ANTIGENS OF P. AERUGINOSA

3.1.1 Growth characteristics of P. aeruginosa isolates from CF lung infection in iron-plentiful and iron-depleted media

The growth characteritics of P. aeruginosa PAAT and PANW in iron-plentiful and iron-depleted media were investigated by monitoring the optical density of the cultures at 470 nm. The doubling time of strain PAAT during the exponential phase was 33 mins during growth in Fe- TSB whereas strain PANW had an exponential doubling time of 38 mins during growth in Fe- TSB. The growth rate of each of these strains was not affected by the concentration of iron in the medium (Fig 3.1). Figure 3.2 presents the growth characteristics of PAAT and PANW in Fe+ CDM and also in Fe- CDM. The exponential growth rates of each of these isolates were considerably slower in CDM as compared to TSB (33 mins in TSB as compared to 60 mins in CDM for PAAT). Onset of stationary phase occurred at lower optical densities in CDM (OD 470 nm of 0.32 in Fe- CDM as compared to 1.8 in Fe- TSB for PAAT). Copious amount of a pale green pigment was present in the supernatants from Fe- CDM stationary phase cultures but not in Fe+ CDM supernatants. Cell pellets from iron-plentiful cultures were salmon pink coloured whereas pellets from iron-depleted cultures were non-pigmented.

3.1.2 Surface structures of P. aeruginosa isolates: electron microscopy

Early stationary phase cells were negatively stained or carbon shadowed prior to electron microscopic examination. The photomicrographs in Figure 3.3 show representative negatively stained cells of the laboratory strain NCTC 6750 and its mucoid variant M7. Some non-erect fimbriae were evident on the surface of P. aeruginosa 6750 cells grown in Fe+ CDM (Fig 3.3a). M7 also appeared to express some non-erect fimbriae when cultured in Fe+ CDM (Fig 3.3b). No

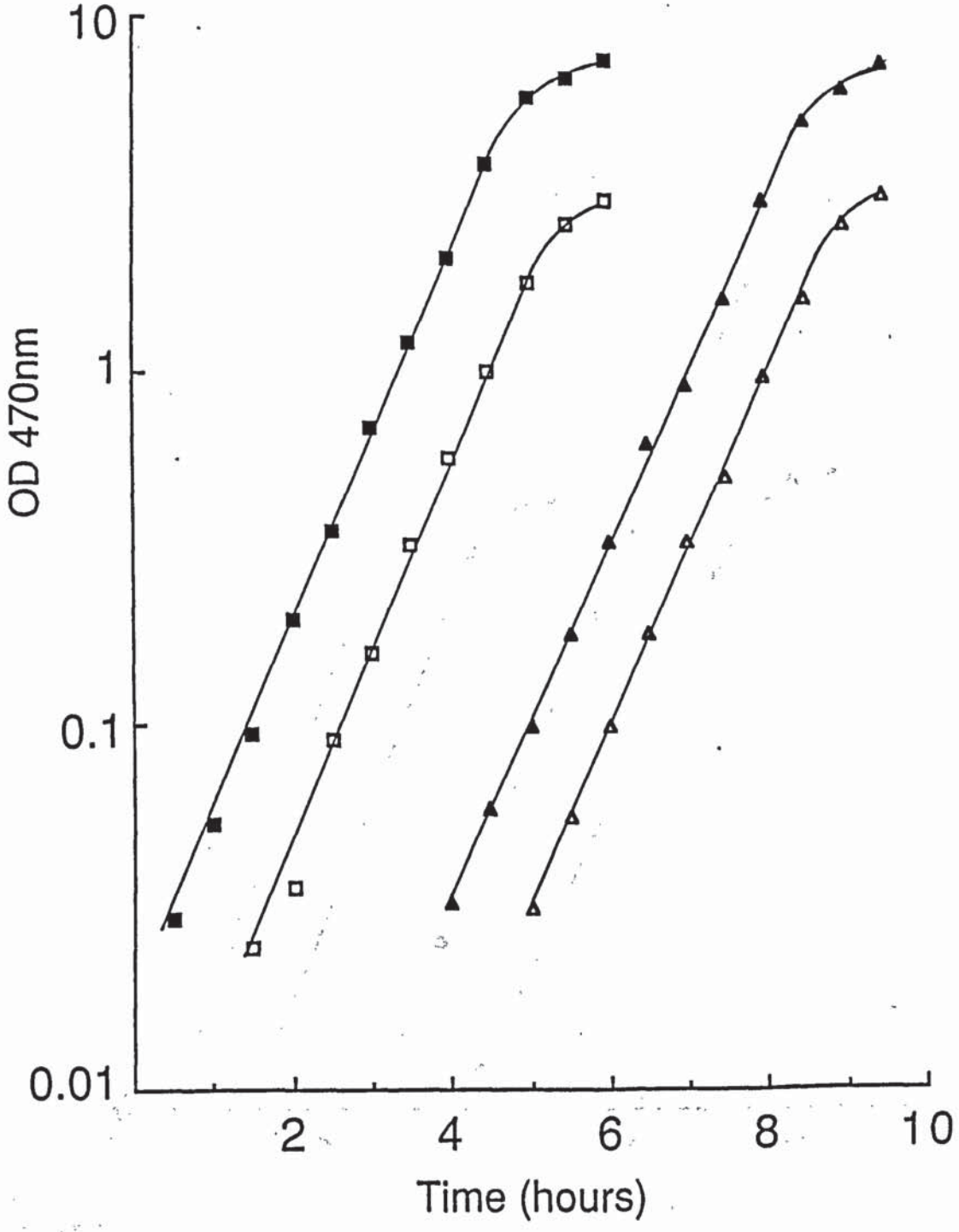


FIGURE 3.1

GROWTH OF P. AERUGINOSA PAAT IN Fe+ TSB (-■-) AND Fe- TSB (-□-) AND OF P. AERUGINOSA PANW IN Fe+ TSB (-▲-) AND Fe- TSB (-Δ-). GROWTH WAS MONITORED BY CHANGES IN OD AT 470 nm. EXPONENTIAL DOUBLING TIME AND OD AT ONSET OF STATIONARY PHASE

-■- 33 minutes; OD 2.8

-□- 33 minutes; OD 1.8

-▲- 38 minutes; OD 4.1

-Δ- 38 minutes; OD 2.0

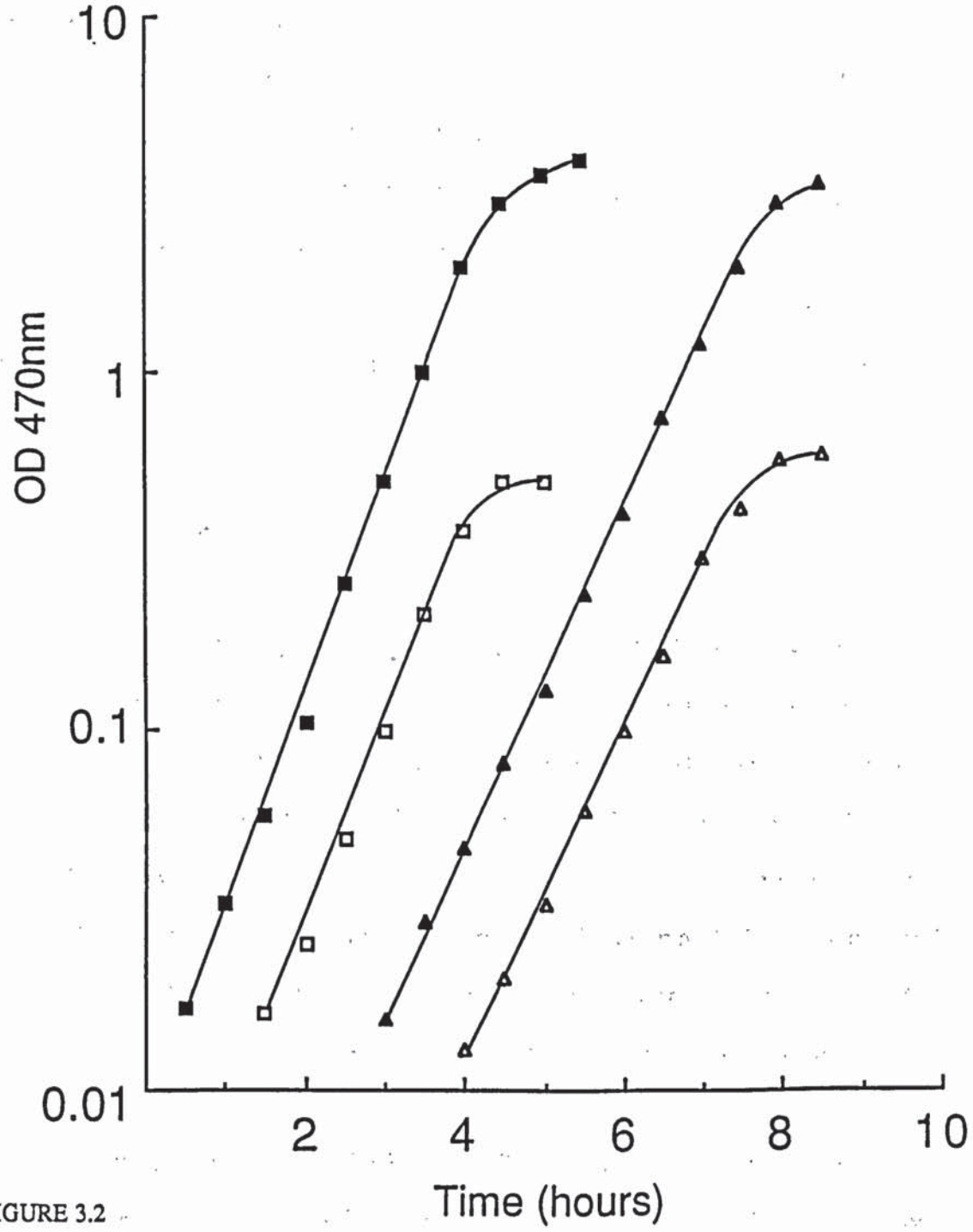


FIGURE 3.2

GROWTH OF P. AERUGINOSA PAAT IN Fe+ CDM (-■-) AND Fe- CDM (-□-) AND OF P. AERUGINOSA PANW IN Fe+ CDM (-▲-) AND Fe- CDM (-Δ-). GROWTH WAS MONITORED BY CHANGES IN OD AT 470 nm. EXPONENTIAL DOUBLING TIME AND OD AT ONSET OF STATIONARY PHASE:

-■- 60 minutes; OD 2.4

-□- 60 minutes; OD 0.32

-▲- 100 minutes; OD 2.5

-Δ- 100 minutes; OD 0.42

appendages were evident on the surface of PA 6750 or M7 grown in Fe- CDM (Fig 3.3c and d). Some electron dense material was present on the surface of M7 cells grown in Fe- CDM (Fig 3.3d). Figure 3.4 shows typical negatively stained cells of non-mucoid P. aeruginosa PANW (Fig 3.4a) and a stable mucoid strain, PAAA (Fig 3.4b) isolated from the sputum of CF patients and cultivated in Fe- CDM. Both strains were flagellated and appeared to produce a glycocalyx which contained electron-dense crystals. Flagella and glycocalyx material were also evident in photomicrographs of carbon-shadowed preparations of non-mucoid PAAT (Fig 3.4c) and mucoid PAJM (Fig 3.4d). These and all other isolates from CF lung infection examined were flagellated but did not appear to express fimbriae.

3.1.3 Effect of iron-depletion on P. aeruginosa OM proteins and LPS

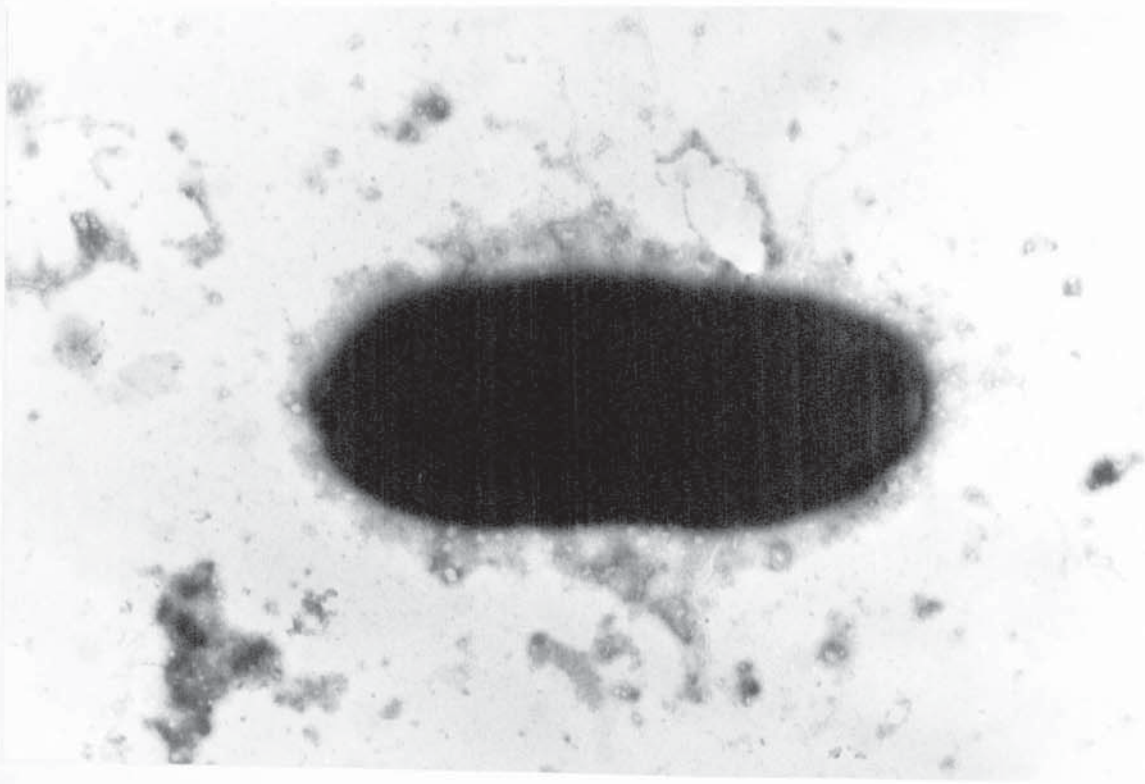
Figure 3.5a shows the protein profile of sarkosyl-extracted OMs prepared from early stationary phase cells of PANW grown in Fe+ TSB (lane 1) and Fe- TSB (lane 2) and PAAT grown in Fe+ TSB (lane 3) and Fe- TSB (lane 4). The OM protein profiles of each of these isolates after growth in Fe+ TSB were similar to the published profiles of P. aeruginosa (Mizuno and Kageyama, 1978; Hancock and Carey, 1979). Several OM proteins with apparent molecular weights of 76 K, 83 K and 85 K were induced following growth in Fe- TSB. These proteins were designated IRMPs.

Figure 3.5b shows that the IRMP of 76 K was not completely repressed in each of these isolates by growth in Fe+ CDM (lanes 1 and 3). Possibly the low solubility of iron in simple salts media makes iron-plentiful conditions difficult to achieve. An additional low molecular weight protein of 22.5 K was induced. Anwar *et al* (1984) reported that two proteins with apparent molecular weights of 22 K and 17 K were induced in the OM of a P. aeruginosa isolate grown in chelexed nutrient broth supplemented with iron. These low molecular weight proteins may be involved in a low affinity iron-uptake system. The IRMPs were expressed less in the OM of

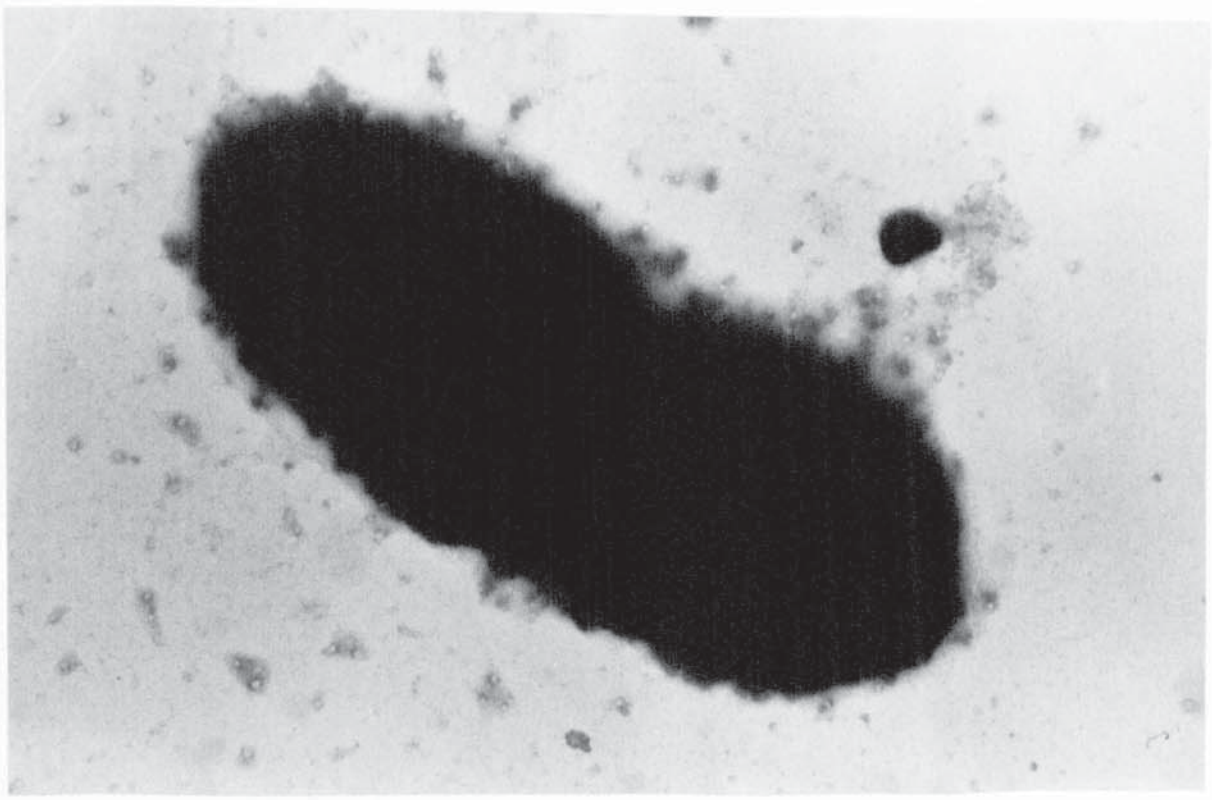
FIGURE 3.3, a - d

ELECTRON MICROGRAPHS SHOWING THE SURFACE STRUCTURE OF NON-MUCOID P. AERUGINOSA 6750 AND ITS MUCOID VARIANT M7 REVEALED BY NEGATIVE STAINING. EARLY STATIONARY PHASE CELLS OF P. AERUGINOSA 6750 AND M7 GROWN IN Fe+ CDM ARE SHOWN IN FIGURES (a) AND (b) RESPECTIVELY. EARLY STATIONARY PHASE CELLS OF P. AERUGINOSA 6750 AND M7 GROWN IN Fe- CDM ARE SHOWN IN FIGURES (c) AND (d) RESPECTIVELY.

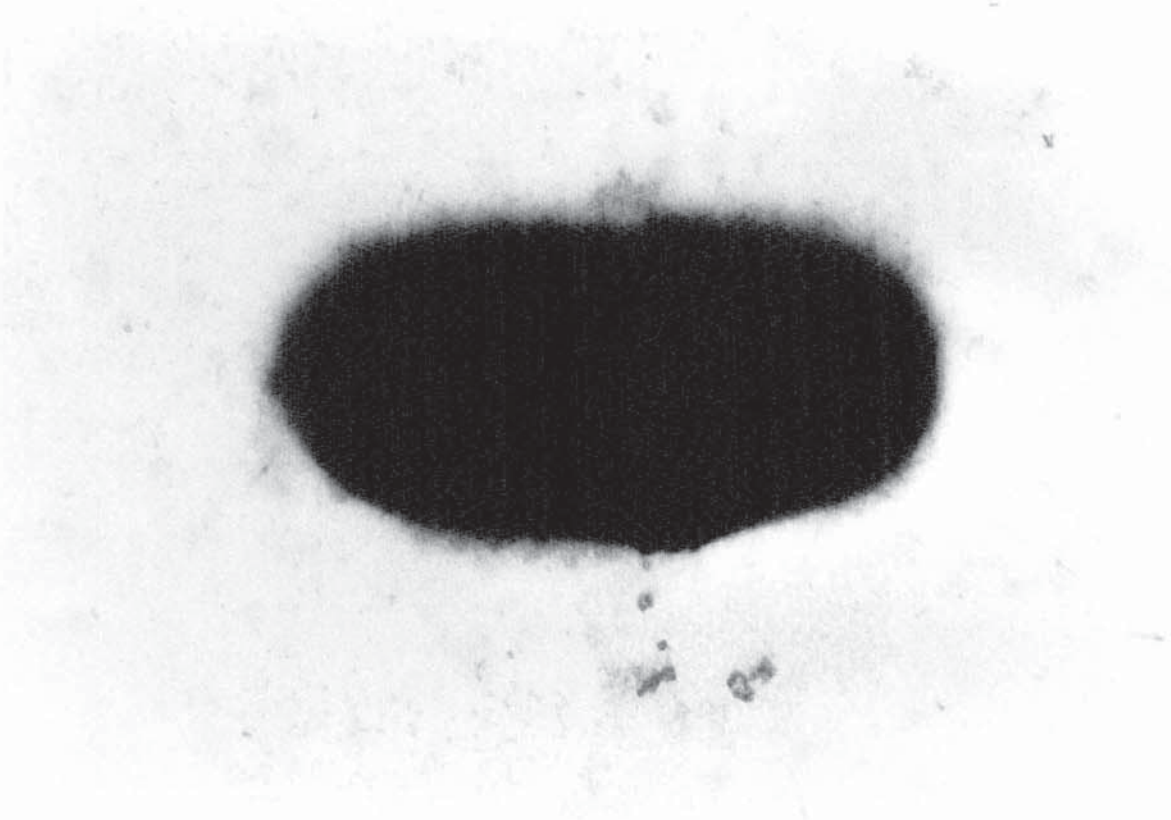
a



b



c



d

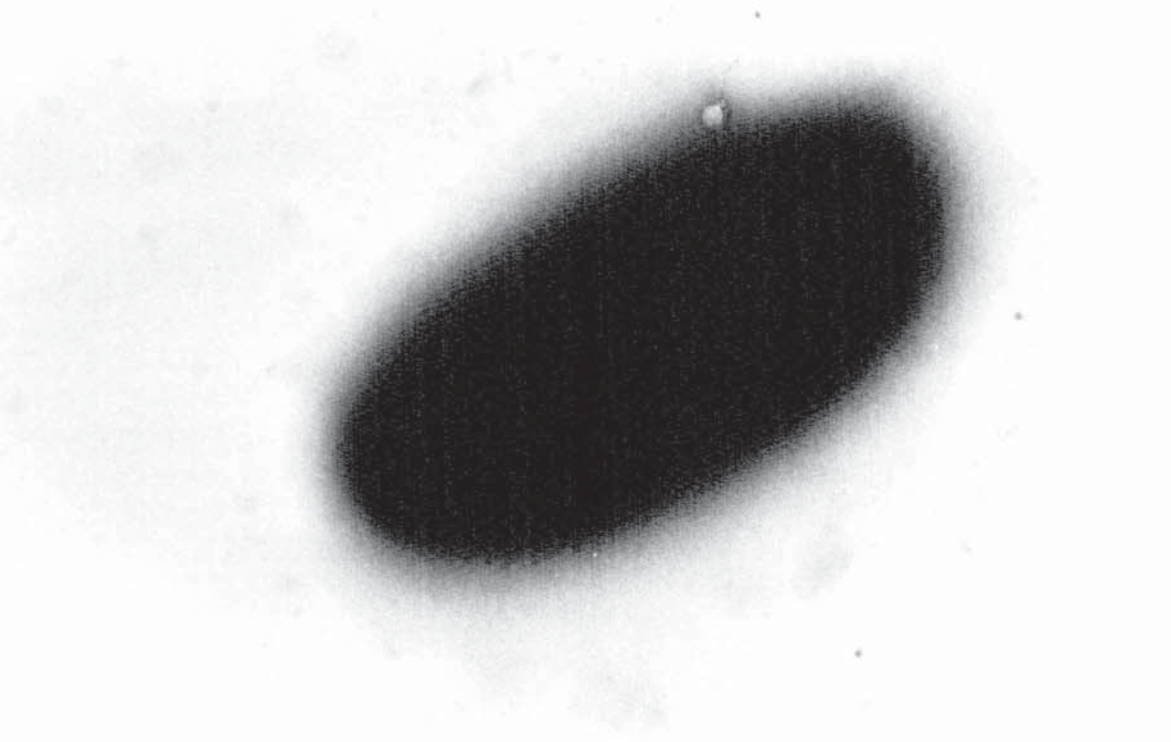
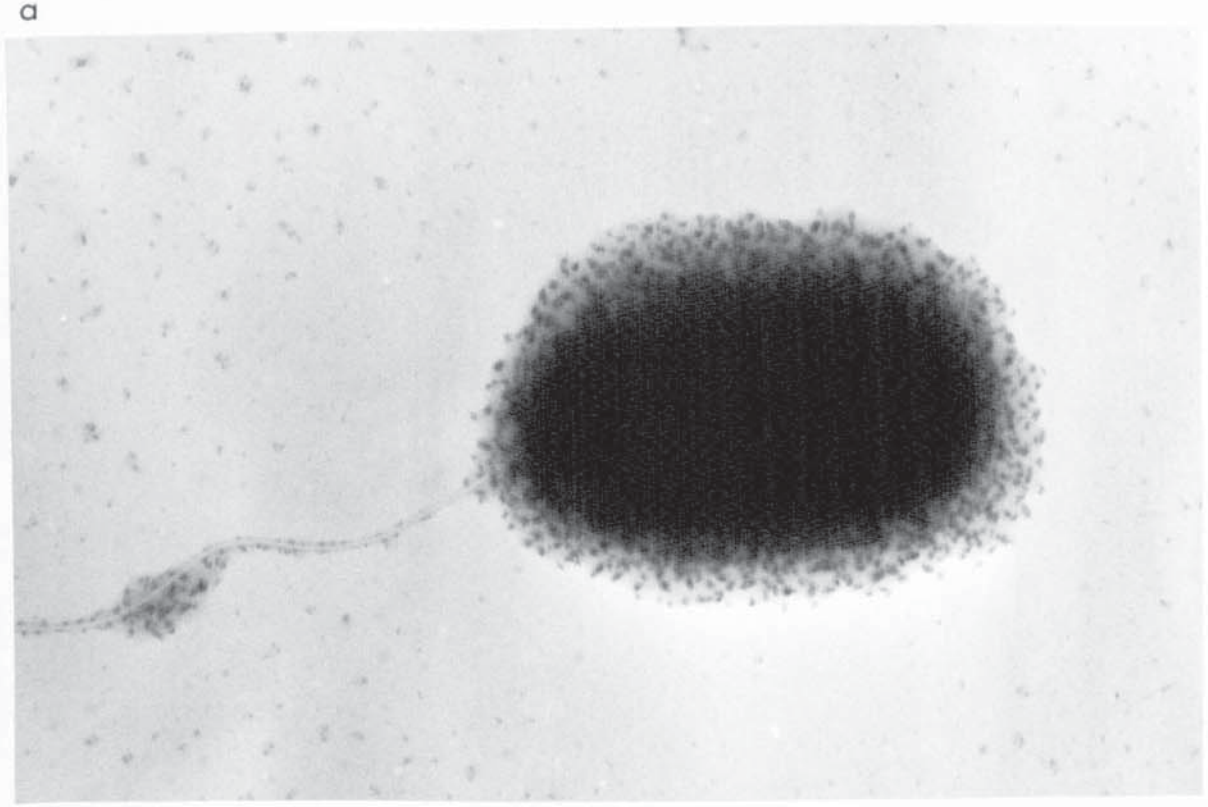


FIGURE 3.4, a-d

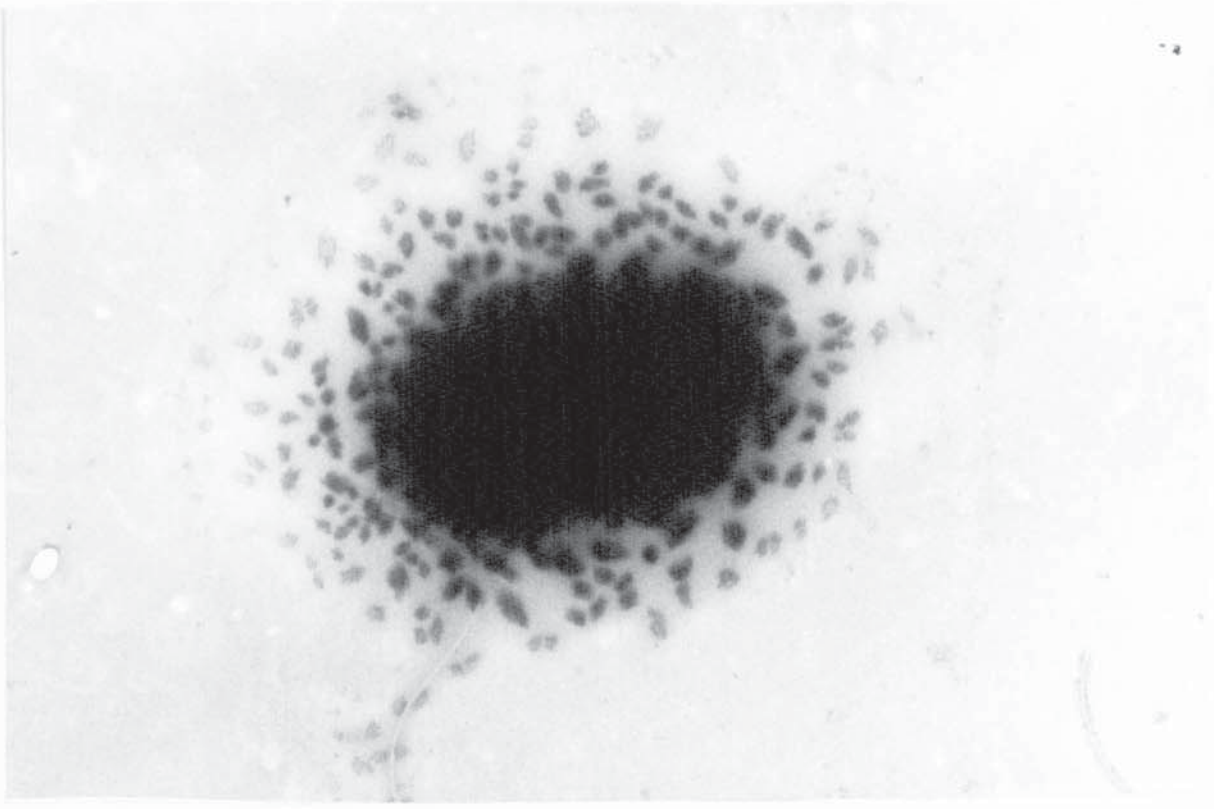
ELECTRON MICROGRAPHS SHOWING THE SURFACE STRUCTURES OF NON-MUCOID AND MUCOID P. AERUGINOSA ISOLATES FROM CF LUNG INFECTION REVEALED BY NEGATIVE-STAINING AND SHADOWING TECHNIQUES.

NEGATIVE-STAINED EARLY STATIONARY PHASE CELLS OF PANW (a) AND PAAA (b) GROWN IN Fe- CDM AND CARBON SHADOWED EARLY STATIONARY PHASE CELLS OF PAAT (c) AND PAJM (d) GROWN IN Fe- CDM ARE SHOWN. PANW AND PAAT HAVE A NON-MUCOID MORPHOLOGY WHEN GROWN ON THE SURFACE OF AGAR PLATES. PAAA AND PAJM ARE STABLE MUCOID STRAINS.

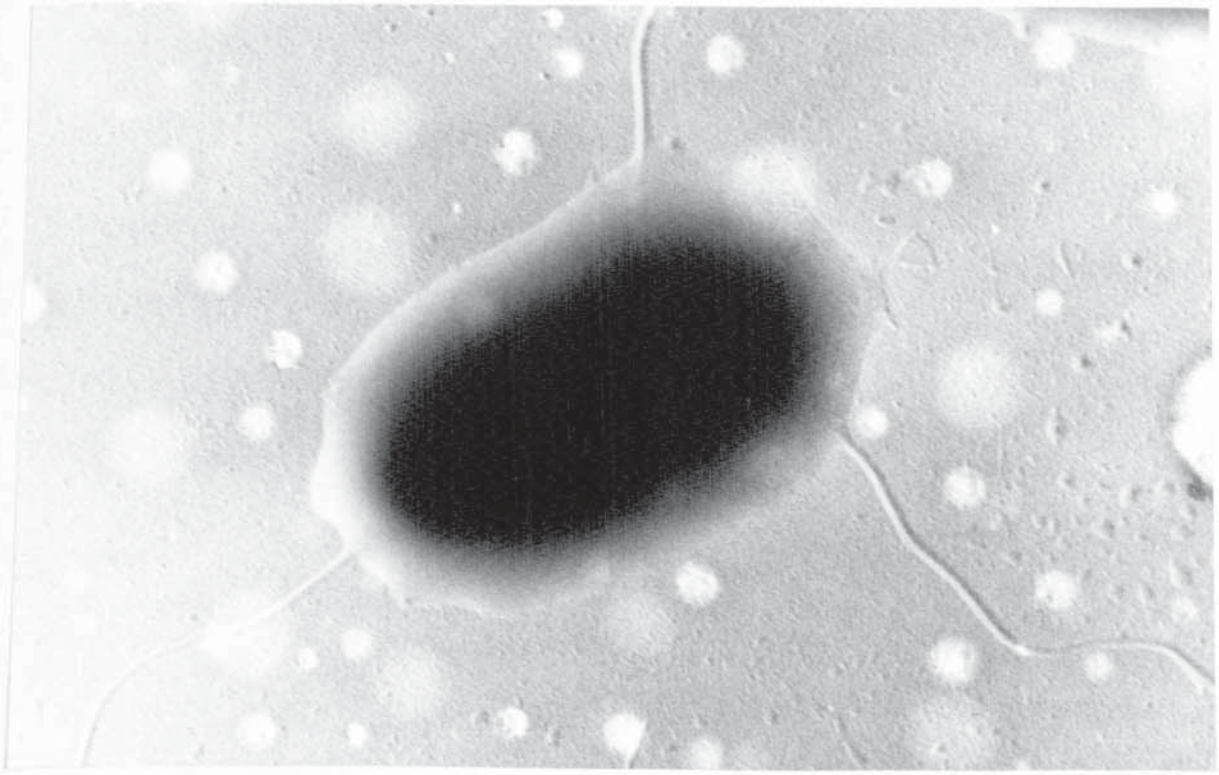
a



b



c



d

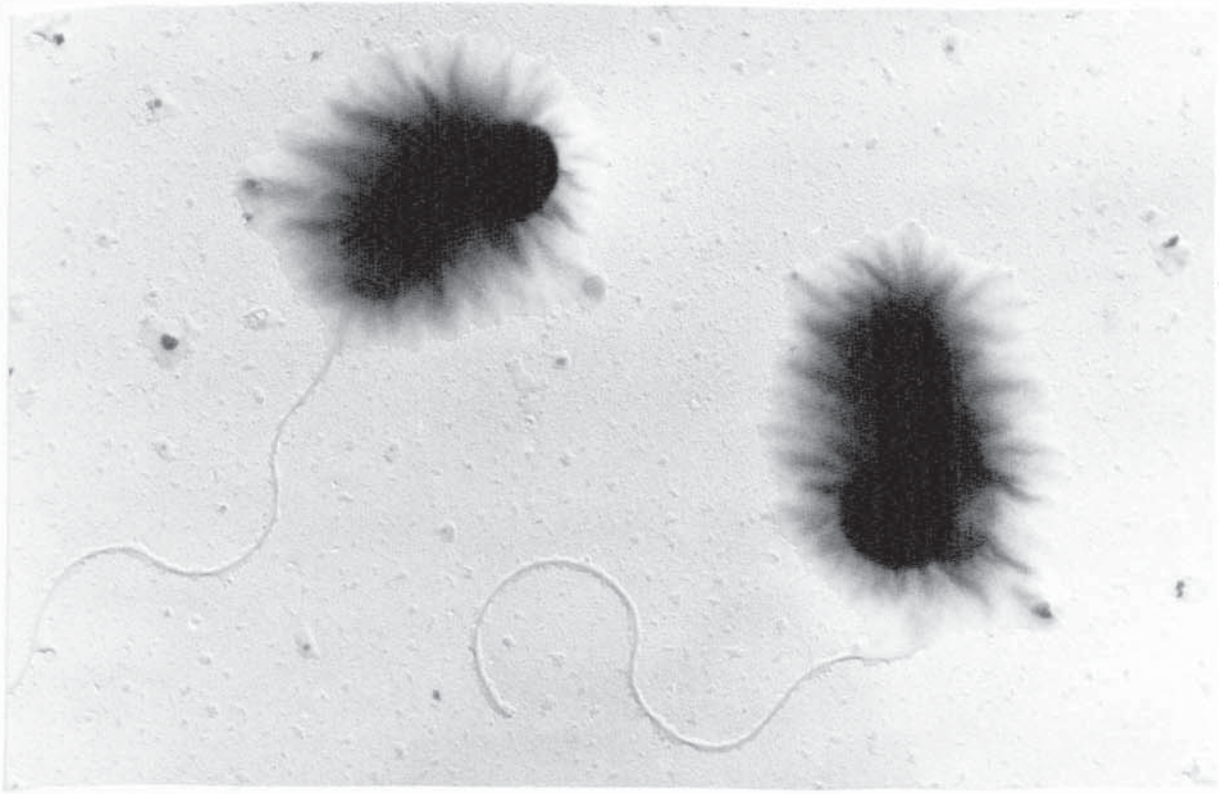


FIGURE 3.5a

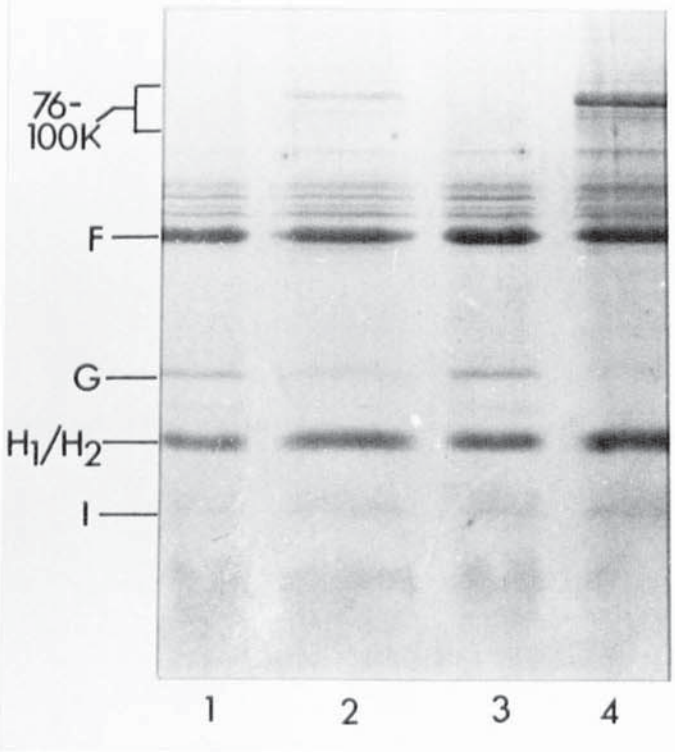
THE OM PROTEIN PROFLE OF P. AERUGINOSA PANW GROWN IN Fe+ TSB (LANE 1) AND Fe-TSB (LANE 2) AND OF P. AERUGINOSA PAAT GROWN IN Fe+ TSB (LANE 3) AND Fe- TSB (LANE 4).

FIGURE 3.5b

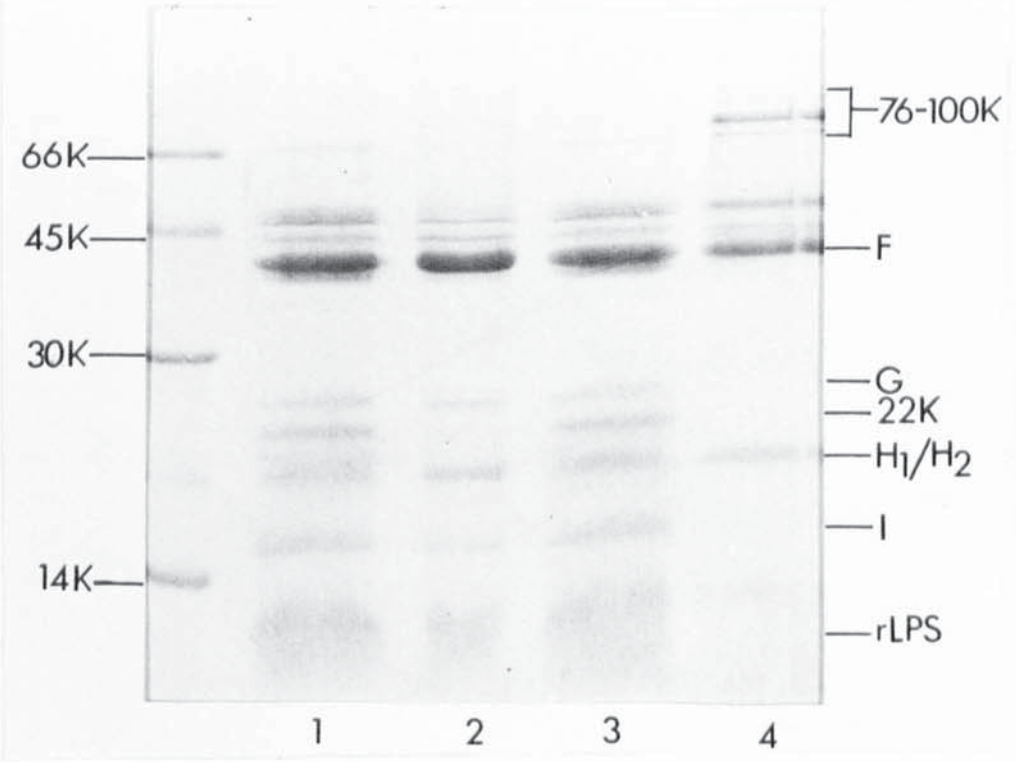
THE OM PROTEIN PROFILES OF P. AERUGINOSA PANW GROWN IN Fe+ CDM (LANE 1) AND Fe- CDM (LANE 2) AND OF P. AERUGINOSA PAAT GROWN IN Fe+ CDM (LANE 3) AND IN Fe- CDM (LANE 4). MIGRATION OF STANDARD MOLECULAR WEIGHT PROTEINS BOVINE AL BUMIN (66K), OVALBUMIN (45K), CARBONIC ANHYDRASE (30K) AND LYSOZYME (14K) ARE SHOWN IN THE LEFT HAND COLUMN.

THE LETTERS IN THE RIGHT HAND COLUMN SHOW PROTEINS LABELLED ACCORDING TO STANDARD SCHEMES OF HANCOCK AND CAREY, (1979) AND MIZUNO AND KAGEYAMA (1978). THE MOLECULAR WEIGHTS OF PROTEINS ARE EXPRESSED IN KILODALTONS.

a


76-
100K
F
G
H1/H2
I
1
2
3
4

b


66K
45K
30K
14K
76-100K
F
G
22K
H1/H2
I
rLPS
1
2
3
4

Fe- CDM-grown cells (Fig 3.5b, lanes 2 and 4) as compared to Fe- TSB-grown cells. Protein G was expressed less in the OM of iron-depleted cells as compared to iron-plentiful cells.

Extracted LPS can be resolved by SDS-PAGE and the carbohydrate component can subsequently be visualised by staining techniques, the most sensitive of which is silver staining after periodic acid oxidation (Tsai and Frasch, 1982). Characterisation of LPS is not restricted to isolated fractions; by the technique of Hitchcock and Brown (1983) a small amount of whole cells can be solubilised and proteins digested by incubation in a buffer containing proteinase K enzyme prior to analysis by SDS-PAGE. This technique has been applied to a range of Gram-negative organisms, including OM preparations of P. aeruginosa (Engels *et al*, 1985).

Methods for OM preparation based on insolubility in detergents work by selectively leaving or extracting protein-LPS complexes (Poxton *et al*, 1985), and may therefore not be representative of the cell surface components. An SDS-PAGE system was developed in the course of this study which allowed optimal resolution and staining of whole cell digests of P. aeruginosa isolates from CF lung infection. The use of this system was evaluated by resolving the LPS of proteinase K digests of whole cells lysates of each of the 17 a serotypes (IATS) of P. aeruginosa (Fig 3.6, lanes 1-17) and one polyagglutinating strain, PA 6750 (lane P). Good resolution of the polysaccharide components of all these strains was obtained.

Figure 3.7a and 3.7b show the SDS-PAGE silver stained profiles of proteinase K digests of early stationary phase cells of two nontypable P. aeruginosa strains PANW and PAAT respectively. Cells were cultivated in Fe- TSB (lanes 1-3) Fe+ TSB (lanes 4-6), Fe- CDM (lanes 7-8) or Fe+ CDM (lanes 9 and 10). Each series of lanes represent single, double and triple loadings of the same sample. The LPS core and 0–antigen structures of each isolate grown in iron-depleted conditions were more darkly stained than the LPS from iron-plentiful cells.

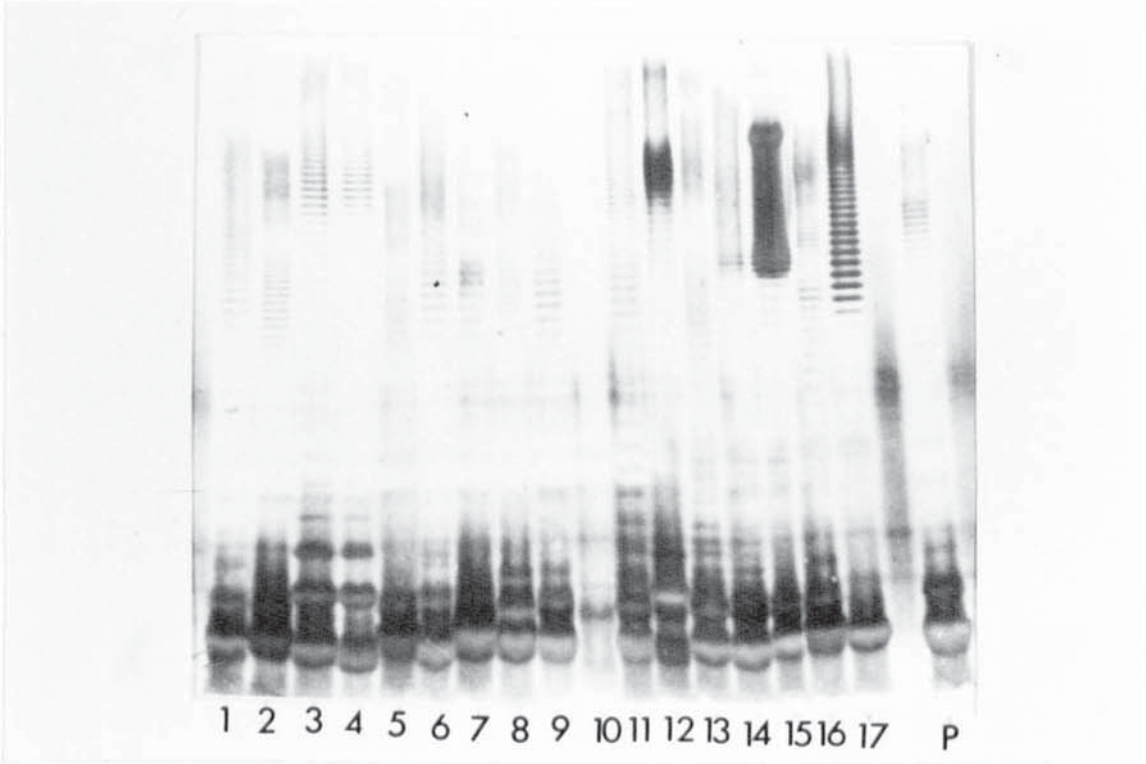


FIGURE 3.6

THE SDS-PAGE SILVER STAINED PROFILES OF PROTEINASE K DIGESTS OF WHOLE CELLS OF A REPRESENTATIVE OF EACH OF THE 17 O-SEROTYPES OF P. AERUGINOSA (IATS) (LANES 1-17) AND OF ONE POLYAGGLUTINATING STRAIN (LANE P) ALL GROWN IN Fe- CDM. THE CELLS WERE SUSPENDED TO AN OD OF 10 AT 470 nm BEFORE DIGESTION AND 15 µl LOADED IN EACH LANE.

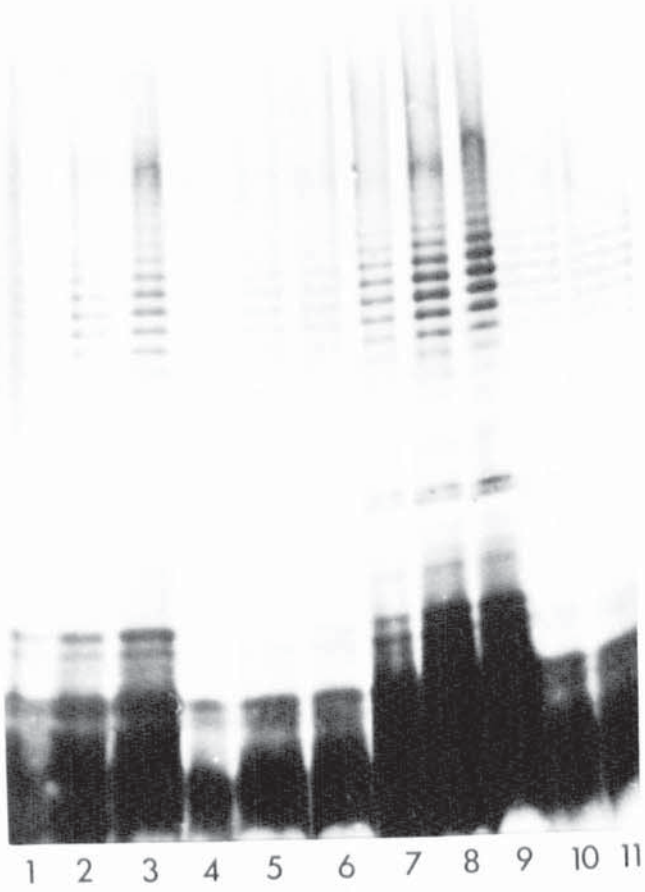
FIGURE 3.7a

THE SDS-PAGE SILVER STAINED PROFILES OF PROTEINASE K DIGESTS OF WHOLE CELLS OF P. AERUGINOSA PANW GROWN IN Fe- TSB (LANES 1-3), Fe+ TSB (LANES 4-6), Fe- CDM (LANES 7-9) AND Fe+ CDM (LANES 10 AND 11). CELLS WERE RESUSPENDED TO AN OD 470 nm OF 10 AND AFTER PROTEINASE K DIGESTION 5, 10 AND 15 µl WERE LOADED IN CONSECUTIVE LANES.

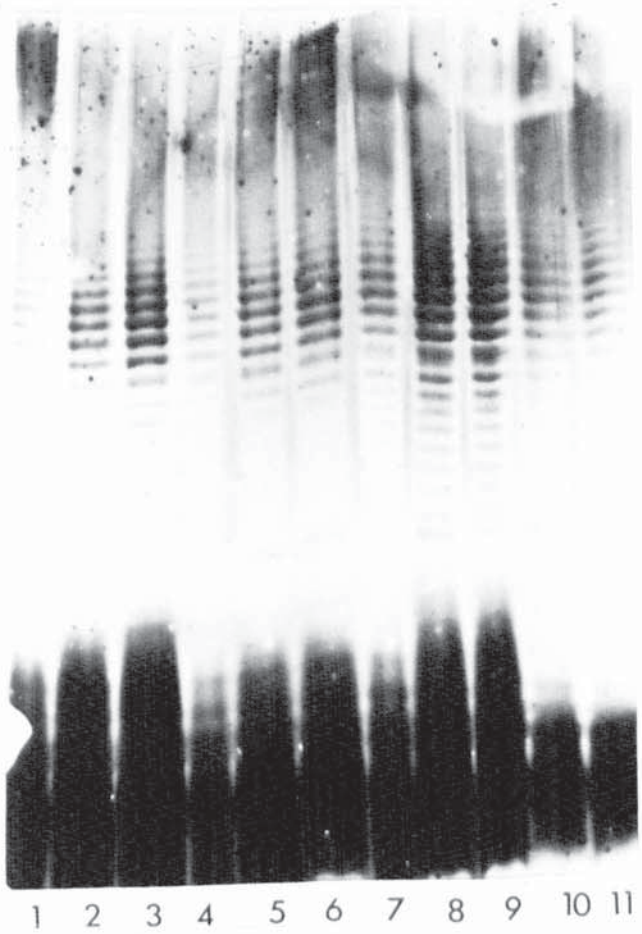
FIGURE 3.7b

THE SDS-PAGE SILVER STAINED PROFILES OF PROTEINASE K DIGESTS OF WHOLE CELLS OF P. AERUGINOSA PAAT GROWN IN Fe- TSB (LANES 1-3), Fe+ TSB (LANES 4-6), Fe- CDM (LANES 7-9) AND Fe+ CDM (LANES 10 AND 11). CELLS WERE RESUSPENDED TO AN OD 470 nm OF 10 AND AFTER PROTEINASE K DIGESTION 5, 10 AND 15 µl WERE LOADED IN CONSECUTIVE LANES.

a.



b.



3.1.4 Reaction of antibodies in the sera from CF patients with OM antigens of P. aeruginosa and other Gram-negative bacteria

Immunoblotting with sera from teenage CF patients was used to investigate the antibody response to OM antigens of P. aeruginosa and an index of Enterobacteriaceae strains. Figure 2.8 a shows the OM protein profile of strains of S. marcescens (lane 1), E.coli (lane 2), K.pneumoniae (lane 3), P.mirabilis (lane 4) and P. aeruginosa (lane 5). Strains of Enterobacteriaceae were all recent isolates from clinical urinary tract infections. The strains were cultivated in Fe- TSB and a number of high molecular weight (ca 80K) proteins were induced. The predominant proteins in the Enterobacteriaceae are the porin proteins with a molecular weight of about 40 K.

The SDS-PAGE separated proteins were electrophoretically transferred to NC paper. Figures 3.9a to 3.9e show the immunoblots obtained when these OM antigens were probed with serum from a teenage CF patient whose lungs were not infected with P. aeruginosa (Fig 3.9a), a patient whose lungs were intermittently infected with P. aeruginosa (Fig 3.9b) and patients with chronic P. aeruginosa lung infection (Fig 3.9c to 3.9e).

In Figure 3.9a there is strong reaction with OM protein antigens and rough LPS of the Enterobacteriaceae. Antigens in the OMs of S.marcescens, E.coli and P.mirabilis which were not visualised by staining procedure were recognised on the immunoblot. This may be due to reaction of antibodies in this patient's serum with the 0-antigen of these strains. This patient's serum only reacted faintly with some OM antigens of P. aeruginosa.

Figure 3.9b shows the reaction of antibodies in the serum from a patient whose lungs were colonised with mucoid E.coli and who had intermittent.

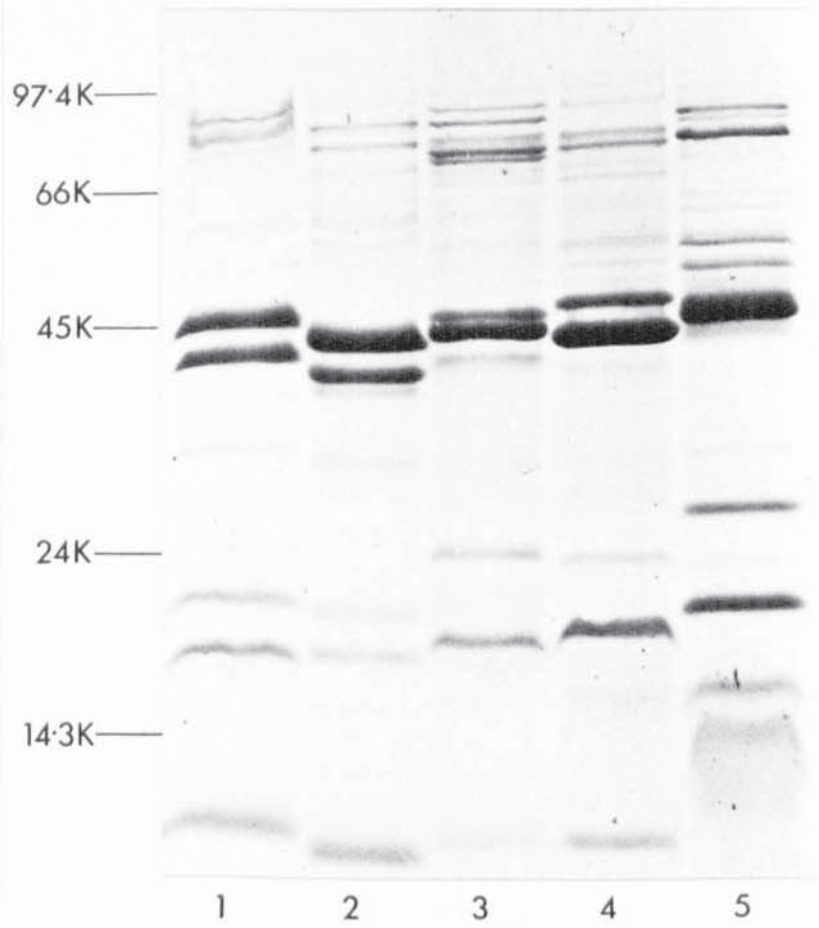


FIGURE 3.8

THE SDS-PAGE OM PROTEIN PROFILES OF STRAINS OF S. MARCESCENS (LANE 1), E. COLI (LANE 2), K. PNEUMONIAE (LANE 3), P. MIRABILIS (LANE 4) AND P. AERUGINOSA (LANE 5) ALL GROWN IN Fe-TSB.

ARROWS IN THE LEFT MARGIN INDICATE MIGRATION OF MOLECULAR WEIGHT PROTEIN STANDARDS.

FIGURE 3.9, a-e

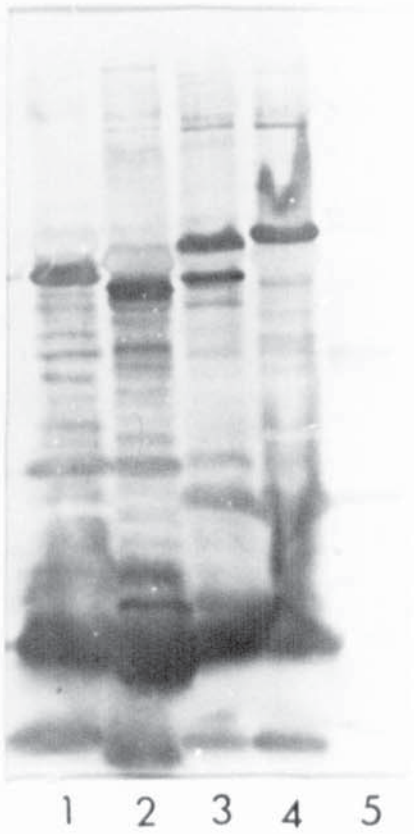
IMMUNOBLOTS OF THE OM ANTIGENS SHOWN IN 3.8 ELECTROPHORECTICALLY TRANSFERRED TO NC PAPER AND REACTED WITH SERA FROM CF PATIENTS. SERA USED WERE OBTAINED FROM PATIENTS WITH

(A) NO HISTORY OF P. AERUGINOSA INFECTION;

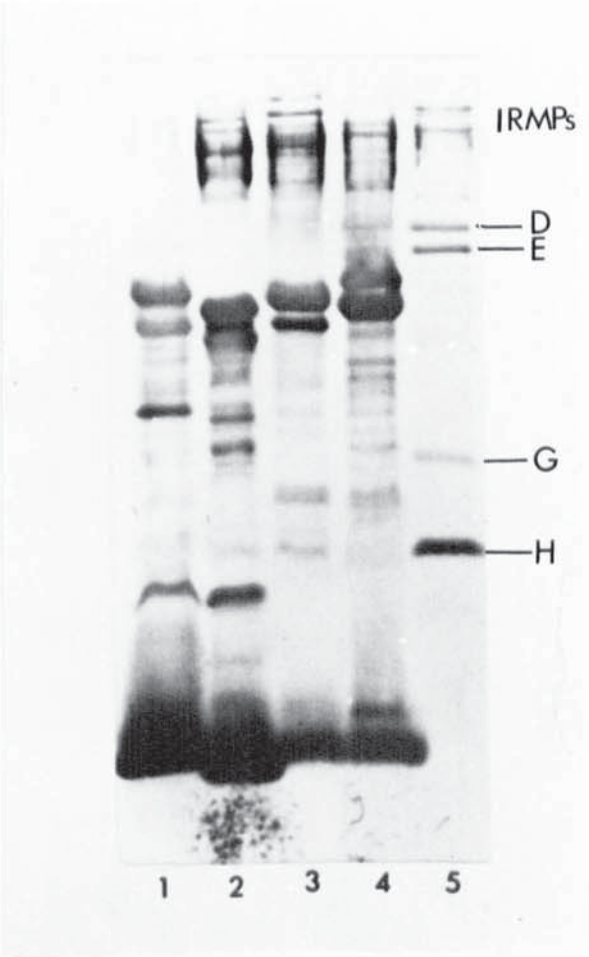
(B) INTERMITTENT P. AERUGINOSA LUNG INFECTION,

(C) - (E) PATIENTS WHOLE LUNGS WERE CHRONICALLY COLONISED WITH P. AERUGINOSA.

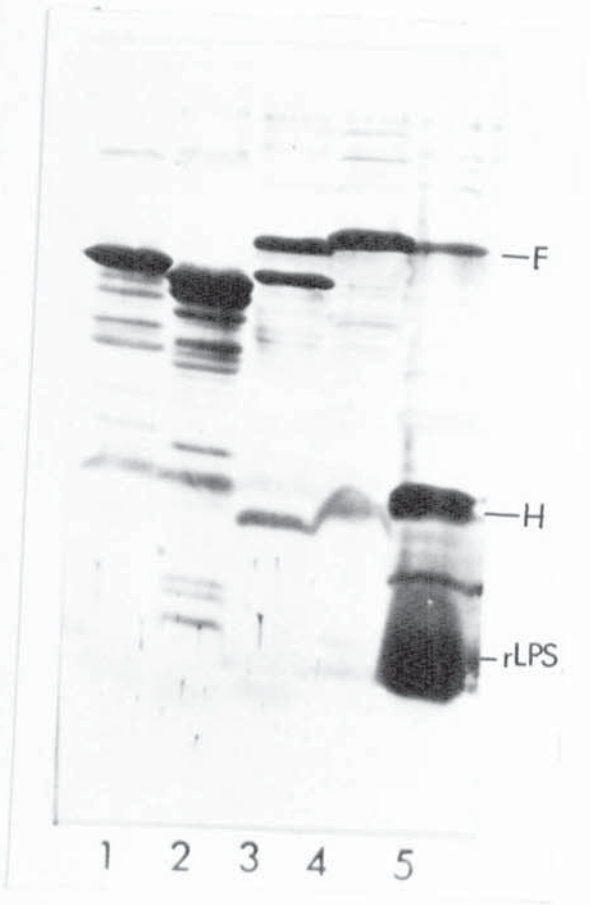
a



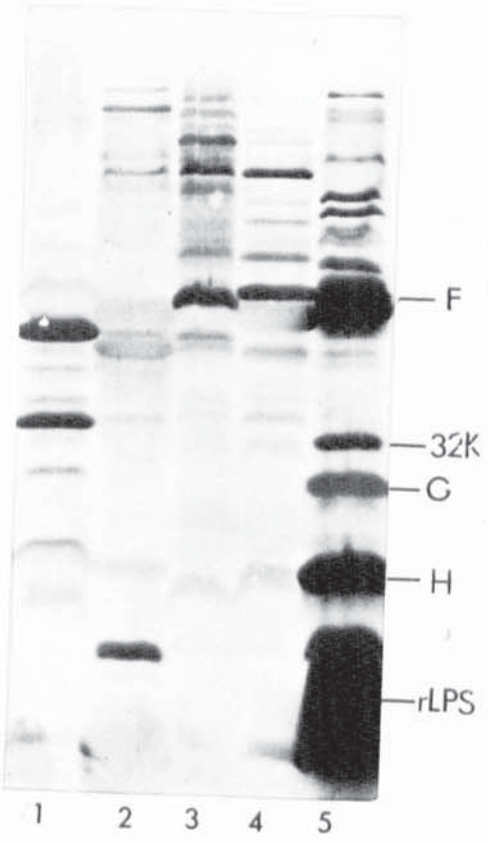
b



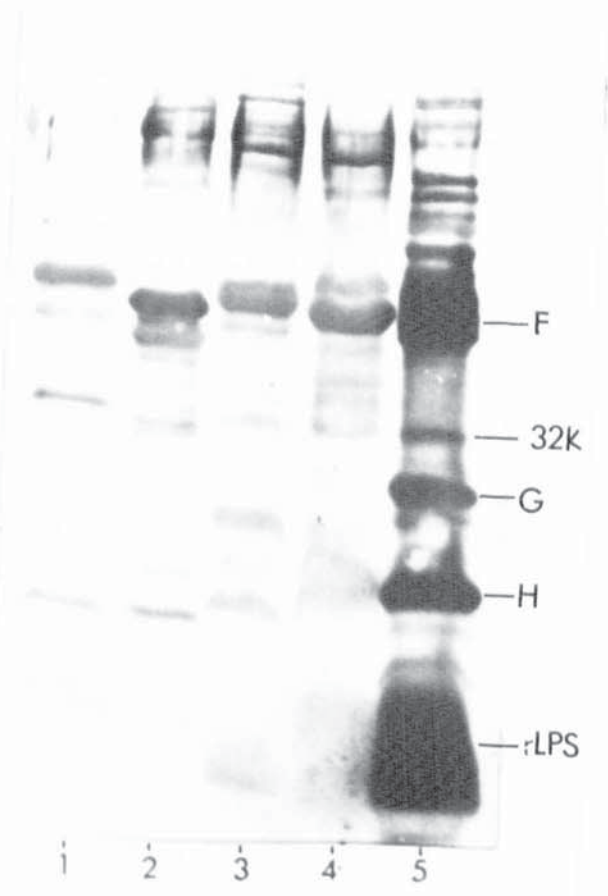
c



d



e



P. aeruginosa lung infection. There was a strong reaction with some of the IRMPs, porin proteins and rough LPS of the Enterobacteriaceae. The patient's serum contained antibodies which reacted with P. aeruginosa IRMPs, OM proteins D, E, G and H1/H2.

Sera from patients whose lungs were colonised with mucoid P. aeruginosa contained antibodies to all the major OM proteins of P. aeruginosa including the IRMPs and also the P. aeruginosa rough LPS. The OM proteins F and H1/H2 were particularly strongly recognised. An additional antigen in the OM of P. aeruginosa which was not visualised by Coomassie blue or amido black staining was immunogenic. This antigen had a molecular weight of 32 K. Sera from two of these patients contained antibodies which reacted strongly with the porins of the Enterobacteriaceae. There was only a faint reaction with the rough LPS of the Enterobacteriaceae.

3.1.5 Discussion

The availability of iron in the body fluids is restricted by high affinity iron-binding proteins lactoferrin and transferrin (Bullen, 1981; Weinberg, 1978). Many Gram-negative bacteria respond *in vitro* to iron-deprivation by the induction of high molecular weight OM proteins together with the production of low molecular weight iron chelators, known as siderophores (Neilands, 1984; Neilands, 1981; Griffiths, 1983). Gram-negative bacteria recovered from human CF lung infection (Brown *et al*, 1984) and urinary tract infections (Shand *et al*, 1985) and also from experimental animal infections (Griffiths *et al*, 1983; Sciortino and Finkelstein, 1983) expressed high molecular weight IRMPs *in vivo*. These findings emphasise the value of studying the physiological and antigenic states of bacteria grown *in vitro* in conditions which mimic the iron-restricted conditions of the body.

The expression of pili by a non-mucoid laboratory strain appeared to be repressed by growth in iron-depleted media. Iron deprivation may favour the

production of glycocalyx material. Both fimbriae and exopolysaccharides are thought to be involved in initiating adherence of P. aeruginosa to inert surfaces and to human tissues. The glycocalyx also plays an important role in the formation of microcolonies of P. aeruginosa (Costerton *et al*, 1981; Costerton *et al*, 1978). Electron microscopic examination of non-mucoid and stable mucoid strains of P. aeruginosa from CF lung infection revealed that all the strains including those which exhibited non-mucoid morphology when grown on the surface of agar plates formed some glycocalyx material particularly when grown in iron-depleted media*. Other workers, also using electron microscopy techniques, recently reported expression of surface glycocalyxes by non-mucoid P. aeruginosa strains (Pier *et al*, 1986). Ten clinical isolates from CF lung infection were examined; they were invariably flagellated but did not express fimbriae when grown in iron-plentiful or iron-depleted batch cultures. This is in contrast with the results of Luzar *et al* (1985) who reported that 7 of the 10 rough strains of P. aeruginosa they examined lacked flagella, as determined by electron microscopy. The strains had all been obtained from CF patients with poor clinical condition. This does not seem to account for the differences in the results of these two studies since the strains used in this present study were obtained from patients with poor clinical status.

Several IRMPs with molecular weights in the range 76K-100K were induced in the OM of two strains from CF lung infection following growth in iron-depleted simple salts media or chelexed tryptone soya broth without added iron. This confirms results of earlier studies which have reported derepression of several high molecular weight OM proteins in P. aeruginosa grown in iron-depleted media (Brown *et al*, 1984; Ohkawa *et al*, 1980; Meyer *et al*, 1979; Mizuno and Kageyama, 1978). Differences were noted in the number and also in the amounts of IRMPs expressed by the two strains included in this present study, even though they were cultivated under identical growth conditions.

Enumeration of IRMPs in studies using one-dimensional SDS-PAGE is

* The relevance of these studies to an alginate glycocalyx is questionable; ruthenium red staining has shown glycocalyx only in alginate producing P.aeruginosa (Govan & Harris, 1986)

likely to be incomplete since Cody and Gross (1987) demonstrated that IRMPs of the same molecular weight could be separated by two-dimensional SDS-PAGE. There has been great diversity reported in the molecular weight of the IRMPs expressed by strains of P. aeruginosa and also of the IRMPs expressed by root-colonising Pseudomonas spp. (Cody and Gross, 1987; de Weger *et al*, 1986). The diversity of the siderophores produced by Pseudomonas spp. is though to account for the great diversity of IRMPs reported in these studies.

Ohkawa *et al* (1980) also reported repression of protein G in the OM of one P. aeruginosa strain grown in iron-depleted media. The expression of this protein in the OM of other strains was not affected by the concentration of iron in the growth medium. These investigators concluded that factors other than the availability of iron were responsible for repression of protein G. However, protein G was expressed less in the OM of both the isolates included in this present study when grown in iron-depleted simple salts media and iron-depleted complex laboratory media as compared to iron-plentiful media. This may suggest that the concentration of iron in the media is a factor which affects the expression of OM protein G.

A low molecular weight (14K) protein involved in the uptake of iron-pyochelin complexes has been reported (Sokol, 1984; Sokol and Woods, 1983). No evidence of this protein was found in OMs prepared in this study from cells cultivated in either iron-depleted simple salts medium or chelexed tryptone soya broth. In the experimental protocol adopted by Sokol and Woods (1983), OMs were prepared from PAO 1, grown on simple salts medium with no added iron, using sucrose density centrifugation. The OMs were incubated with [^{59}Fe]-pyochelin and after denaturing OMs were analysed by SDS-PAGE. Bound [^{59}Fe]-pyochelin was selected by autoradiography. OMs were also transferred to NC paper which was incubated with [^{59}Fe]-pyochelin and bound iron was again visualised by autoradiography. This procedure assumes that bonds between Fe-pyochelin and the protein involved in its uptake are stable during denaturing and that breakdown of the siderophore-protein complexes does not occur.

Sokol and Woods (1986) more recently reported that monoclonal antibodies to the ferri-pyochelin binding protein reacted with a 14K protein in the OM of P. aeruginosa serotype strains. However, the electrophoretic conditions (1 Amp for 30 mins) used to transfer the SDS-PAGE separated proteins to NC paper was probably insufficient to effect transfer of high molecular weight proteins. Recent experiments carried out in these workers laboratories indicated that the 14K protein is a breakdown fragment of a high molecular weight protein (Dr PA Sokol, Session 10, ASM 87th Annual Meeting, Atlanta, 1987).

Magazin *et al* (1986) demonstrated that 2.4 kilobases of DNA encode the ferric pseudobactin receptor protein in a Pseudomonas B10, a plant pathogen, which is an 85K molecular weight outer membrane protein. Evidence that the ferri-pyoverdin$_{pss}$ binding protein of a P. syringae pv. syringae strain is a 74K protein was also recently provided by Cody and Gross (1987) in studies using a mutant strain which was unable to synthesize pyoverdin$_{pss}$. Proof that the high molecular weight IRMPs of P. aeruginosa function as receptors for iron-siderophore complexes requires isolation of the siderophore complexes or cloning of the relevant genes and elucidation of their role in iron uptake.

The SDS-PAGE silver stained profile of proteinase K digests of P. aeruginosa whole cell lysates showed two distinct regions; a fast migrating low molecular weight region which represents the core LPS component and a ladderlike series of more slowly migrating bands which represent 0-antigen structures with different numbers of repeating units. Variations in the number of O-polysaccharide repeating units are responsible for the marked heterogeneity of SDS-PAGE profiles of LPS from smooth strains of Enterobacteriaceae (Jann *et al*, 1975; Palva and Mäkelä, 1980; Goldman and Leive, 1980; Hitchcock and Brown, 1983).

Polyagglutinating and nontypable P. aeruginosa strains are prevalent among isolates from CF lung infection (Hancock *et al*, 1983). This feature is associated with deficient production of 0-antigen side chains (Hancock *et al*, 1983; Ojenyi *et*

al, 1985; Pitt *et al*, 1986). Whole cells of two nontypable strains from CF lung infection were incubated with proteinase K and their LPS components were resolved by SDS-PAGE. The silver stained gels indicated that both isolates expressed core LPS and 0–antigen structures. Hancock *et al* (1983) reported that 10 μg of purified LPS from a number of nontypable or polyagglutinating strains was the minimum amount required for visualisation of their 0-antigen structure by SDS-PAGE. Also, with many isolates no smooth LPS was visualised when 30 μg of purified LPS were resolved. Using these loadings the amount of rough LPS was in vast excess. The sample loadings used in this study were chosen to allow complete visualisation of the 0-antigen structure. Assuming that LPS represents 10% of the dry weight of P. aeruginosa cells the samples loaded in this study were equivalent to 3-9 μg of LPS. The differences reported in this study as compared to that in Hancock's laboratory may be due to the greater sensitivity of the SDS-PAGE-silver stain procedure used here and possibly strain differences. Also, Hancock *et al* (1983), included EDTA in the sample denaturing buffer; EDTA is known to stabilise rough LPS.

The results of this study indicate that SDS-PAGE can be used to investigate phenotypically induced alterations in P. aeruginosa LPS. The effect of nutrient depletion on P. aeruginosa LPS is investigated further in section 3.4.

The antigenicity of the OM antigens of P. aeruginosa and strains of the Enterobacteriaceae was investigated by immunoblotting with serum from teenage CF patients. Strains of S. marcescens, E. coli, K. pneumoniae and P. mirabilis were chosen as representative of Gram-negative bacteria which are part of the normal gut flora and which cause acute and chronic pneumoniae. Several species of enteric bacteria secrete the siderophore enterochelin when grown in iron-depleted media (Neilands, 1984; Griffiths, 1983). There is also evidence that P. aeruginosa can utilise enterochelin although it does not produce the siderophore (Liu and Shokrani, 1978). Some enteric species appear to share a common siderophore binding protein since anitserum to the 81K ferric enterobactin receptor protein of E. coli reacted with

an IRMP from iron-restricted Salmonella typhimurium, and also from K. pneumoniae; however, antisera to the 81K enterochelin receptor did not react with OM proteins of iron-depleted P. aeruginosa (Chart and Griffiths, 1985). The immunoblotting results presented in this study indicate the specificity of the antibody response to P. aeruginosa OM proteins, including the IRMPs, and also to LPS.

3.2 SURFACE ANTIGENS OF *IN VIVO*-GROWN P. AERUGINOSA: ANTIBODY RESPONSE IN THE LUNGS AND SERA FROM INFECTED AND IMMUNISED RATS

Chronic experimental lung infection with mucoid P. aeruginosa PAO 579 was established using the rat model developed by Cash *et al*, (1979). Fourteen days post-infection the *in vivo*-grown bacteria were recovered directly and without subculture from the lungs. The composition and antigenicity of sarkosyl-extracted OMs prepared from these cells was investigated using sera and lung fluid from infected and immunised rats.

3.2.1 Antibody response in the lungs of rats infected with PAO 579

Figure 3.10 shows the Coomassie blue stained (Fig 3.10a) and amido black stained (Fig 3.10b) OM protein profiles of mucoid PAO 579 isolated directly and without subculture from the lungs of infected rats (lane 3) and of the same isolate cultivated in Fe- TSB(lane 2) and Fe+ TSB (lane 1). The OMs were separated using 14% w/v acrylamide gels. Complete transfer of the SDS-PAGE separated proteins to NC paper was confirmed by staining the polyacrylamide gel, after immunoblotting, with Coomassie blue stain. Three proteins with molecular weights of 76K, 83K and 85K were strongly expressed in the OM of *in vivo*-grown and Fe-TSB-grown PAO 579. Two further proteins with molecular weights of approximately 90K and 100 K were also expressed and were detected by the more sensitive amido black stain. These protein were repressed following growth in iron-plentiful media and were therefore IRMPs. Protein G was expressed less in the OM of *in vivo*-grown as compared to *in vitro*-grown cells. Only minor differences were noted in the expression of the major OM proteins D, E, F, H1/H2 and I as revealed by Coomassie blue or amido black staining.

The OMs described in Figure 3.10 were transferred to NC paper and probed with lung fluid from rats sacrificed 14 days post-infection. Figure 3.11a shows that

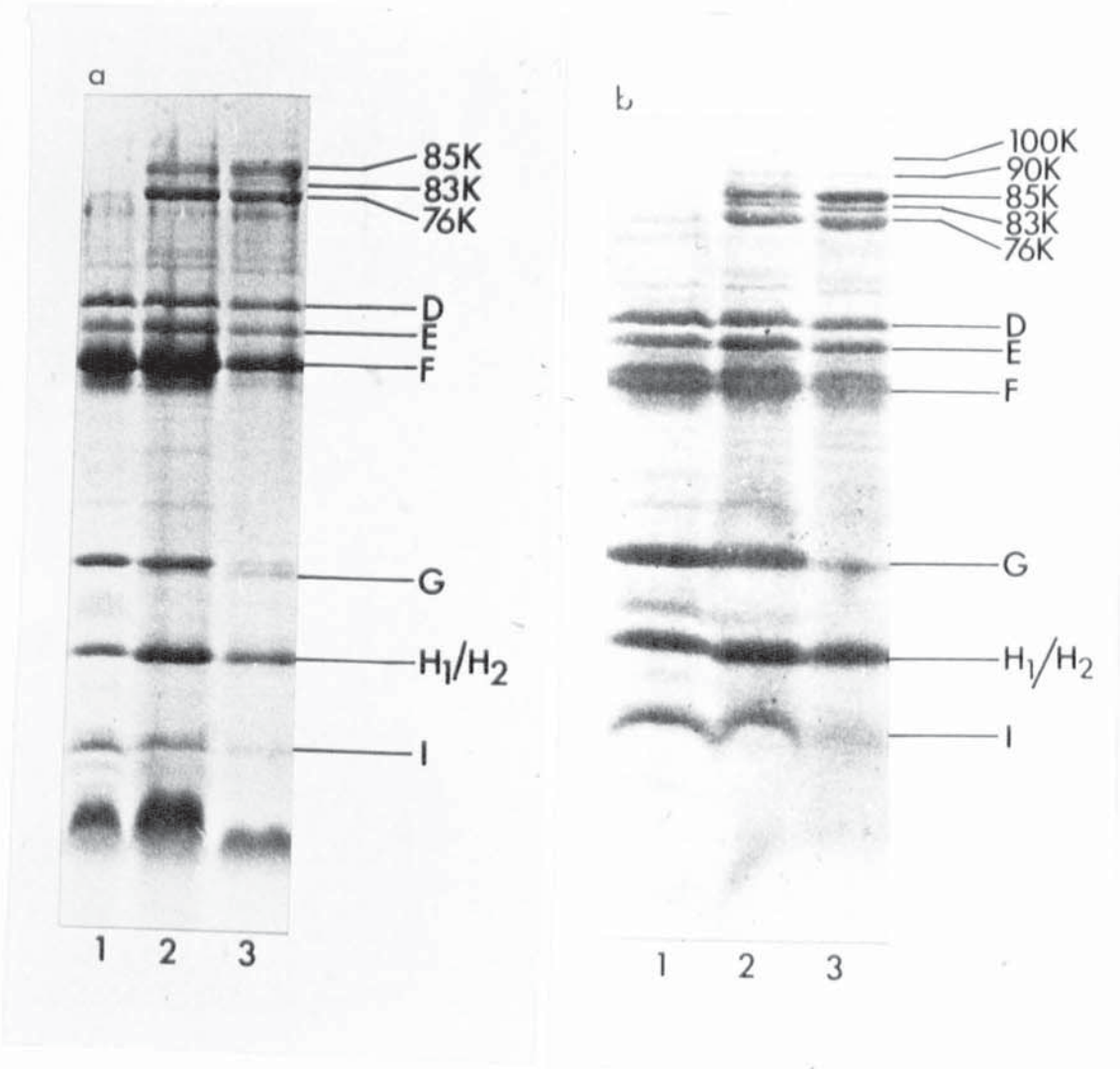


FIGURE 3.10a

THE SDS-PAGE OM PROTEIN PROFILES OF P. AERUGINOSA PAO 579 ISOLATED DIRECTLY FROM THE LUNGS OF INFECTED RATS (LANE 3) AND THE SAME ISOLATE GROWN IN Fe- TSB (LANE 2) AND Fe+ TSB (LANE 1) LABORATORY MEDIA. PROTEINS WERE SEPARATED USING 14% w/v ACRYLAMIDE GELS AND VISUALISED WITH COOMASSIE BLUE STAIN. PROTEINS ARE LABELLED ACCORDING TO THE SCHEMES OF HANCOCK AND CAREY (1979) AND MIZUNO AND KAGEYAMA (1978). MOLECULAR WEIGHTS OF THE IRMPs ARE SHOWN IN KILODALTONS (K).

FIGURE 3.10b

THE OM PROTEIN PROFILES OF THE ANTIGENS SHOWN IN 3.10a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND VISUALISED WITH AMIDO BLACK STAIN.

the lung fluid contained IgG which reacted strongly with the IRMP of 76K , a 54K antigen (labelled Fla) and the porin protein F. Proteins H1/H2 in the OM of *in vivo*-grown and Fe- TSB-grown PAO 579 were also strongly recognised. The antigen labelled "Fla" is probably flagellin protein as it has an apparent molecular weight similar to that assigned to isolated flagellin proteins from P. aeruginosa (flagellar type-b strains) by other workers (Allison *et al*, 1985) and electron microscopic examination revealed that PA0 579 was flagellated (not shown).

Lung fluid also contained IgG which reacted with protein G in the OM of *in vitro* but not *in vivo*-grown PAO 579. Presumably protein G was expressed in the OM or the inocula used to establish the infection and an immune response was developed to this antigen. There was a fainter reaction with the other IRMPs and OM proteins D, E and I. The antigen running close to the bottom of the gel (labelled rLPS), which may represent the core region of LPS, was weakly recognised by IgG.

The IRMPs were recognised by IgA in the lung fluid from infected rats (Fig 3.11b). IgA also reacted with all the major OM proteins except proteins F and I. The binding of antibody to the background of the high molecular weight region of lanes 2 and 3 on this immunoblot may be due to reaction of lung IgA with the 0-antigen of PAO 579 LPS (see 3.2.2).

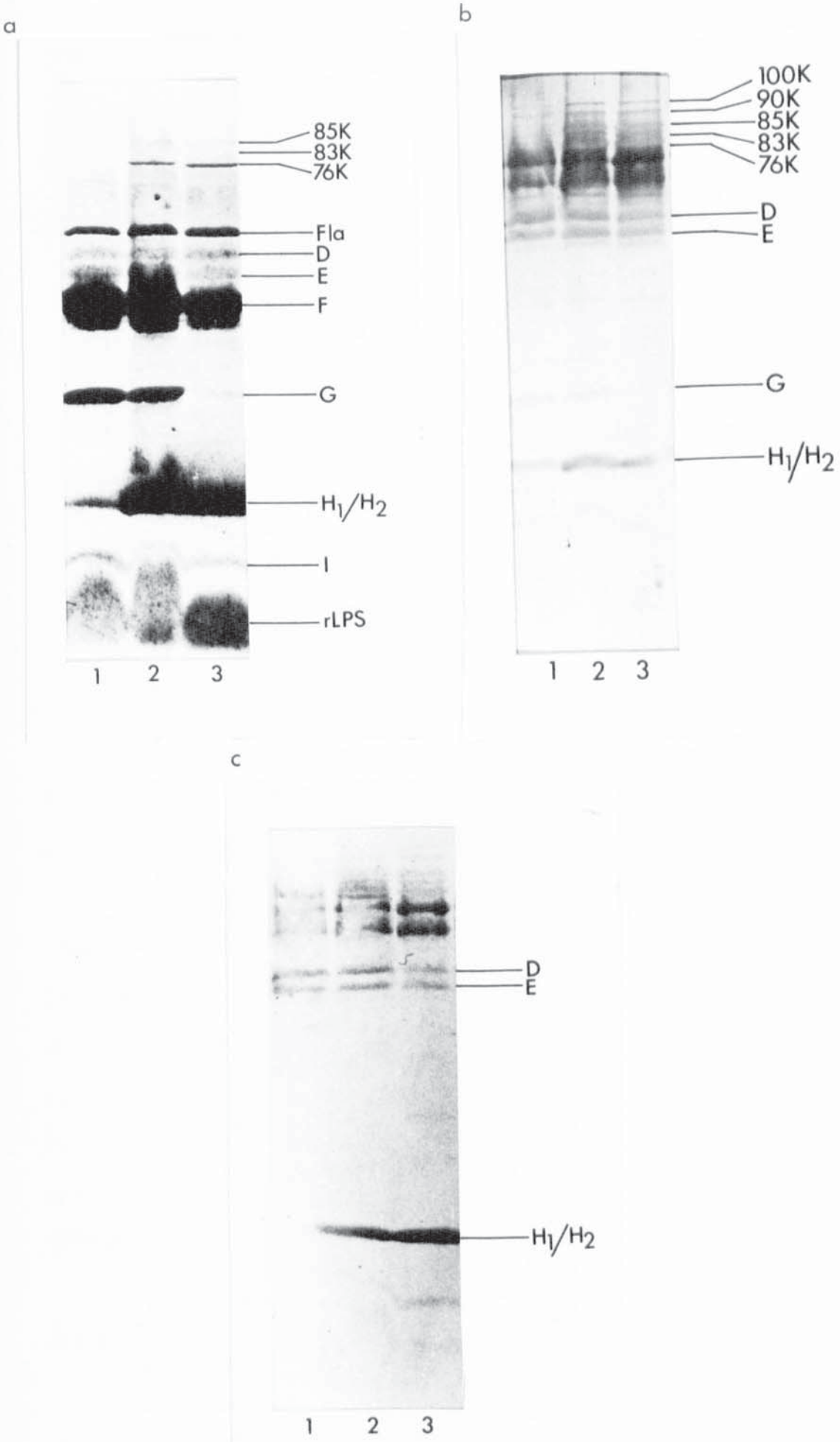
Figure 3.11c shows the reaction of IgM in lung fluid from infected rats with PAO 579 OM antigens. Immunoblotting results suggest the IgM response in the lung was much less than with the other classes of immunoglobulins. Again OM proteins H1/H2 of *in vivo* -grown and Fe- TSB-grown PAO 579 were recognised.

3.2.2 Antibody response in sera from rats infected with PAO 579

Sera from infected rats was absorbed with extracted LPS from the homologous strain prior to use in immunoblotting to avoid immunodetection of antigens due to co-migrating LPS (Poxton *et al*, 1985). Figures 3.12a, 3.12b and 3.12 c show the reaction of IgG, IgA and IgM respectively in LPS-absorbed sera

FIGURE 3.11, a - c

IMMUNOBLOTS OF THE OM ANTIGENS SEPARATED IN 3.10a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LUNG FLUID FROM RATS INFECTED WITH PAO 579. THE REACTIONS OBTAINED WITH IgG (a), IgA (b) and IgM (c) ARE SHOWN.


a
85K
83K
76K
Fla
D
E
F
G
H1/H2
I
rLPS
1 2 3
b
100K
90K
85K
83K
76K
D
E
G
H1/H2
1 2 3
c
D
E
H1/H2
1 2 3

obtained from rats 14 days post-infection with OM antigens of PAO 579. Serum IgG reacted with all the major OM proteins including the IRMPs and flagellin protein of *in vivo*-grown cells. The IRMPs and proteins H1/H2 of *in vivo*-grown and Fe-TSB-grown PAO 579 were the antigens most strongly recognised by IgG in LPS-absorbed sera. The concentration of the polymerising agents used in the gel system had been reduced to allow separation of OM proteins H1 and H2. Consequently protein G migrated with an apparently lower molecular weight. By comparison with the Coomassie blue stained profile of replicate portions of the gel it was confirmed that serum IgG reacted with OM proteins H1 and H2. The reaction of IgG in sera with protein F was weaker than the protein F-antibody reaction obtained with lung fluid. Serum IgM reacted with the IRMPs, proteins D, E and H1/H2 of PAO 579 (Fig 3.12c). The IRMPs were also recognised by IgA in sera (fig 3.12b).

The antigenicity of the LPS present in the OM of the samples described in Figure 3.10 was investigated by immunoblotting with whole sera from infected rats. Proteins present in the OM samples were digested by incubation with buffer containing proteinase K prior to electrophoresis. Figures 3.13a and 3.13b show the reaction of IgG and IgM respectively with PAO 579 LPS. The LPS 0-antigen profile of *in vivo*-grown (lane 3) and Fe- TSB-grown (lane 2) PAO 579 were similar. The LPS 0-antigen profile of OMs prepared from PAO 579 grown in Fe+ TSB (lane 1) showed less heterogenity in the number of 0-antigen bands. A serum IgA response to PAO 579 LPS was not detected by immunoblotting. Results obtained using ELISA techniques indicated that the titre of IgA in the sera from infected rats was much lower than the other classes of immunoglobulins and that the IgA titre to PAO 579 LPS was low (see section 3.2.4).

3.2.3 Antibody response of rats immunised with PAO 579 grown in Fe- TSB

Rats were immunised intraperitoneally with formalin-fixed early stationary phase cells of PAO 579. The inoculum was cultivated in Fe- TSB as the OM antigen

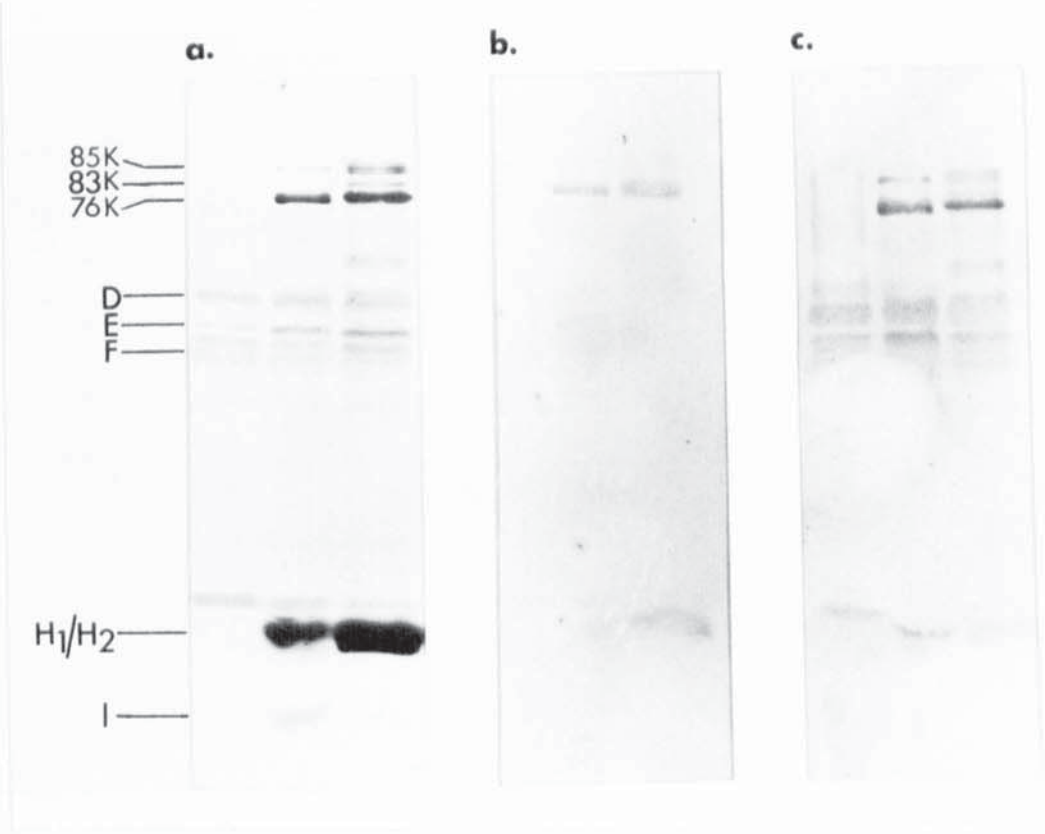


FIGURE 3.12, a-c

IMMUNOBLOTS OF THE OM ANTIGENS SEPARATED IN 3.10a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERA FROM RATS INFECTED WITH PAO 579. THE REACTIONS OBTAINED WITH IgG (a), IgA (b) and IgM (c) ARE SHOWN.

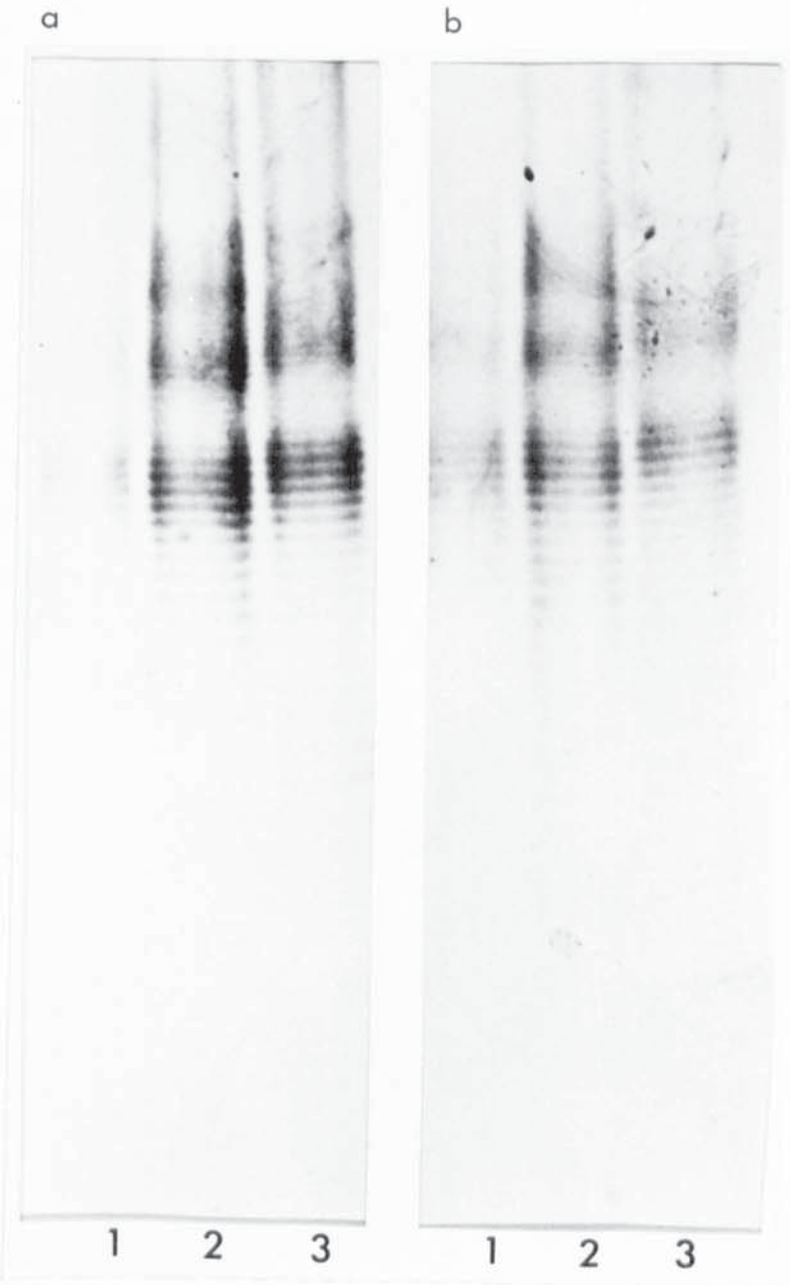


FIGURE 3.13, a and b

IMMUNOBLOTS OBTAINED WITH PROTEINASE K DIGESTS OF OMs OF PAO 579 ISOLATED DIRECTLY FROM THE LUNGS OF INFECTED RATS (LANE 3) AND THE SAME ISOLATE GROWN IN Fe- TSB (LANE 2) AND Fe+ TSB (LANE 1) AFTER ELECTROPHORETIC TRANSFER TO NC PAPER AND REACTION WITH WHOLE SERA FROM RATS INFECTED WITH PAO 579. THE REACTIONS OBTAINED WITH IgG (a) and IgM (b) ARE SHOWN. SAMPLES CONTAINED EQUAL AMOUNTS OF PROTEIN PRIOR TO DIGESTION WITH PROTEINASE K.

profile of iron-depleted cells most closely mimicked the OM protein and LPS antigens of PAO 579 directly isolated from the lungs of infected rats.

Figure 3.14a shows the reaction of IgG antibodies in LPS-absorbed sera from these immunised rats with OM antigens of PAO 579. IgG reacted with all the major OM proteins, including the IRMPs of PAO 579. The reaction with all these proteins, and especially protein F, was stronger than obtained with sera from infected rats. Figures 3.14b and 3.14c show the reaction of IgA and IgM in immunised rat sera with PAO 579 OM proteins. The specificities of the antigen-antibody reactions were similar to those obtained in immunoblots probed with sera from infected rats.

Figure 3.15 shows the reaction of lung fluid from immunised rats with OM antigens of PAO 579. Though IgG reacted predominantly with LPS the OM proteins were also recognised . However it is possible that reaction of antibodies with co-migrating LPS, may contribute to recognition of OM proteins as LPS-specific antibodies had not been absorbed. The reaction of IgM in immunised rat lung fluid with PAO 579 LPS was so strong that reaction with the OM proteins was obscured (data not known). Immunoblotting did not reveal the presence of IgA in the lungs of immunised rats to PA0 579 OM antigens.

3.2.4 Antibody response to whole cell surface antigens examined by ELISA

The whole bacterial cell ELISA measures antibody binding to surface-exposed antigenic determinants, both proteins and LPS (Borowski *et al*, 1984). IgG, IgA and IgM titres in whole and LPS-absorbed sera from infected rats to Fe+ TSB- and Fe- TSB-grown cells of PAO 579 were quantified. Comparison of the end-point titres indicated the IgG and IgM were the major classes of immunoglobulins present in the sera obtained from infected rats 14 days post-infection (Fig 3.16). Titres of IgA in whole serum to PAO 579 surface antigenic determinants were more than 10-fold lower than either IgG or IgM. In all cases the

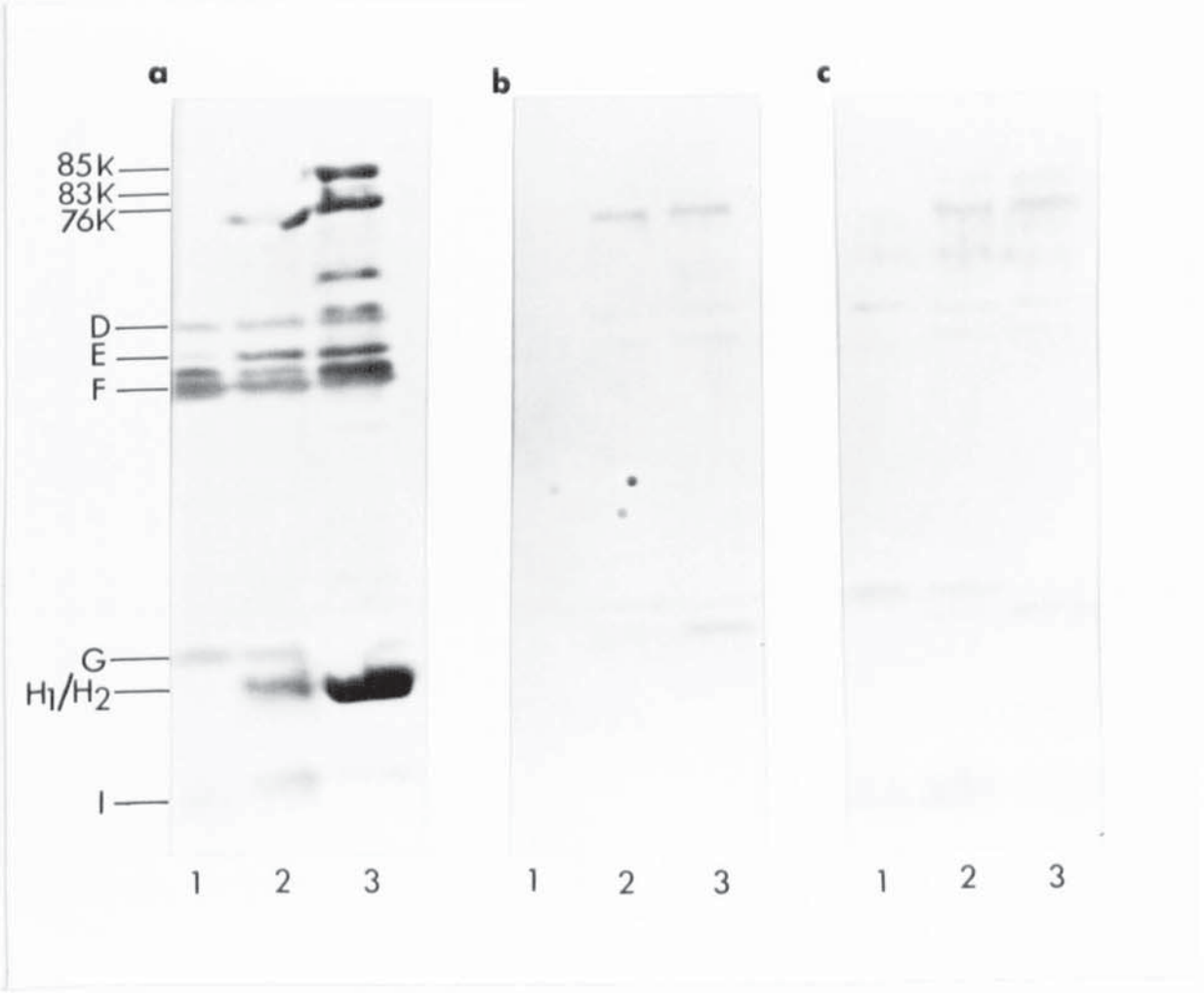


FIGURE 3.14, a - c

IMMUNOBLOTS OF THE OM ANTIGENS SEPARATED IN 3.10a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERA FROM RATS IMMUNISED WITH PAO 579 GROWN IN Fe-TSB. THE REACTIONS OBTAINED WITH IgG (a), IgA (b) and IgM (c) ARE SHOWN.

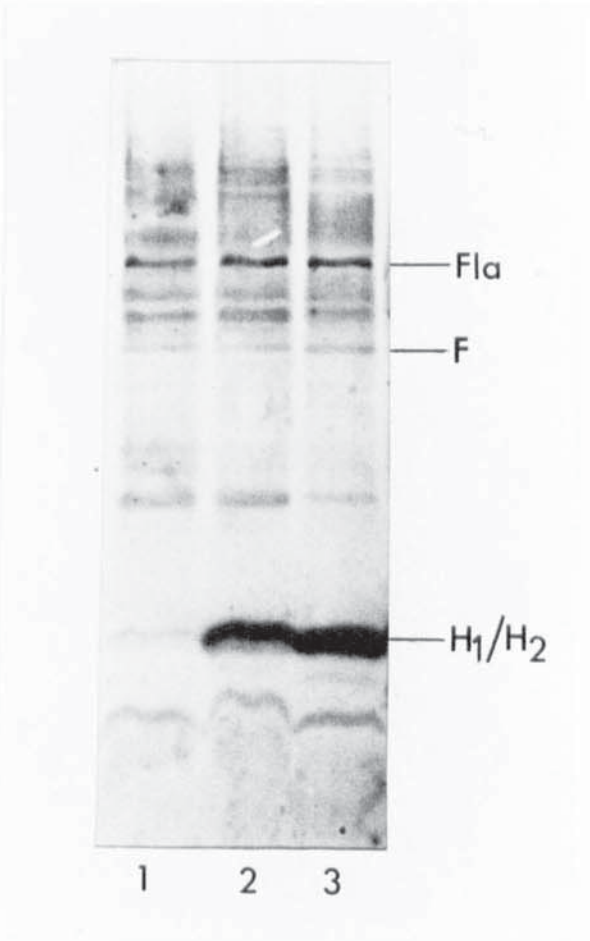


FIGURE 3.15

IMMUNOBLOT OF THE OM ANTIGENS SEPARATED IN 3.10a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LUNG FLUID FROM RATS IMMUNISED WITH PAO 579 GROWN IN Fe-TSB. THE REACTION WITH IgG IS SHOWN.

end point-titres to Fe- TSB-grown antigen were higher than to Fe+ TSB-grown antigen. Absorption of serum with LPS from the homologous strain reduced the IgG and IgM titres by factors of 3.3 to 4.6 fold. The IgA titres were reduced by 1.7 to 2-fold after LPS absorption (these figures were based on mean titre values). These ELISA data reflect the results obtained by immunoblotting.

Similar results were obtained when the antibody titres of immunised rat sera were quantified (Fig 3.17). However, the titres of each class of immunoglobulin were at least 8-fold higher in immunised rat sera as compared to infected rat sera. It was noted that the end-point IgG titre in LPS-absorbed serum to Fe- grown-PAO 579 was lower than to Fe+ grown cells. The reason for this is not clear. The proportion of IgA, relative to the other classes of immunoglobulins, was also reduced in immunised rat sera.

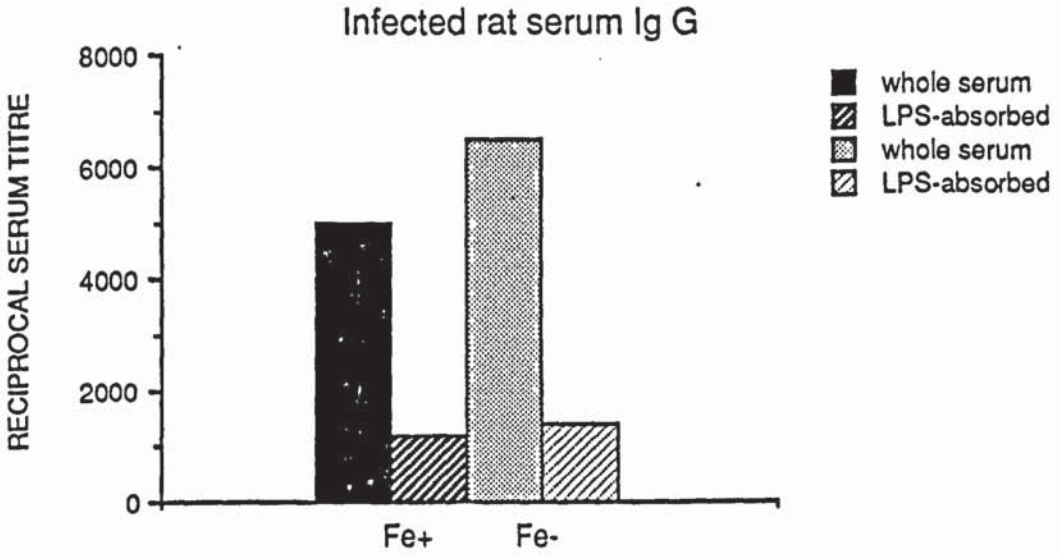
ELISA results indicated that sera from a group of control rats contained a low titre of IgM (end-point titre $4x10^2$) to PAO 579 surface antigens. Antibodies of the IgA and IgG classes in sera from control rats to PAO 579 were not detected in this ELISA.

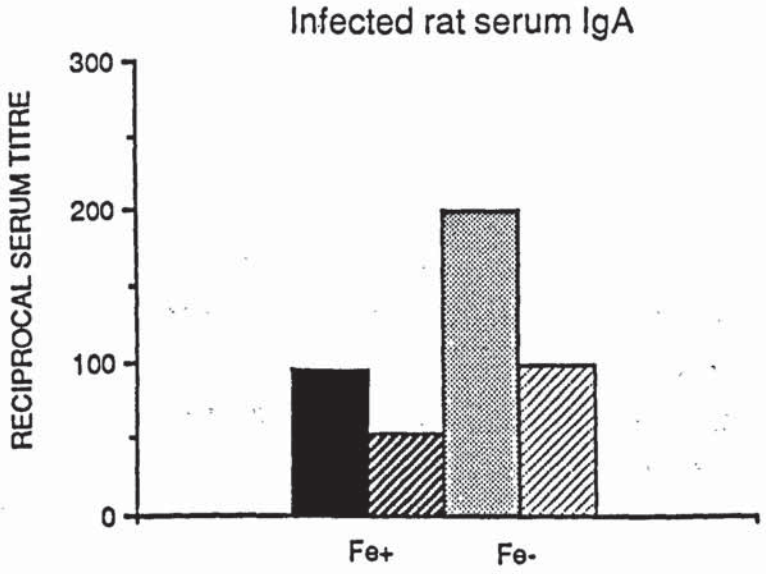
3.2.5 Reaction of antibodies with OM antigens of P. aeruginosa serotype strains

The IATS defines 17 0-serotypes of P. aeruginosa (Bergan, 1975). The strain used in this experimental infection was a nontypable mucoid variant of PAO 381 - a serotype 5 strain. Outer membranes were prepared by sarkosyl-extraction from each of the 17 0-serotypes of P. aeruginosa, all grown in iron-depleted media (Fig 3.18). A number of IRMPs were expressed in the OM of each of these isolates. Protein F and H1/H2 were highly conserved in all these serotype strains. Greater variability was noted in the apparent molecular weights of proteins D and E (molecular weight range 42-50K) and also in the expression of protein G. The migration, and therefore the apparent molecular weight, of protein I appeared to be

FIGURE 3.16

COMPARISON OF THE ANTIBODY TITRES OF WHOLE AND LPS-ABSORBED SERA FROM RATS INFECTED WITH PAO 579. ELISA WELLS WERE COATED WITH WHOLE CELLS OF PAO 579 GROWN IN Fe+ TSB OR FE- TSB.. THE TITRES ARE EXPRESSED AS THE RECIPROCAL OF THE SERUM DILUTION WHICH GAVE AN ABSORBANCE OF 0.1 UNITS ABOVE BLANK WELLS. THE END-POINT TITRES OF IgG, IgA AND IgM ARE SHOWN.


Infected rat serum Ig G
RECIPROCAL SERUM TITRE
8000
6000
4000
2000
0
Fe+
Fe-
whole serum
LPS-absorbed
whole serum
LPS-absorbed


Infected rat serum IgA
RECIPROCAL SERUM TITRE
300
200
100
0
Fe+
Fe-

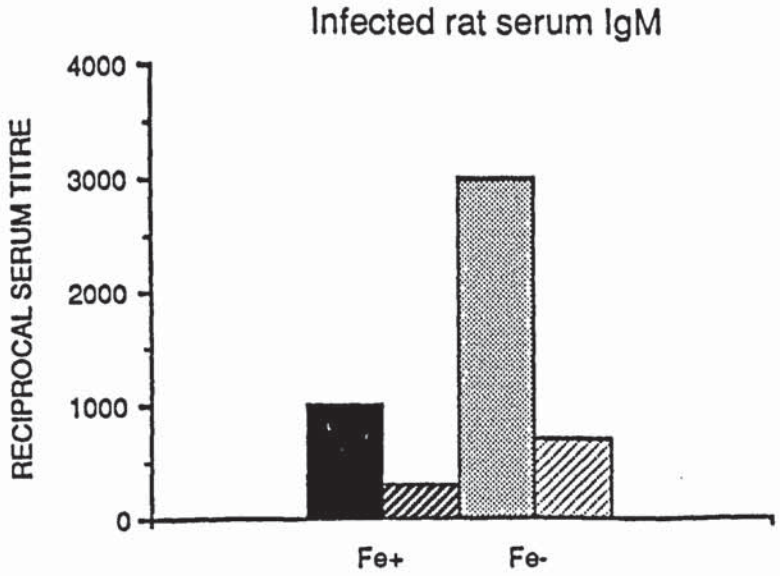
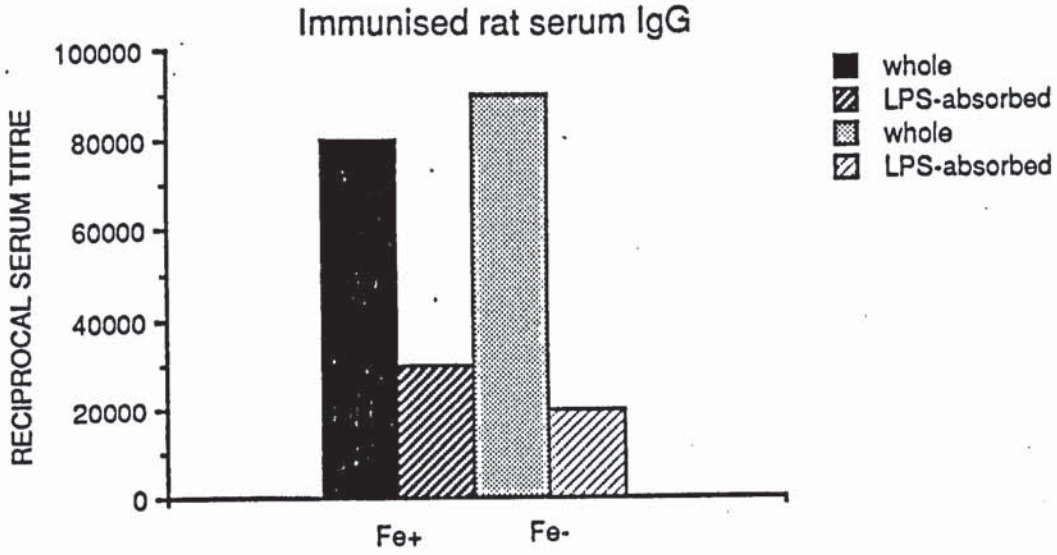
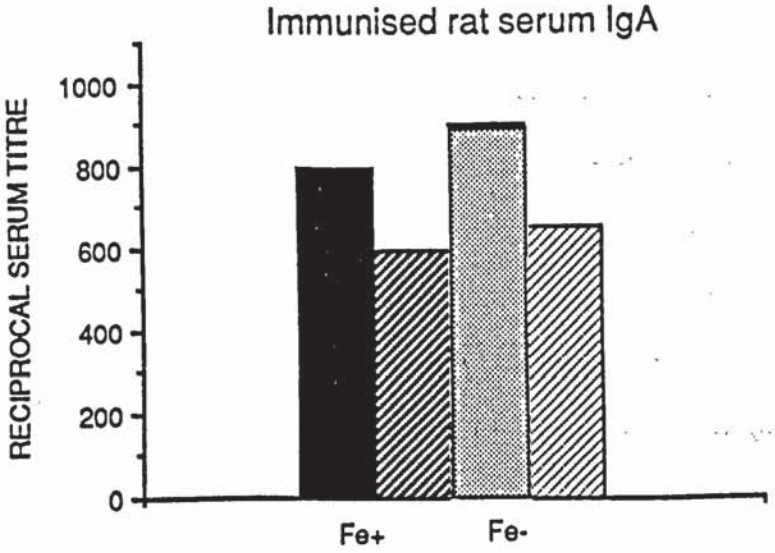
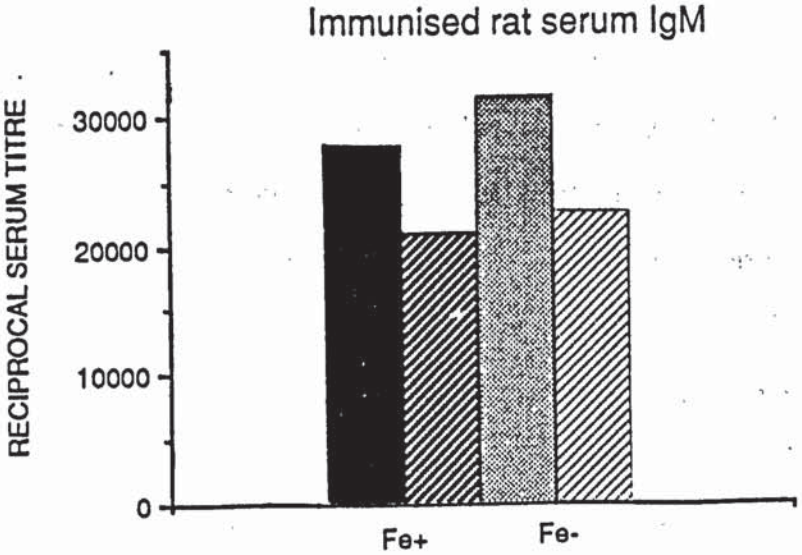

Infected rat serum IgM
RECIPROCAL SERUM TITRE
4000
3000
2000
1000
0
Fe+
Fe-

FIGURE 3.17

COMPARISON OF THE ANTIBODY TITRES OF WHOLE AND LPS-ABSORBED SERA FROM RATS IMMUNISED WITH PAO 579 GROWN IN Fe- TSB. ELISA WELLS WERE COATED WITH WHOLE CELLS OF PAO 579 GROWN IN Fe+ TSB OR Fe- TSB. THE TITRES ARE EXPRESSED AS THE RECIPROCAL OF THE SERUM DILUTION WHICH GAVE AN ABSORBANCE OF 0.1 UNITS ABOVE BLANK WELLS. THE END-POINT TITRES OF IgG, IgA AND IgM ARE SHOWN.


Immunised rat serum IgG
RECIPROCAL SERUM TITRE
100000
80000
60000
40000
20000
0
Fe+
Fe-
whole
LPS-absorbed
whole
LPS-absorbed


Immunised rat serum IgA
RECIPROCAL SERUM TITRE
1000
800
600
400
200
0
Fe+
Fe-


Immunised rat serum IgM
RECIPROCAL SERUM TITRE
30000
20000
10000
0
Fe+
Fe-

affected by the rough LPS component.

Figure 3.19 shows the immunoblots obtained by probing the OM antigens described in Figure 3.18 with lung fluid from infected rats after transfer of the antigens to NC paper. IgG to the IRMPs reacted with these antigens in the OM of all the serotype strains. Similarly, antibodies to proteins F, H1/H2 and I reacted strongly with each of these antigens in the OM of most serotype strains. Coomassie blue staining of the SDS-polyacrylamide gel after electrophoretic transfer revealed that protein F in the OM of serotypes 1-5 had not transferred completely to the NC paper which may account for the faint reaction of antibodies with these proteins on this immunoblot.

An antigen which probably represents flagellin protein in the OM of serotypes 1,2, 3, 4, 5, 12, and 16 was strongly recognised on this immunoblot. Flagellin protein, if expressed, may have been sheared from the other serotypes during OM preparation. Alternatively, lack of antibody reaction may be due to differences in the antigenic determinants in these flagellins. Antibodies developed to PAO 579 core LPS reacted with this antigen in most serotype strains.

Figure 3.19b shows the reaction of IgG in whole sera from infected rats with the OM antigens of the serotype strains. Whole sera was used to allow investigation of the cross-reactivities of protein- and LPS-specific antibodies with other 0–serotypes of P. aeruginosa. The IRMPs of all the serotype strains were only faintly recognised on this immunoblot. Antibodies to proteins F and I reacted with all the serotype strains whilst antibodies to proteins H1/H2 reacted with all serotype strains except serotype 6. The intensity of the antibody reactions with OM antigens of different serotype strains appeared somewhat heterogeneous. For example, the reaction with proteins H1/H2 of serotypes 1, 2, 8, 12 and 17 was stronger than with other serotypes. This difference may be due to variations in the amount of H1 and H2 expressed by these strains or to differences in their antibody combining sites.

The strong reaction with serotype 5 was probably due to reaction of IgG with

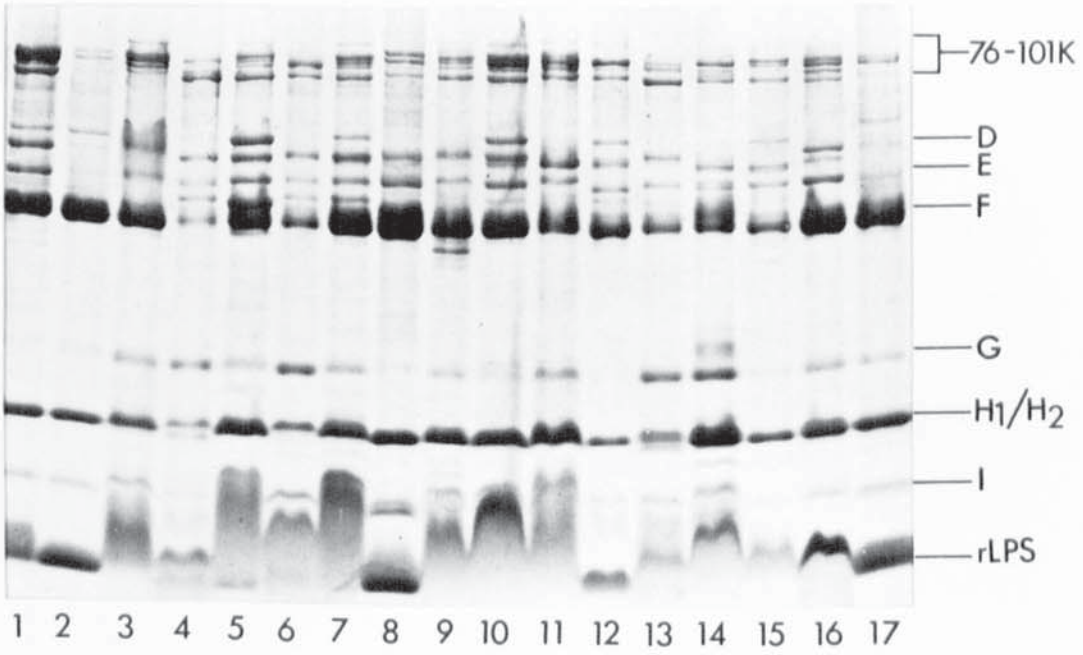


FIGURE 3.18

THE OM PROTEIN PROFILES OF A REPRESENTATIVE OF EACH OF THE 17 0–SEROTYPES OF P. AERUGINOSA (LANES 1-17) ALL GROWN IN IRON-DEPLETED MEDIA. 14% w/v ACRYLAMIDE GELS WERE USED AND PROTEINS WERE VISUALISED WITH COOMASSIE BLUE STAIN. THE MOLECULAR WEIGHT RANGE OF THE IRMPs IS INDICATED IN KILODALTONS (K).

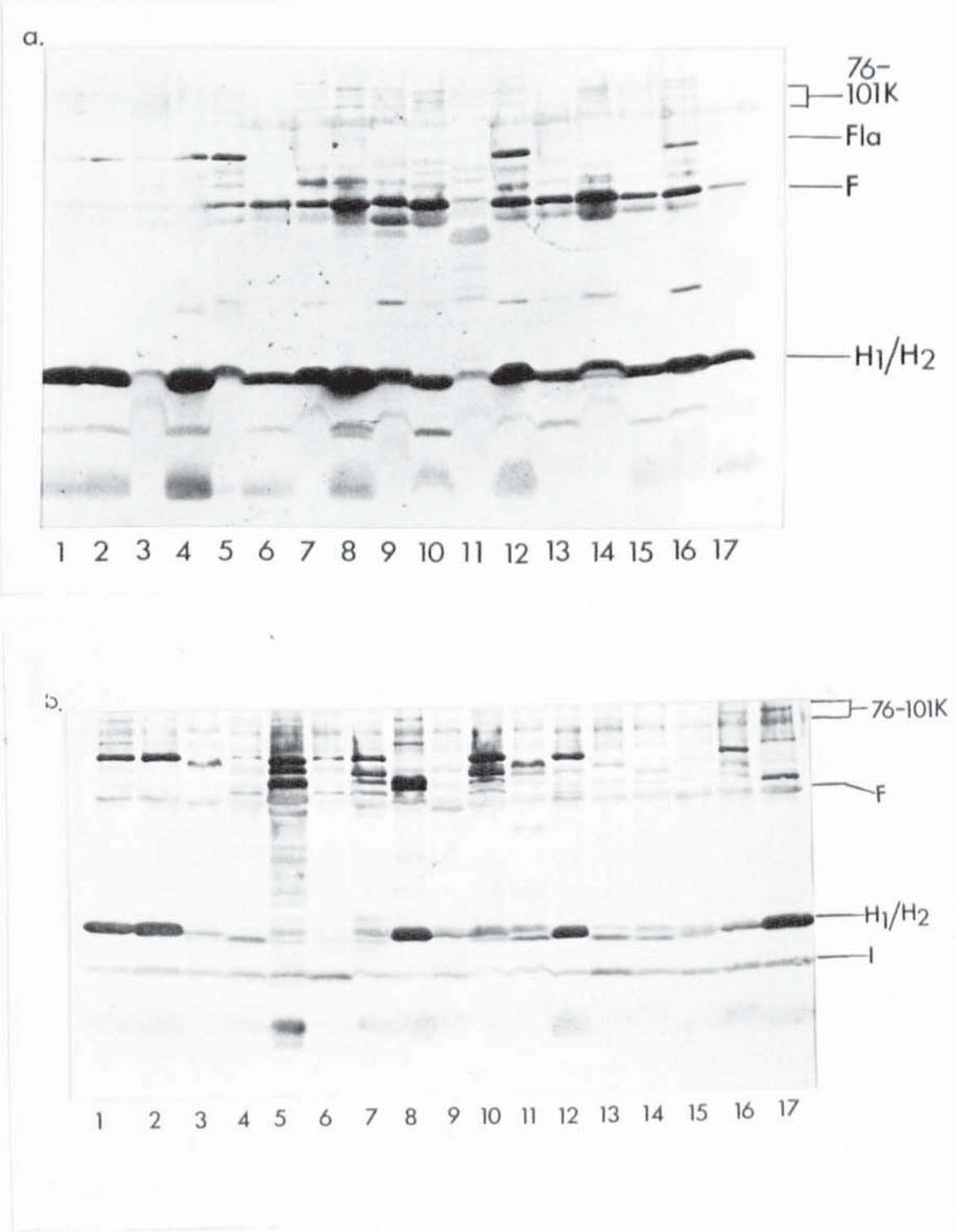


FIGURE 3.19, a and b

IMMUNOBLOTS OF THE OM ANTIGENS SEPARATED IN 3.18 ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LUNG FLUID (A) AND WHOLE SERA (B) FROM RATS INFECTED WITH PAO 579. THE REACTION OBTAINED WITH IgG IS SHOWN.

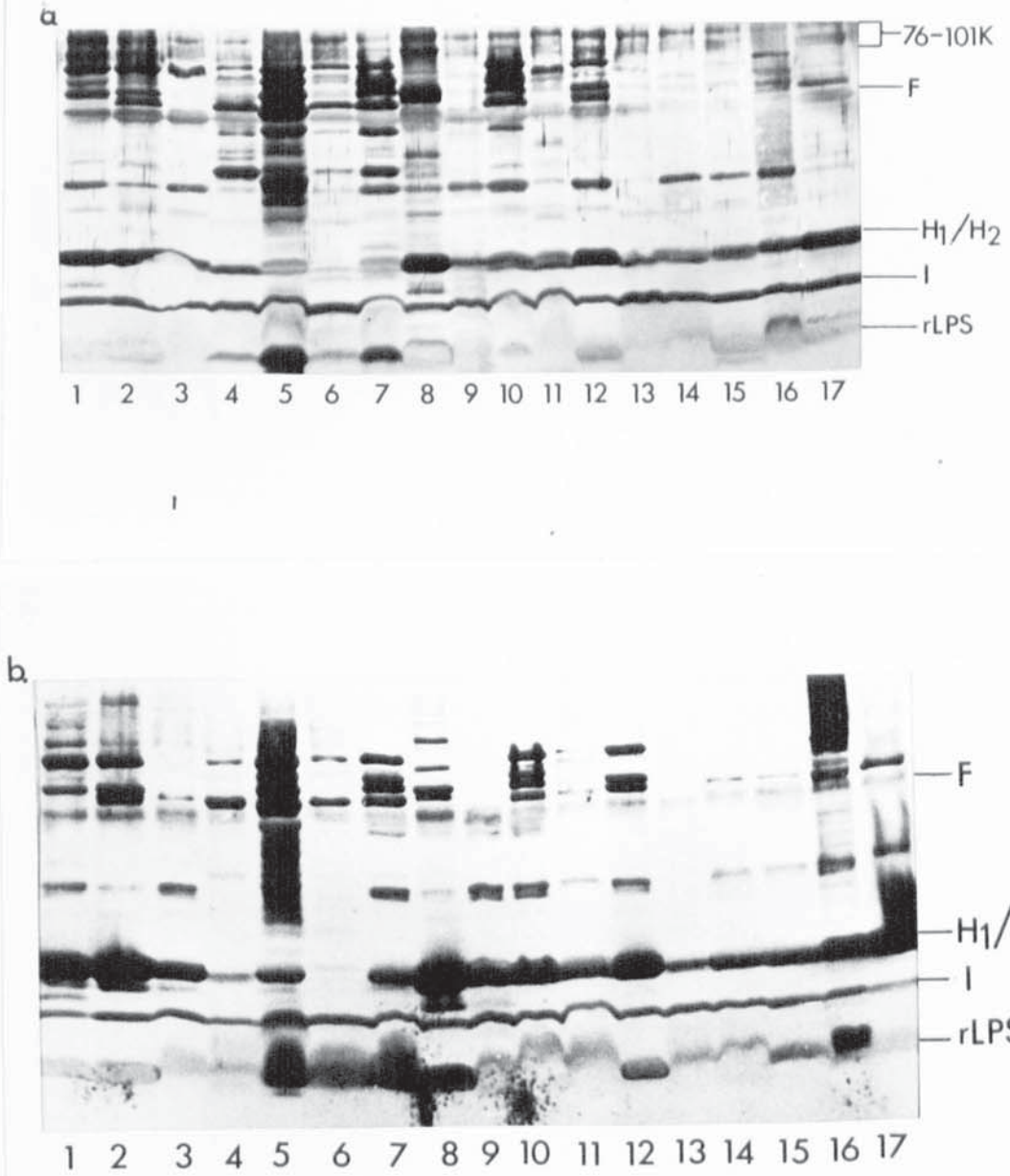


FIGURE 3.20, a and b

IMMUNOBLOTS OF THE OM ANTIGENS DESCRIBED IN 3.18 AFTER ELECTROPHORETIC TRANSFER TO NC PAPER AND INCUBATION WITH WHOLE SERA (A) AND LUNG FLUID (B) FROM RATS IMMUNISED WITH PAO 579 GROWN IN Fe- TSB. THE IgG RESPONSE IS SHOWN.

the LPS 0-antigen of this serotype strain.

IgG in sera from immunised rats reacted more strongly with the major OM proteins, and other OM antigens, of these serotype strains (Fig 3.20a) than IgG in sera from infected rats. The antibody antigen reaction was somewhat heterogeneous, however, antibodies to OM proteins H1/H2 and I appeared to react with these antigens in the OM of all the serotype strains. The proteins of serotypes 13-16 were only faintly recognised. The transfer of these antigens to NC paper was subsequently confirmed by visualisation with amido black stain.

The pattern of cross-reaction of serum IgG was mirrored when the IgG response in the lungs of immunised rats was investigated (Fig 3.20b). The reaction obtained with lung fluid was less intense than was obtained with serum.

Antibodies of the IgG class in sera from infected or immunised rats directed to PAO 579 LPS reacted with the 0-antigen of serotype 5, and to a lesser extent serotype 16, strains. Serum IgM reacted faintly with serotype 5 and strongly with serotype 16 strains (not shown). Similarity in the O-antigens of P. aeruginosa IATS serotypes 2, 5 and 16 has been reported on the basis of agglutination tests with typing sera (Kasuma, 1978).

3.2.6 Reaction of antibodies with OM antigens of an index of strains of Enterobacteriaceae

The OM antigens of the index of strains of Enterobacteriaceae and P. aeruginosa described in section 3.1.5 were reacted with lung fluid and sera from infected rats. Figure 3.21a shows that IgG in lung fluid from infected rats to proteins H1/H2 appeared to cross-react with an antigen of similar molecular weight in the OM of S. marcescens, K. pneumoniae, P. mirabilis and to a lesser extent E. coli. Sera from infected rats also contained IgG which reacted with this low molecular weight antigen in the OM of the strains of Enterobacteriaceae (Fig 3.21b).

The specificity of the antibody response to P. aeruginosa was confirmed by

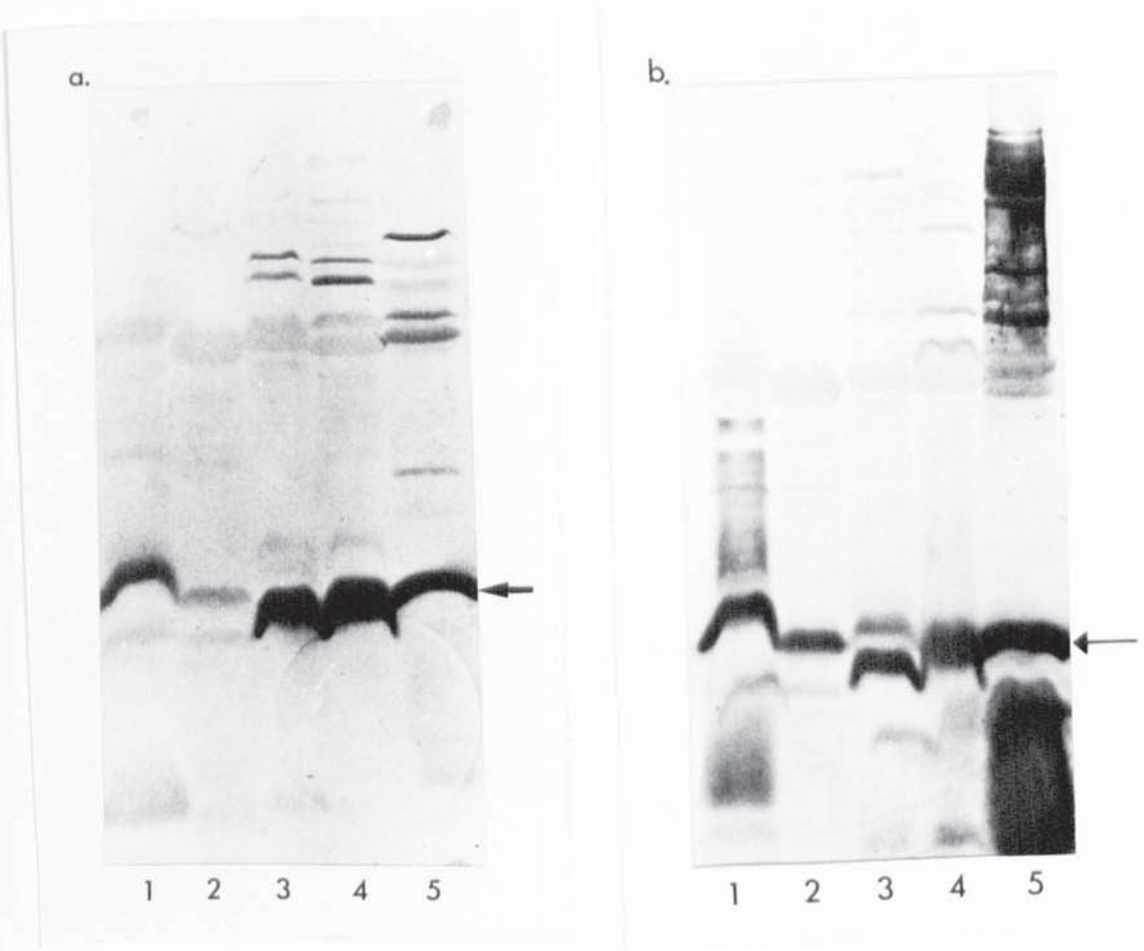


FIGURE 3.21, a and b

IMMUNOBLOTS OF THE OM ANTIGENS OF S. MARCESCENS (LANE 1) E. COLI (LANE 2), K. PNEUMONIAE (LANE 3), P. MIRABILIS (LANE 4) AND PAO 579 (LANE 5) (AS DESCRIBED IN FIG 3.8) AFTER ELECTROPHORETIC TRANSFER TO NC PAPER AND REACTION WITH FLUIDS FROM RATS INFECTED WITH PAO 579. THE REACTIONS OBTAINED WITH IgG IN LUNG FLUID (A) AND SERA (B) ARE SHOWN. THE CROSS-REACTING ANTIGEN IS INDICATED WITH AN ARROW.

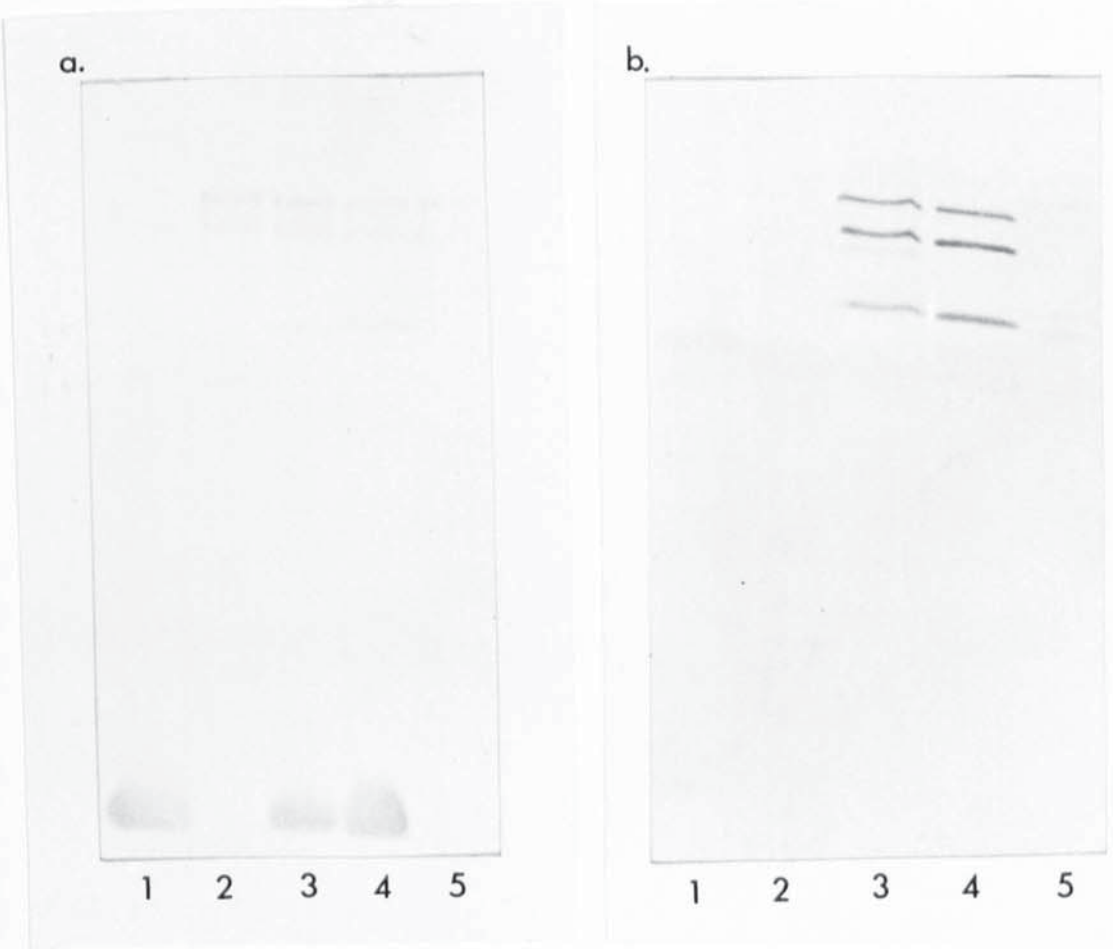


FIGURE 3.22, a and b

IMMUNOBLOTS OF THE OM ANTIGENS OF S. MARCESCENS (LANE 1), E. COLI (LANE 2), K. PNEUMONIAE (LANE 3), P. MIRABILIS (LANE 4) AND PAO 579 (LANE 5) (AS DESCRIBED IN FIG 3.8) AFTER ELECTROPHORETIC TRANSFER TO NC PAPER AND REACTION WITH FLUIDS FROM NON-INFECTED CONTROL RATS. THE REACTION OBTAINED WITH IgG IN LUNG FLUID (A) AND SERA (B) IS SHOWN.

blotting with lung fluid and sera from a group of control rats not infected with P. aeruginosa. Antibodies of the IgG class reacted with some OM proteins of the Enterobacteriaceae but not PAO 579 (Figs 3.22a and 3 .22b).

3.2.7 Discussion

Information regarding the host-bacterium interaction in infection is still greatly lacking, in particular, few studies have investigated the surface antigens of pathogens grown *in situ* in infections (Brown and Williams, 1985a). In this study the local and systemic antibody responses to both protein and LPS surface antigens of *in vivo*-grown P. aeruginosa were investigated using the animal model of chronic lung infection developed by Cash *et al* (1979). The histopathologic changes which occur in the lungs of experimentally infected rats resemble those seen in the lungs of patients with chronic P. aeruginosa pneumoniae (Cash *et al*, 1979). In another study using this animal model direct electron microscopic examination revealed the formation of exopolysaccharide enclosed microcolonies in infected alveoli similar to those seen in bronchoscopy and post-mortem specimens from CF patients (Lam, 1983; Lam *et al*, 1980). In the clinical and experimentally induced infections P. aeruginosa is confined to the lung; bacteraemia and toxaemia do not occur.

These data indicate that mucoid P. aeruginosa grew in the lungs of rats under conditions of iron-deprivation as judged by the expression of IRMPs. The pattern of IRMPs was similar to that previously described for mucoid P. aeruginosa recovered directly without subculture, from the sputum of a CF patient (Brown *et al*, 1984). The same IRMPs were induced following growth of the isolate used in this present study in iron-depleted laboratory media. However protein G was expressed less in the OM of *in vivo*- as compared to *in vitro*-grown cells. The function of protein G has not been described. Results presented in section 3.1.3 suggest that the concentration of iron in the growth environment may alter the expression of this protein in the OM of some P. aeruginosa isolates. Alternatively, features of the lung

environment such as growth rate and the microcolony mode of growth, restriction of nutrients other than iron, or physical factors such as pH or oxygen tension were not mimicked by growth *in vitro* in iron-depleted batch cultures and may account for the differences in the OM antigens of *in vivo*- and *in vitro*-grown cells.

Lungs and serum obtained from rats 14 days post-infection contained IgG, IgA, and IgM to P. aeruginosa. These antibodies were directed to OM proteins and LPS of the infecting strain. The results indicate that the various classes of immunoglobulins were present in the lung in proportions significantly different from those in serum.

The predominant class of immunoglobulin to P. aeruginosa present in the lung and serum was IgG. Intramuscular or intranasal immunisation of rabbits with whole cells or LPS stimulated an IgG response which was detected in both serum and lung fluids (Reynolds, 1974; Reynolds and Thompson, 1973a). IgG promotes clearance of bacteria by opsonising the cells for phagocytosis and also by activating complement. IgM plays an important role in the primary serum response to P. aeruginosa (Reynolds and Thompson, 1973b) and other Gram-negative bacteria. However, the lower respiratory tract secretions do not normally contain detectable amounts of IgM (Reynolds and Newball, 1974).

The antibody response at mucosal surfaces are normally mediated principally by secretory IgA (Tomasi *et al*, 1965). The results of this present study indicate that an IgA response was elicited in the lung during chronic P. aeruginosa lung infection. A secretory IgA response was induced in the lungs of rabbits following intrapulmonary but not intramuscular immunisation with P. aeruginosa (Reynolds, 1974; Reynolds and Thompson, 1973a). This secretory IgA was found to be less efficient than IgG as an opsonin for P. aeruginosa in *in vitro* opsonisation tests, however it did have agglutinating activity specific to the immunising serotype (Reynolds, 1974). The clearest role for secretory IgA in bacterial disease is in prevention of attachment of bacteria to the mucosal surface (Edebo *et al*, 1985;

Tomasi, 1976).

In this present study IgG in lung fluid from infected rats reacted strongly with P. aeruginosa OM proteins F, H1/H2, flagellin and IRMPs. Protein H1 was induced in the OM of P. aeruginosa recovered directly and without subculture from the sputum of a CF patient (Brown *et al*, 1984) and was recognised by antibodies in the patient's own serum (Anwar *et al*, 1984). These present data indicate that protein H1 was also induced in the OM of P. aeruginosa grown *in vivo* in the lungs of infected rats and that an antibody response was elicited to this protein.

The IgG response detected in the serum to protein F appeared weaker than in the lung. Hedstrom *et al* (1984) have previously reported a strong antibody response to P. aeruginosa OM proteins F, H2 and I in the serum of mice infected by subcutaneous injection of live P. aeruginosa. In this animal model of chronic lung infection P. aeruginosa is largely contained within exopolysaccharide enclosed microcolonies in the lung and does not spread to the bloodstream. This may account for the somewhat weaker antibody response to protein F reported here.

Gilleland *et al* (1984) demonstrated that immunisation with purified outer membrane protein F or passive administration of anitsera to protein F protected mice from intraperitioneal challenge with the homologous and heterologous P. aeruginosa strains. The protein F preparations did however contain small amounts of LPS. In a more recent study Matthews-Greer and Gilleland (1987) reported that active immunisation with protein F (extracted from polyacrylamide gels) afforded significant protection of animals in a burned mouse model. To address the problems of LPS contamination these investigators used a protein F preparation containing high concentrations of LPS (7μg/10μg of protein) and compared the protection afforded with this antigen with that obtained by using a control preparation containing 7 μg of LPS. The protein F preparation afforded significant protection above that provided by LPS against subsequent challenge with heterologous strains. Active immunisation with OM protein H did not however afford significant protection to mice in this burned mouse model. The antigen preparations used were prepared from cells grown

in nutrient broth. Holder *et al*, (1982) also demonstrated that mice immunised with highly purified flagellar antigens were protected against live challenge with P. aeruginosa in a burn wound model. Antibody to flagellin protein does not opsonise bacteria for phagacytosis however attachment of antibodies to flagellin protein may reduce the mobility of the organism at the site of infection and hence its pathogenicity. The opsonising ability of antibodies to P. aeruginosa major OM proteins has not yet been investigated.

The IRMPs were important targets for IgG and IgA in the lung and all three classes of immunoglobulins in sera from infected rats. The IRMP of 76K was particularly strongly recognised by IgG in the lung. This may suggest that this protein is either exposed at the cell surface or is more strongly expressed by bacteria growing in the lung. An antibody response to the IRMPs or other proteins involved in nutrient uptake would be expected to reduce the growth rate of the pathogen and may contribute to the slow growth rates of bacteria in infection (Brown and Williams, 1985a). Passive administration of antibody to the high molecular IRMPs of E. coli has recently been shown to protect turkeys from peritoneal challenge with the homologous and heterologous strains (Bolin and Jensen, 1987).

An IgG or IgM response in the lung of infected rats to the 0-antigen of the infecting strain was not revealed by immunoblotting. There was some reaction between IgA and an antigen which may have been LPS. However, ELISA studies indicated that serum IgG and IgM were mainly directed to LPS of the infecting strain. It is possible that LPS-specific antibodies were bound to bacteria or to inflammatory cells recruited into the lung in response to infection.

Lung fluid and sera from a group of non-infected control rats contained antibodies which reacted faintly on immunoblots with the OM antigens of several species of Enterobacteriaceae but not P. aeruginosa. It was interesting that lung fluid and sera from infected rats contained IgG which reacted with an antigen of molecular weight similar to that of P. aeruginosa protein H in the OM of S. marcescens, K.

pneumonia, P. mirabilis and to a lesser extent E. coli strains. A peptidoglycan-associated lipoprotein which has a similar biochemical composition to P. aeruginosa protein H has been described in the OM of a number of Gram-negative bacteria (Mizuno, 1979; 1981). However, antisera to P. mirabilis peptidoglycan-associated lipoprotein reacted with this protein in the OM of other strains of Enterobacteriaceae but not P. aeruginosa (Mizuno, 1981). The results of this present study suggest that some strains of Enterobacteriaceae and P. aeruginosa may contain a common antigenic determinant.

The origin of the immunoglobulins present in the lungs of infected rats cannot be ascertained from these data. In passive transfer experiments a small proportion of ^{125}I-labelled IgG (Reynolds and Thompson, 1973a) and an IgG monoclonal antibody to H. influenzae type B LPS (Toews *et al*, 1985) were later detected in bronchial lavage fluids. Transudation of serum antibodies across the inflamed alveolar membranes was increased following intrapulmonary challenge with P. aeruginosa (Pennington and Kuchmy, 1980; Dunn *et al*, 1985). The results of this study indicate that antibodies in the lung were to some extent synthesised locally. IgG, IgA and IgM-containing cells have been demonstrated in human bronchial mucosa and lymph nodes (Tourville *et al*, 1969; Martinez-Tello *et al*, 1968). Furthermore, significantly increased numbers of IgA and to a lesser extent IgG containing cells were present in the lung lymph tissue of patients with chronic bronchopulmonary diseases (including CF patients), than in a group of control patients.

The immunisation schedule used in this present study resulted in high titres of systemic IgG and IgM to P. aeruginosa LPS and also OM proteins. The lungs of immunised rats contained IgG to P. aeruginosa and immunoblotting results indicated that the specificity of these antibodies was essentially identical to that of serum IgG. Other workers have reported similar findings in rats immunised with H. influenzae type B (Toews *et al*, 1985).

Systemic immunisation with P. aeruginosa and H.influenzae type B has been shown to enhance the rate of pulmonary clearance of these organisms in animal models of acute pneumonia (Dunn *et al*, 1985; Toews *et al*, 1985; Pennington and Kuchmy, 1980). Increased clearance rates were probably related to antibody mediated opsonisation of the bacteria (Dunn *et al*, 1985; Toews *et al*, 1985; Pennington and Kuchmy, 1980).

The OM proteins F, H1/H2 and I appeared to be highly conserved in all the serotype strains confirming the results of other workers (Mutharia *et al*, 1982; Mizuno and Kageyama, 1978). The results presented here indicated that whilst antibodies to these proteins reacted with these antigens in other serotype strains the intensity of the antibody-antigen reaction varied. Mutharia *et al* (1982) reported that these OM proteins were antigenically similar as hyperimmune sera reacted homogenously with other serotype strains. This difference may be due to variations in the titre of antibodies in the sera used in these studies. Nevertheless, vaccines based on OM proteins may avoid the problems of serotype specificity and toxicity associated with LPS-containing vaccines. The data presented here indicated that proteins H1 and H2 are important targets for immunoglobulins present in the lungs and sera of rats with P. aeruginosa lung infection and therefore these proteins may be ideal candidates for vaccine development. Pennington *et al* (1986) reported that passive administration of monoclonal antibody to protein F did not protect mice from acute pneumonia. This result is surprising considering that the porin was strongly recognised by IgG in the lung. Clearly, consideration must be given to development of the appropriate antibodies of the required specificity at the mucosal membrane. The iron uptake system may provide a suitable alternative mechanism for immunological intervention in P. aeruginosa infection.

The pulmonary clearance of the mucoid strain used in this study and its non-mucoid parent strain were previously compared (Govan *et al*, 1983). Intrapulmonary killing of the mucoid strain was less efficient than that of the non-mucoid strain unless the inocula were washed before introduction into the lungs. The

alginate produced by mucoid strains of P. aeruginosa may interfere with efficient opsonisation (Baltimore and Mitchell, 1980) or phagocytosis (Schwartzmann and Boring, 1971) of bacteria in the lung. The sheer size of the microcolony may interfere with phagocytosis by host defence cells in the lung since macrophages could not phagocytise *in vitro*-grown microcolonies of P. aeruginosa. Single cells of a related strain of P. aeruginosa were efficiently phagocytised by these macrophages (Costerton *et al*, 1979). Release of toxins and other substances from these "frustrated" macrophages and PMNs may generate an inflammatory response and contribute to tissue damage in the lung (Govan and Harris, 1986; Høiby *et al*, 1986).

3.3 ANTIBODY RESPONSE TO P. AERUGINOSA ANTIGENS EXAMINED LONGITUDINALLY USING THE RAT MODEL OF CHRONIC LUNG INFECTION AND ALSO WITH SERA FROM CLINICAL INFECTIONS

3.3.1 Enumeration of bacteria recovered from lungs of rats infected with P. aeruginosa 492a

The lungs of rats were inoculated with an agar bead suspension containing early stationary phase cells of P. aeruginosa 492a (referred to as PA 492a) grown in Fe+ TSB (Group A) or Fe- TSB (Group B). The inoculum introduced into the lungs of rats in group A and group B were estimated to contain 9×10^6 and 3×10^7 viable P. aeruginosa respectively. Rats from each group were sacrificed 1, 4, 7, 14, 21 and 28 days post-infection and the lungs of individual rats were homogenised in 30 ml PBS. Viable counts were estimated following serial dilution by plating on Fe-TSA, NA and PIA. The counts obtained on Fe- TSA and NA were similar with slightly higher counts recorded on Fe-TSA. Counts on PIA were consistently around one half of a log cycle lower than on NA and Fe- TSA; this was not due to enumeration of bacteria other then P. aeruginosa . Figure 3.23 shows the mean number (± s.e.m.) P. aeruginosa c.f.u. enumerated on Fe- TSA ml^{-1} of lung homogenate from rats from group A(Fig 3.23a) and group B (Fig 3.23b) at each time of sacrifice. These data suggest that the bacteria multiplied rapidly during the first 24 hours following infection. The number of c.f.u. enumerated continued to increase until around day 7 after which time some clearance of P. aeruginosa occurred. In an earlier study sections from rats' lungs infected with P. aeruginosa in agar beads were examined 10 days after infection using transmission electron microscopy. Aggregates of bacteria were found among lung tissues and all the bacteria in the aggregate showed similar morphology which indicated that they were from one parenteral strain (Lam, 1983).

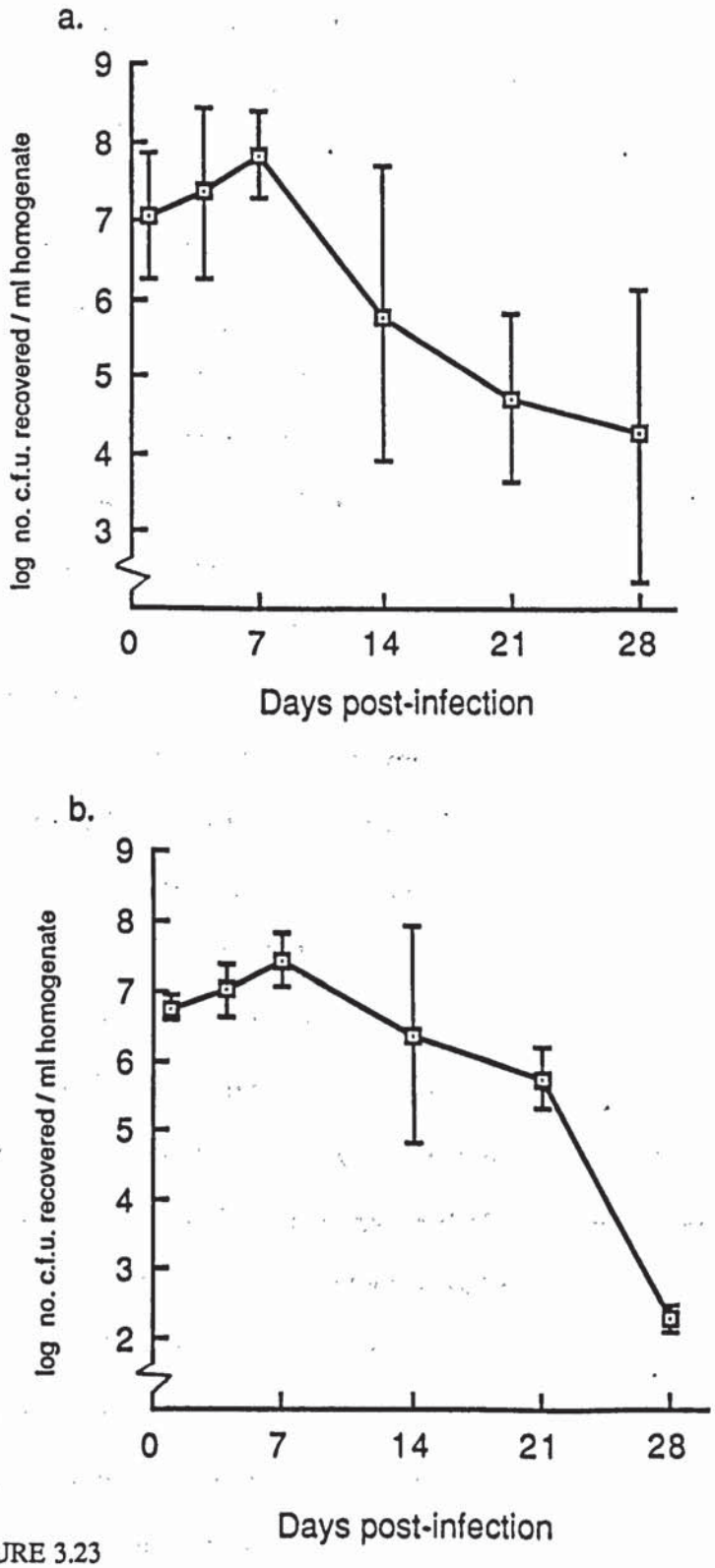


FIGURE 3.23

COLONY FORMING UNITS (c.f.u.)† OF P. AERUGINOSA RECOVERED FROM THE LUNGS OF RATS INFECTED WITH PA 492a GROWN IN Fe+ TSB (a) OR Fe-TSB (b). THE MEAN (± s.e.m.) NUMBER OF C.F.U. ENUMERATED AT DAYS 1, 4, 7, 14, 21 AND 28 DAYS POST-INFECTION FOR EACH GROUP ARE SHOWN.

† c.f.u are counted as c.f.u./ml lung homogenate

3.3.2 Longitudinal antibody response in experimental rat lung infection examined by CIEWIG

The PAO 1 antigen for CIE, which was prepared by disruption of the cells with pressure and subsequent sonication, contained 36 anode migrating antigens, which form precipitins with hyperimmune antibody (Lam *et al*, 1983). The PAO 1 antigen-antibody precipitin pattern is shown in Figure 3.24a. In this system the heavily stained precipitin marked 3l has been identified as the LPS 0-antigen (Lam *et al*, 1983). Antigens for CIE were prepared from PA 492a grown in Fe+ TSB and in Fe- TSB using the procedure of Lam *et al* (1983). Standard hyperimmune polyvalent antibody to PAO 1 formed precipitins with many antigens of the 492a antigen system. Notably precipitin no 3l was missing (Fig 3.24b). When OMs from PA 492a were included in the intermediate gel a number of precipitin limes of identity were formed and several peaks dropped out of the reference system (Fig 3.24c).

Pre-immune serum collected from control rats prior to infection did not form precipitins with the PA 492a antigen (Fig 3.24d).

The presence of precipitating antibodies in sera from rats sacrificed 1, 4, 7, 14 , 21 and 28 days post-infection was investigated. Figures 3.25 and 3.26 shows the precipitins formed with PA 492a antigen grown in Fe+ TSB with sera from rats in group A (Fe+ inoculum) and group B (Fe- inoculum) respectively. Sera obtained from rats 1 and 4 days post-infection did not form precipitins in CIE with PA 492a antigens. Sera from two of the three rats in each group sacrificed 7 days post-infection formed one or two precipitins with PA 492a antigen. Sera from 2 of the group A rats and 1 group B rat sacrificed 14 days post-infection formed only one weak precipitin with the Fe+ 492a antigen. Subsequent sera from the remaining rats formed 2 or more precipitins with PA 492a antigen. Of these, two darkly staining precipitins were most notable. An increasing number of precipitins were formed with sera from rats sacrificed 21 and 28 days post-infection. The area under the darkly stained precipitin peaks decreased with increasing duration of infection which

indicates a stronger antibody response to these antigens.

Figures 3.27 and 3.28 show the precipitins formed with PA 492a antigen grown in Fe- TSB with these same sera. The pattern of precipitin formation was very similar to that obtained with antigen prepared from PA 492a grown in Fe+ TSB. Additional precipitins were formed with sera from some rats sacrificed 28 days after infection and Fe- antigen as compared to Fe+ antigen. The variation in number of precipitin peaks formed with serum from each rat sacrificed at one point during the infection (see for example Fig 3.26g - 3.26i, day 21 sera) probably reflects differences in individual animal's antibody responses.

CIEs were also performed with sera collected from CF patients hospitalised due to acute exacerbations of pulmonary infection. The lungs of patient 1 were colonised with P. aeruginosa for 6 months prior to serum collection whereas the other patients lungs were colonised with P. aeruginosa for a least 5 years. These sera formed between 1 and 61 precipitins with the PA 492a antigen (Fig 3.29). Sera from most patients formed a greater number of precipitins with PA 492a antigens grown in Fe- TSB compared to antigen grown in Fe+ TSB. Sera from patients 2, 3 and 4 formed less than 15 precipitins, yet their lungs had been colonised with P. aeruginosa for more than 5 years.

3.3.3 Longitudinal antibody response in experimental rat lung infection examined by immunoblotting

3.3.3.1 Serum

Mucoid PA 492a was isolated from the lungs of rats sacrificed 1, 4, 7, 14, 21 and 28 days post-infection. The OM protein and LPS (whole cells digested with proteinase K) profiles of these sequential isolates (taken from Fe- TSA) after one subculture in Fe-TSB appeared identical when examined by Coomassie blue or silver staining procedures. The antigens of PA 492a isolated 7 days post-infection were examined after one further subculture by immunoblotting with sera and lung homogenate from infected rats.

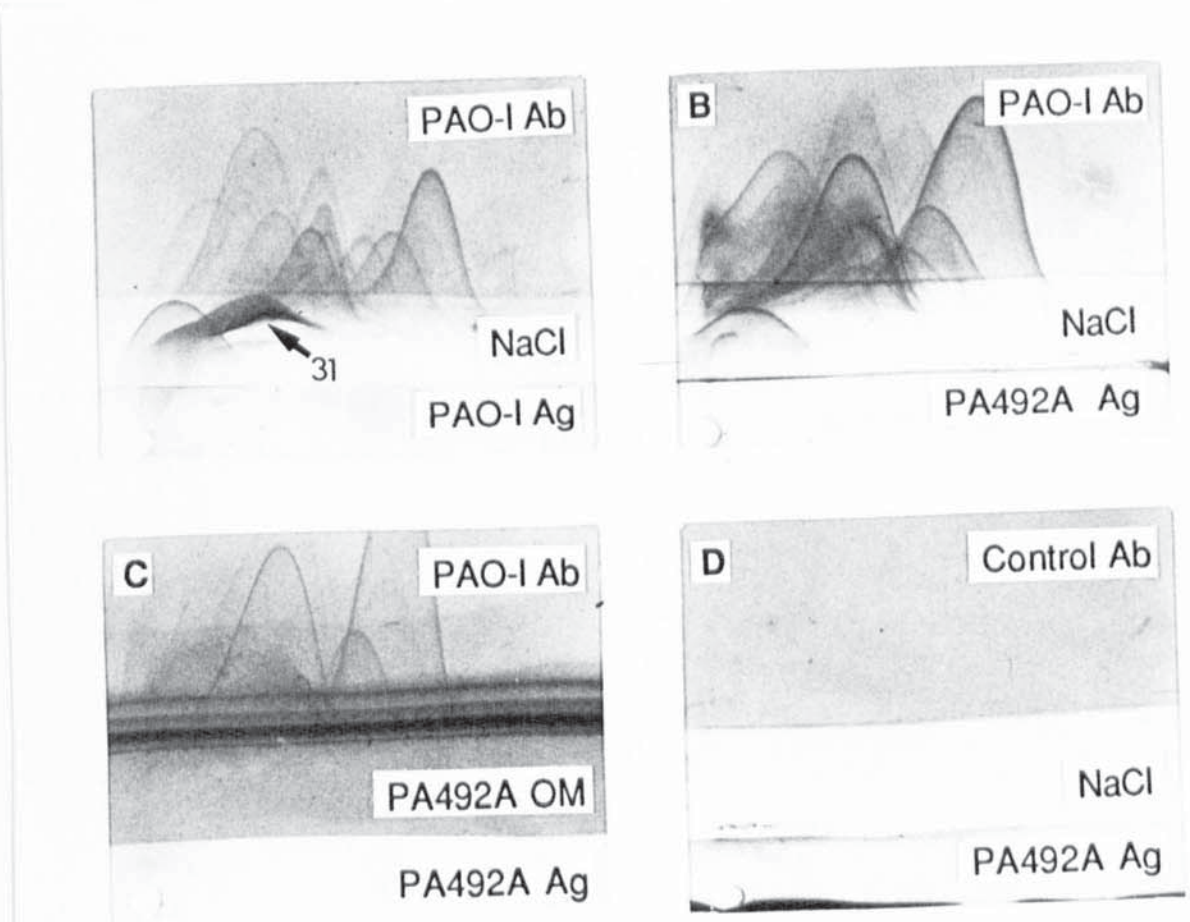


FIGURE 3.24

CROSSED IMMUNOELECTROPHORESIS WITH AN INTERMEDIATE GEL WITH 6 µl (10 µg protein) OF PA01 ANTIGEN OR PA 492a ANTIGEN, AGAINST PAO1 ANTIBODY OR PREIMMUNE CONTROL SERUM (16.7 µl/cm^2). [a] PAO 1 (Ag)-ANTIBODY (Ab) PRECIPITATION PATTERN; THE ARROW MARKS ANTIGEN NO 31 THE O-ANTIGEN PRECIPITIN OF PAO 1; [b] PA 492a Ag PRECIPITINS WITH PAO 1 Ab; [c] CROSSED-LINE IMMUNOELECTROPHORESIS WITH PA 492a Ag AND PAO 1 Ab AS IN [b] WITH PA 492a OMs (8.4 µl/cm^2) IN THE INTERMEDIATE GEL – LINES OF IDENTITY WERE FORMED INDICATING THE PRESENCE OF OM COMPONENTS IN THE STANDARD Ag; [d] CROSSED IMMUNOELECTROPHORESIS WITH PA 492a Ag AND PRE-IMMUNE SERUM. SALINE WAS INCLUDED IN THE INTERMEDIATE GEL IN [a], [b] AND [d] AS CONTROL.

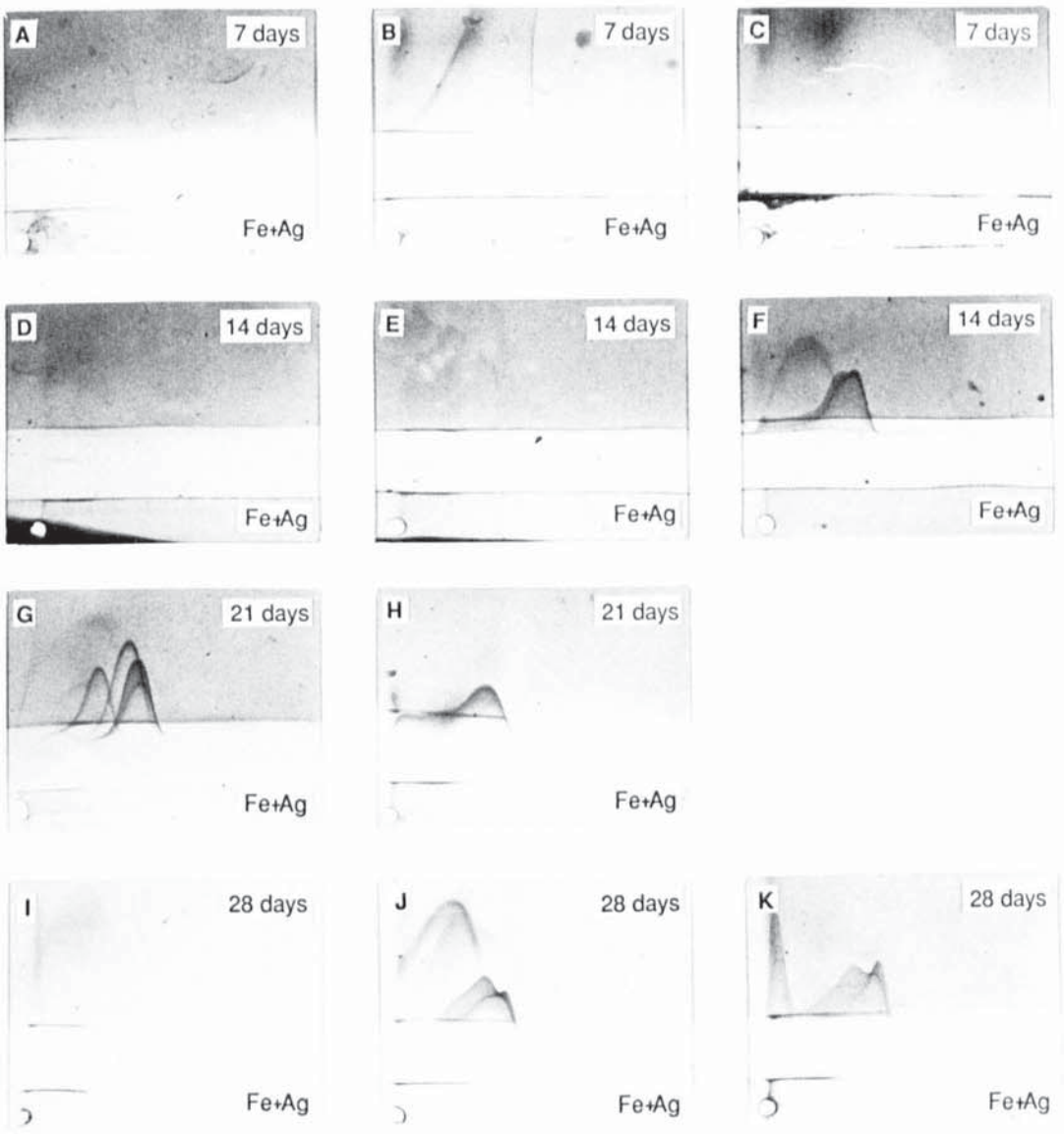


FIGURE 3.25, a - k

ANTIBODY RESPONSE OF RATS INFECTED WITH PA 492a GROWN IN Fe+ TSB TO PA 492a ANTIGENS, CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL OF 6 µl Fe+ Ag AGAINST WHOLE SERA FROM RATS SACRIFICED 7 (a-c), 14 (d-f), 21 (g and h) AND 28 (i-k) DAYS POST-INFECTION. SERUM WAS INCORPORATED IN THE SECOND DIMENSION AT 16.7 µl/cm^2.

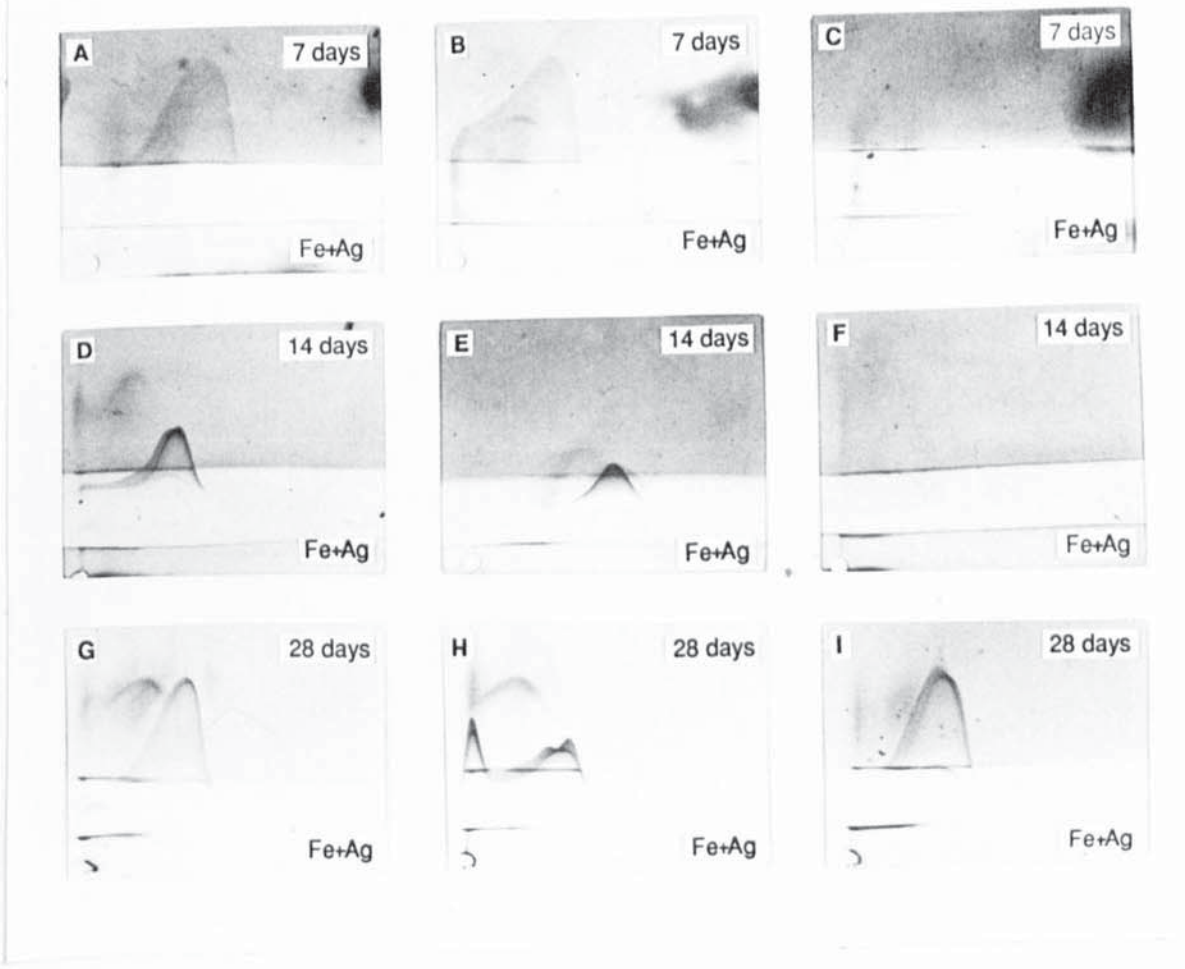


FIGURE 3.26, a - i

ANTIBODY RESPONSE OF RATS INFECTED WITH PA 492a GROWN IN FE- TSB TO PA 492a ANTIGENS, CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL OF 6 µl Fe+ Ag AGAINST WHOLE SERA FROM RATS SACRIFICED 7 (a-c), 14 (d-f), AND 28 (g-i) DAYS POST-INFECTION. SERUM WAS INCORPORATED IN THE SECOND DIMENSION AT 16.7 µl/cm^2.

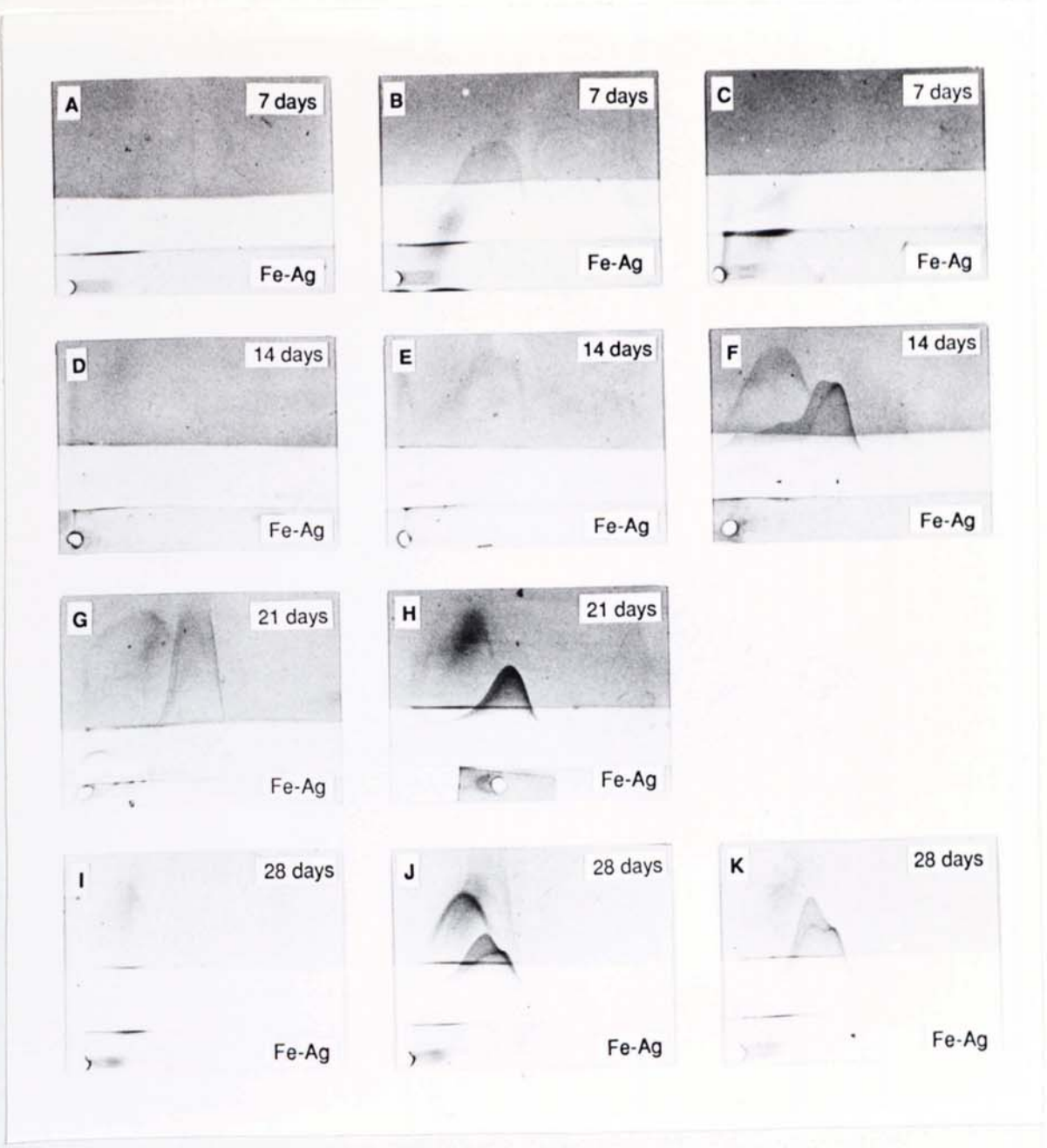


FIGURE 3.27, a - k

ANTIBODY RESPONSE OF RATS INFECTED WITH PA 492a GROWN IN Fe+ TSB TO PA 492a ANTIGENS, CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL OF 6µl Fe- Ag AGAINST WHOLE SERA FROM RATS SACRIFICED 7 (a-c), 14 (d-f), 21 (g and h) and 28 (i-k) DAYS POST-INFECTION. SERUM WAS INCORPORATED IN THE SECOND DIMENSION AT 16.7 µl/cm^2.

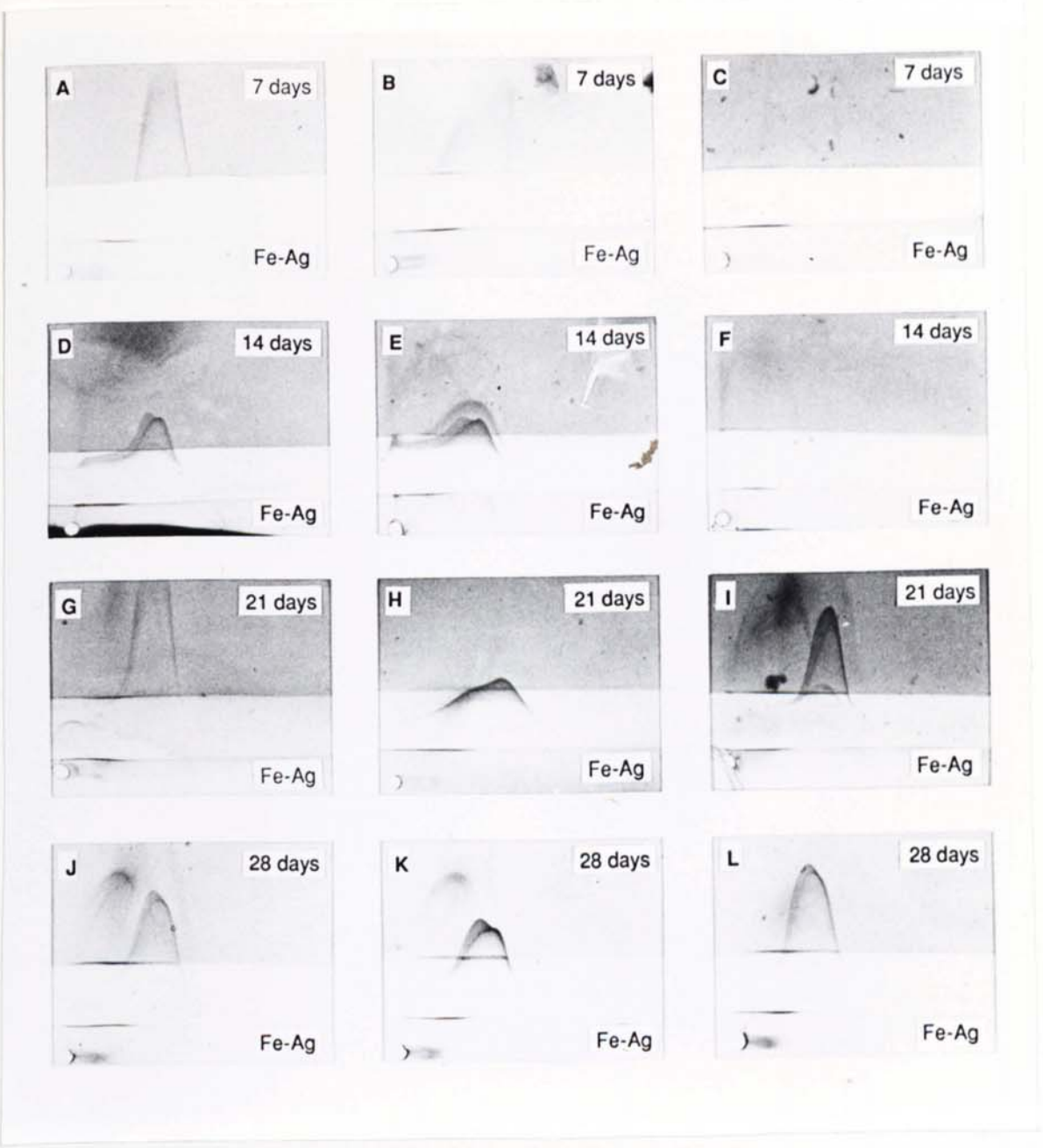


FIGURE 3.28, a - l

ANTIBODY RESPONSE OF RATS INFECTED WITH PA 492a GROWN IN Fe- TSB TO PA 492a ANTIGENS, CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL OF 6µl Fe- Ag AGAINST WHOLE SERA FROM RATS SACRIFICED 7 (a-c), 14 (d-f), 21 (g-i) and 28 (j-l) DAYS POST-INFECTION. SERUM WAS INCORPORATED IN THE SECOND DIMENSION AT 16.7 µl/cm^2.

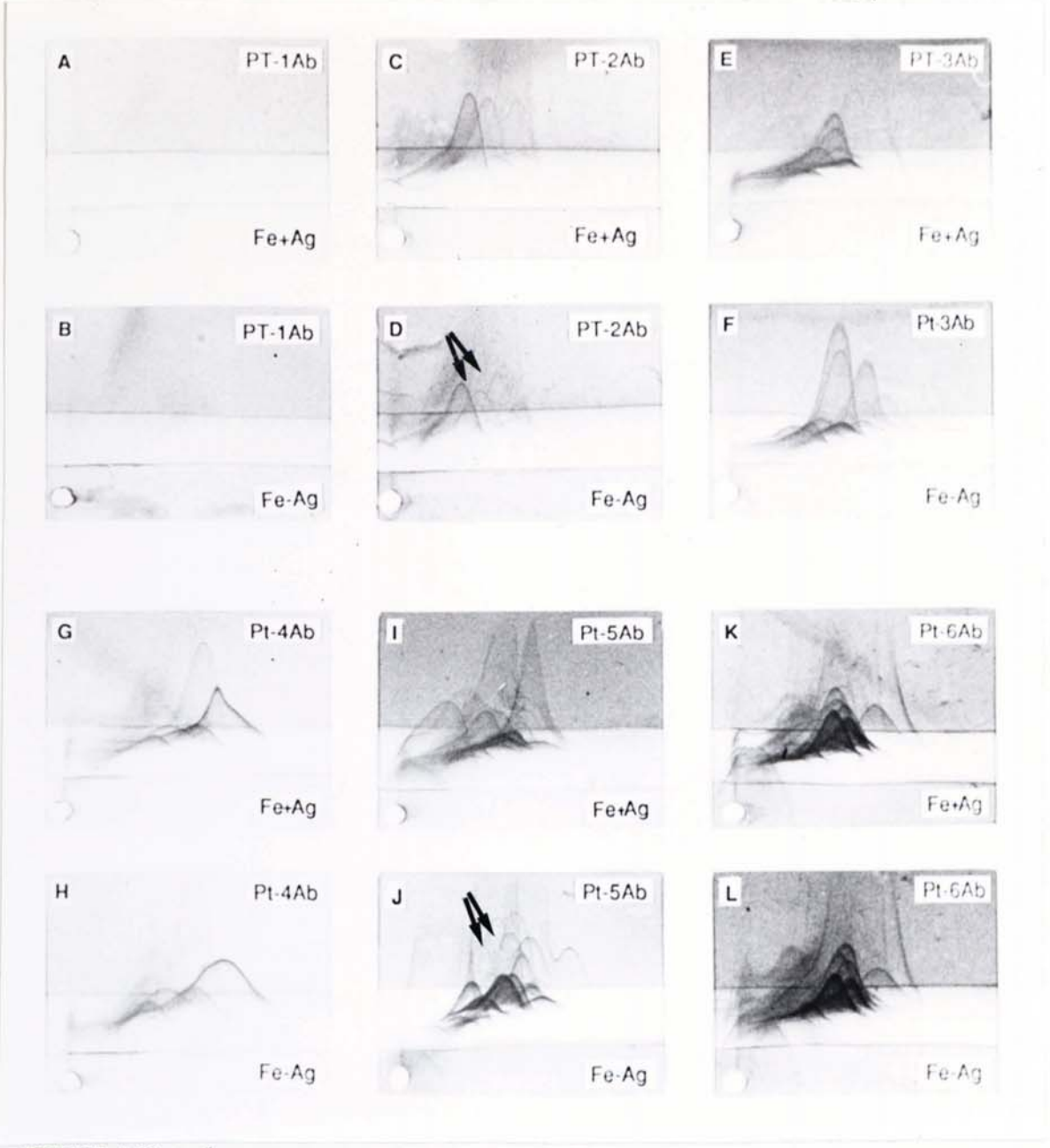


FIGURE 3.29, a - l

ANTIBODY RESPONSE IN SERA FROM SIX CF PATIENTS WHOSE LUNGS WERE COLONISED WITH P. AERUGINOSA. CROSSED IMMUNOELECTROPHORESIS WITH 6 µl Fe+ PA 492a Ag (a, c, e, g, i and k) AND Fe- PA 492a (b, d, f, h, j and l) AND PATIENTS SERA INCORPORATED IN THE SECOND DIMENSION AT 16.7 µl/cm^2. SERUM FROM PATIENT 1 WAS OBTAINED 6 MONTHS AFTER ONSET OF CHRONIC P. AERUGINOSA LUNG INFECTION. P. AERUGINOSA WAS CONSISTENTLY ISOLATED FROM THE LUNGS OF PATIENTS 2, 3, 4, 5 AND 6, OVER A 5 YEAR PERIOD. SERA FROM PATIENTS 2, 3 AND 4 FORMED RELATIVELY FEW PEAKS WITH PA 492a Ag. THE ARROW MARKS THE MIGRATION OF THREE FAINT PRECIPITINS WHICH WERE PRESENT IN CIEs WITH Fe-Ag BUT NOT Fe+ Ag WITH SEVERAL PATIENTS SERA. THESE MAY REPRESENT PRECIPITINS CONTAINING THE IRMPs.

Figure 3.30 shows the Coomassie blue stained protein profiles of OMs prepared from PA 492a grown in Fe+ TSB (lane 1) and Fe- TSB (lane 2) and of solubilised whole cells of PA 492a grown in Fe+ TSB (lane 3) and Fe- TSB (lane 4). Antigens were separated using 10% (w/v) acrylamide gels. Three IRMPs were induced in the OM of Fe- TSB grown PA 492a. These IRMPs were induced to a much less extent in PA 492a as compared to PAO 579 (Fig 3.10) under identical growth conditions. The whole cell protein profile of PA 492a grown in Fe+ TSB and Fe- TSB appeared similar.

The OM antigens of PA 492a grown in Fe-TSB was electrophoretically transferred to NC paper and reacted with sera from individual rats in Group A and Group B collected 4, 7 and 14 days post-infection. Figure 3.31 showed that the serum IgG reaction with PA 492a antigens was similar for both groups of animals. Sera from all rats sacrificed at each time interval after infection were pooled prior to use in further immunological assays.

Figure 3.32a shows the reaction of IgG in sera obtained from rats sacrificed, 1 (lane 2), 4 (lane 3), 7 (lane 4), 14 (lane 5), 21 (lane 6) and 28 (lane 7) days post-infection with OM antigens of PA 492a grown in Fe-TSB. All sera were absorbed with LPS extracted from PA 492a prior to use. The protein antigens were identified by reference to the profile revealed by amido black (lane 1) and Coomassie blue staining. The concentrations of the polymerising agents used in these gels were reduced to allow resolution of OM proteins H1 and H2. A weak IgG response was elicited to the IRMPs and OM proteins D, E, G and H1 by day 4 post-infection. Sera collected from rats 14 days post-infection reacted with flagellin protein, protein F, and the lipoproteins H2 and I in addition to the other major OM proteins. The IgG response to all these proteins but especially to proteins F, H2 and I and flagellar protein appeared stronger on the immunoblots reacted with sera obtained 21 and 28 days post-infection.

Figure 3.32b shows the reaction of IgM in the same LPS-absorbed sera with

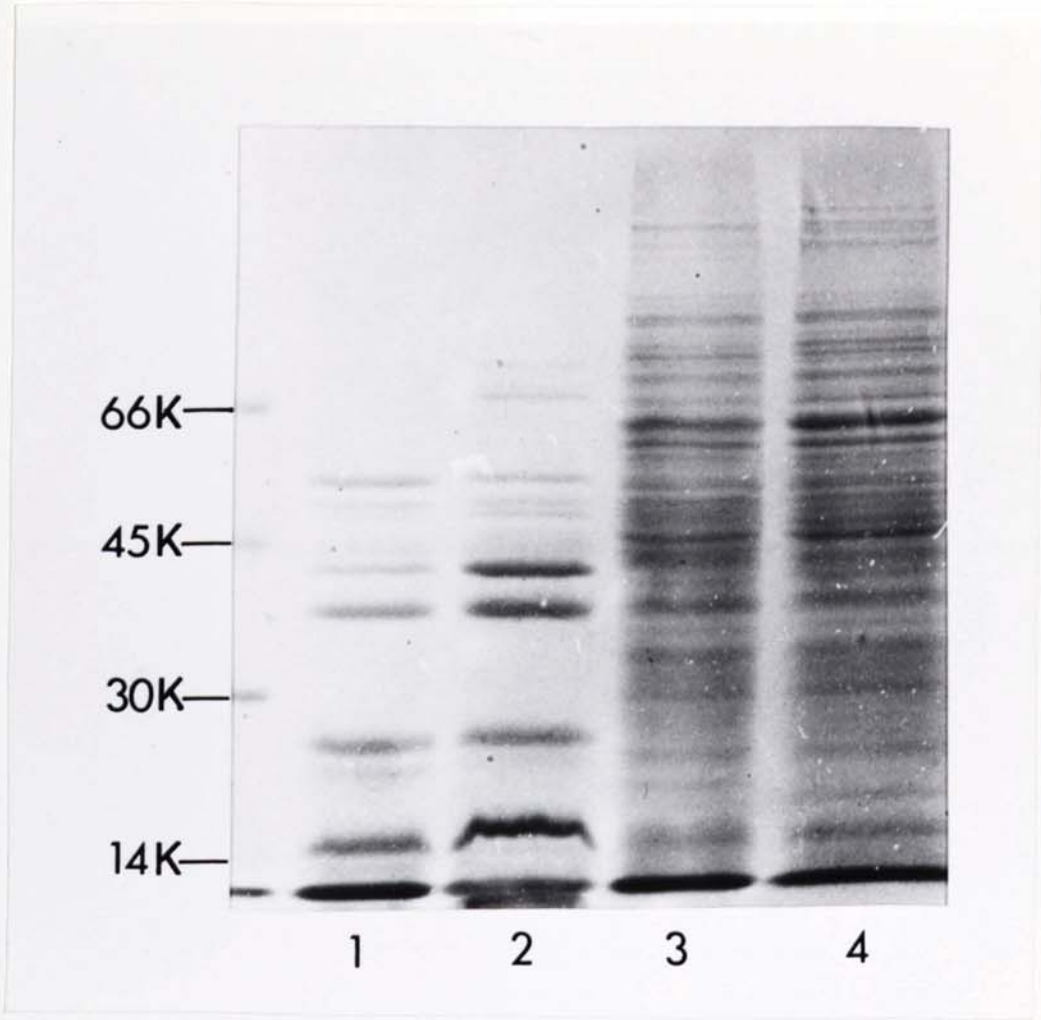


FIGURE 3.30

THE SDS-PAGE PROTEIN PROFILES OF PA 492a SOLUBILISED WHOLE CELLS GROWN IN Fe- TSB (LANE 4) AND Fe+ TSB (LANE 3) AND OF SARKOSYL EXTRACTED OMs PREPARED FROM THE SAME ISOLATE GROWN IN Fe- TSB (LANE 2) AND Fe+ TSB (LANE 1). PROTEINS WERE SEPARATED USING 10% w/v ACRYLAMIDE GELS AND STAINED WITH COOMASSIE BLUE STAIN. MIGRATION OF STANDARD MOLECULAR WEIGHT PROTEINS IS INDICATED ON THE LEFT HAND SIDE.

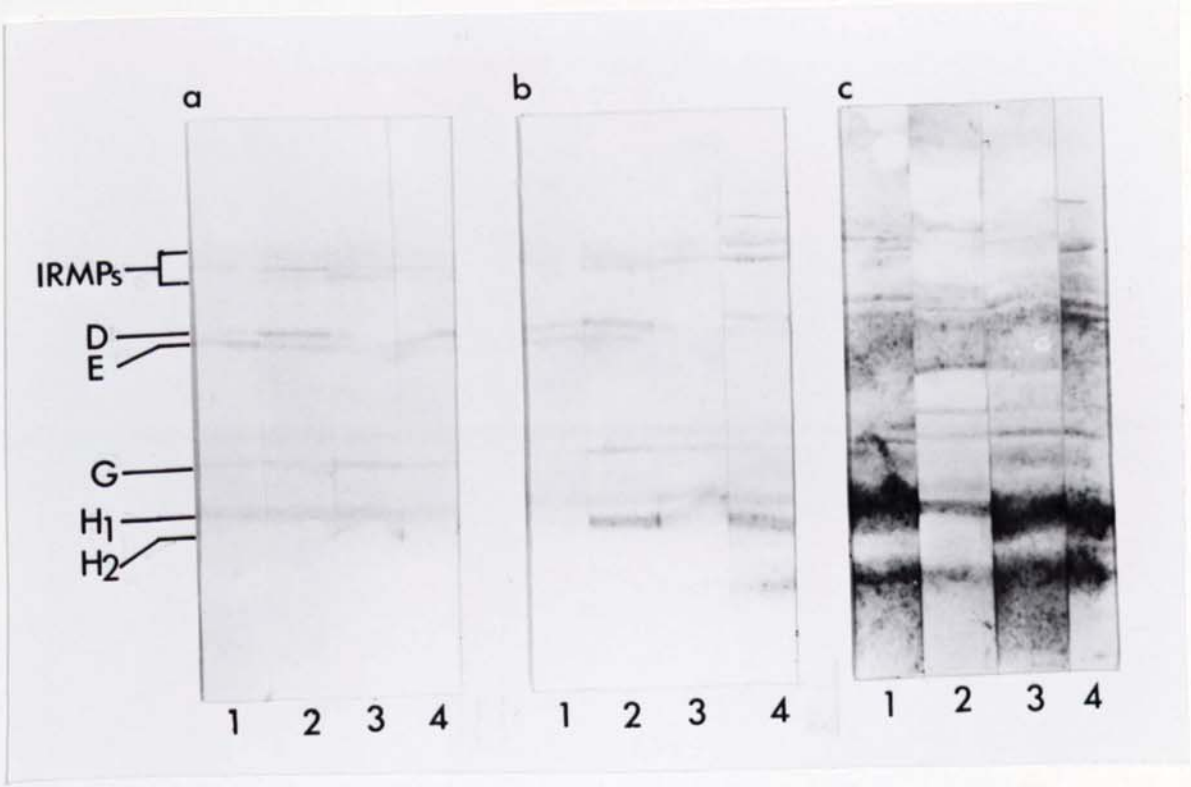


FIGURE 3.31, a-c

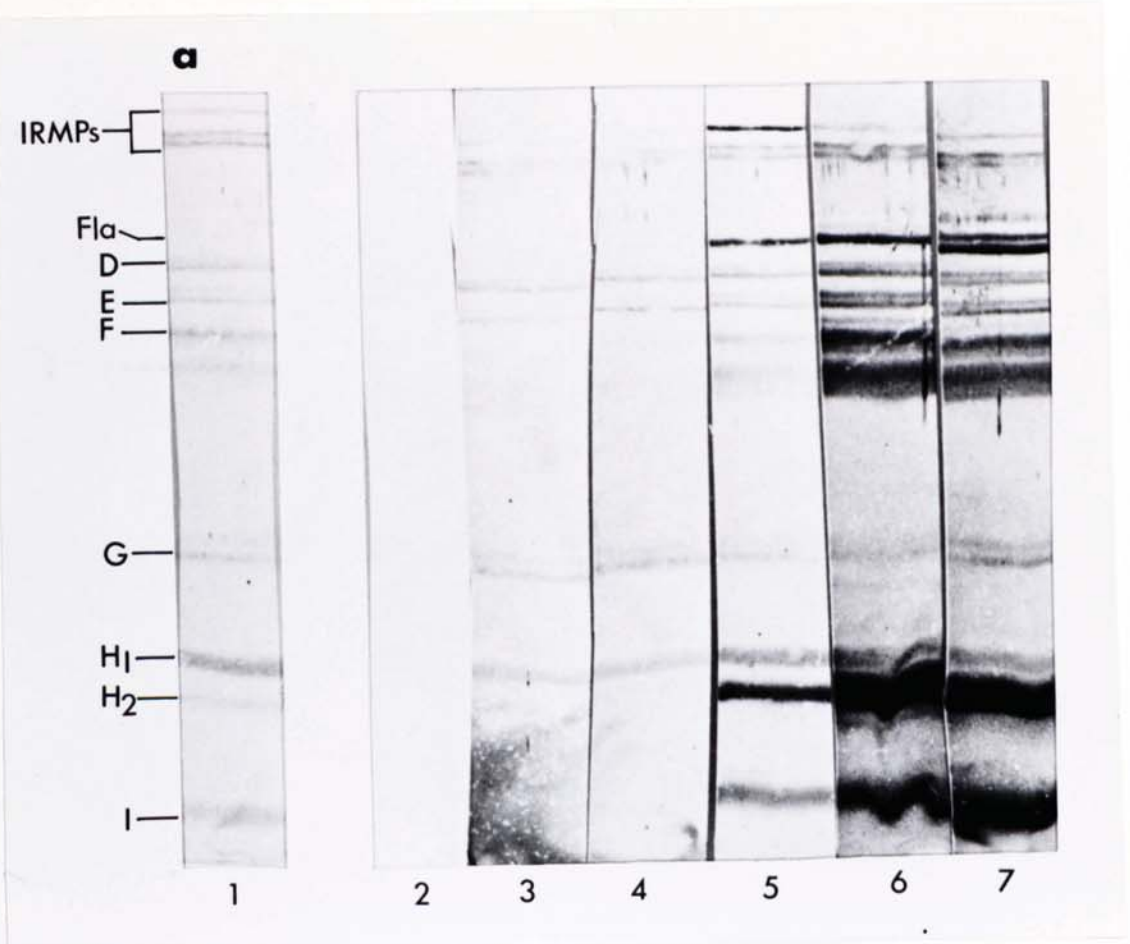
IMMUNOBLOTS OF THE OM ANTIGENS OF P. AERUGINOSA PA 492a GROWN IN Fe- TSB ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH SERA FROM INDIVIDUAL RATS INFECTED WITH PA 492a GROWN IN Fe+ TSB OR Fe- TSB. REACTION WITH SERA OBTAINED 4 (A), 7 (B) AND 14 (C) DAYS POST-INFECTION ARE SHOWN. SERA WERE OBTAINED FROM RATS INFECTED WITH PA 492a GROWN IN Fe+ TSB (LANES 1 AND 2) AND Fe- TSB (LANES 3 AND 4). THE REACTION OBTAINED WITH IgG IS SHOWN.

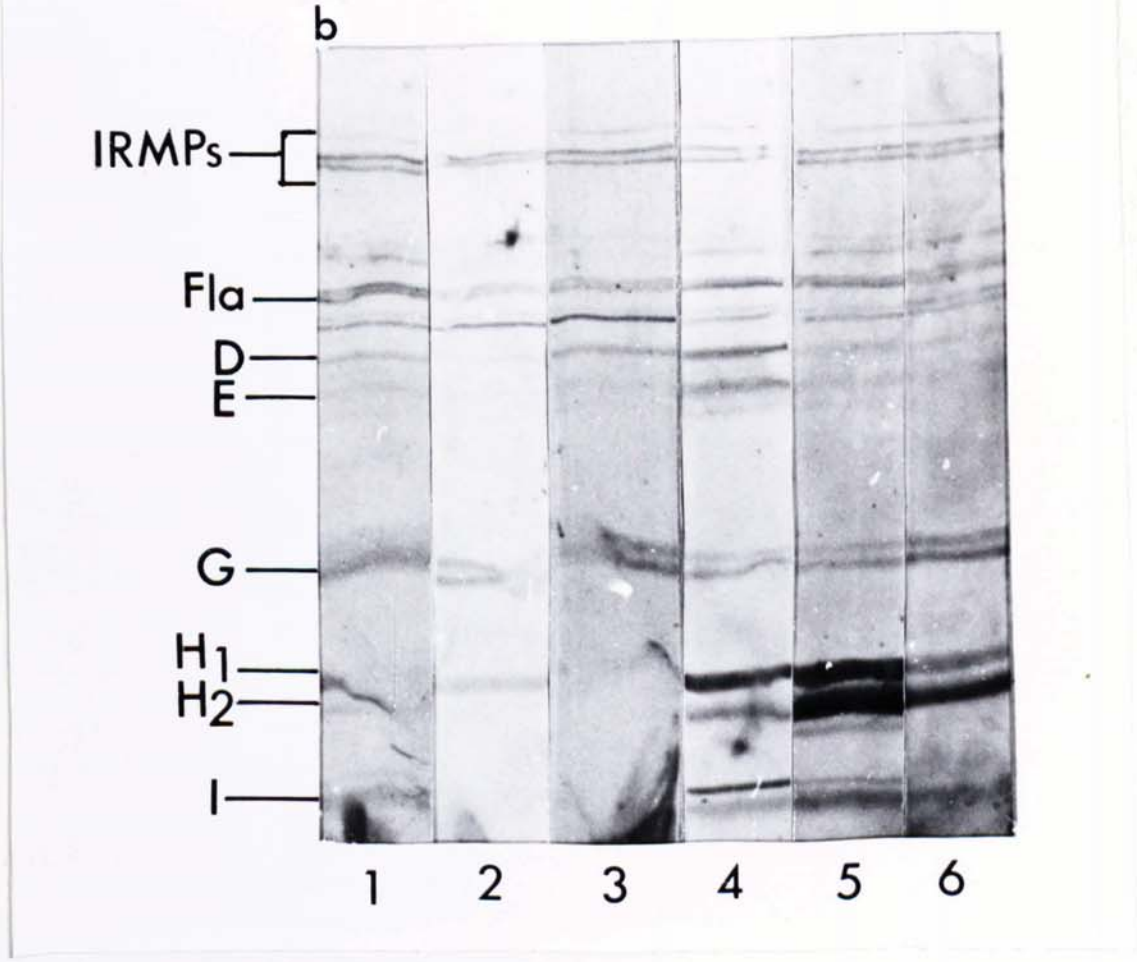
FIGURE 3.32a

STRIP IMMUNOBLOT OBTAINED WITH OM ANTIGENS OF PA 492a GROWN IN FE- TSB ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERA FROM RATS SACRIFICED 1 (LANE 2), 4 (LANE 3), 7 (LANE 4), 14 (LANE 5), 21 (LANE 6) AND 28 (LANE 7) DAYS AFTER INFECTION WITH PA 492a. THE OM PROTEIN PROFILE REVEALED BY STAINING A PORTION OF THE NC PAPER WITH AMIDO BLACK IS SHOWN IN LANE 1. THE REACTION OBTAINED WITH IgG IS SHOWN.

FIGURE 3.32b

REACTION OF IgM ANTIBODIES IN LPS-ABSORBED SERA FROM RATS SACRIFICED 1 (LANE 1), 4 (LANE 2), 7 (LANE 3), 14 (LANE 4), 21 (LANE 5) AND 28 (LANE 6) DAYS AFTER INFECTION WITH PA 492a.


a
IRMPs
Fla
D
E
F
G
H1
H2
I
1
2
3
4
5
6
7


b
IRMPs
Fla
D
E
G
H1
H2
I
1
2
3
4
5
6

PA 492a OM antigens. A weak IgM response was detected in sera obtained 1 day post-infection and in all subsequent sera. As with IgG there was a faint IgM response to proteins H2 and I and to some extent with protein F in sera obtained 14 days post-infection.

Figure 3.33 shows the reaction of IgG in serially diluted sera obtained 7 (Fig 3.33a) and 21 days (Fig 3.33b) post-infection. Considering day 21 sera, reaction of IgG with proteins F and G was not obtained below a dilution of 1 in 400. By contrast, reaction with the lipoproteins H2 and I was not obtained below a dilution of 1 in 25,600 and 1 in 1600 respectively. Comparison of figures 3.33a and 3.33b indicate that the qualitative differences in antigens recognised by sequential sera samples were not due to differences in antibody titres. The IRMPs, flagellar protein and protein H1 were the major OM protein antigens seen in the early stages of infection.

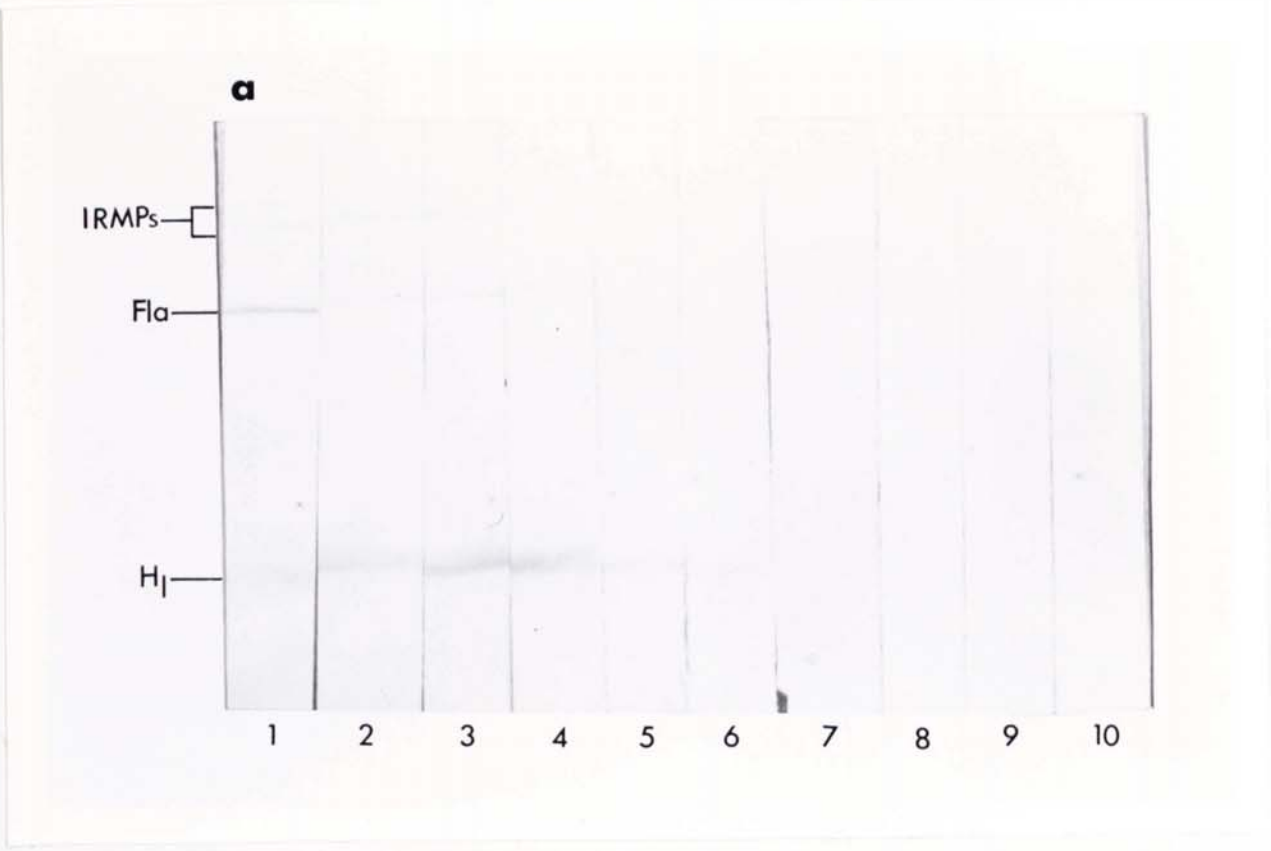
Figure 3.34a shows the reaction of IgG in sequential LPS-absorbed serum samples with solubilised whole cell antigens of PA 492a grown in Fe-TSB. Antibodies in day 1 (lane 2), 4 (lane 3), and 7 (lane 4) sera reacted with antigens of molecular weight greater than 41K which may include the IRMPs and OM proteins D and E. Day 14 sera (lane 5) reacted intensely with more antigens including antigens of molecular weight less than 41K. Subsequent sera reacted more strongly with the same antigens that were recognised by antibodies in sera from rats sacrificed 14 days post-infection. Sera from control (non-infected) rats reacted faintly with a number of whole cell antigens (lane 1). The full complement of IgM antibodies to PA 492a appeared to be present in sera obtained 7 days after infection (Fig 3.34b). Fewer antigens were recognised by IgM than by IgG in sera collected in the latter half of the infection.

To investigate similarities in the antigens of PA 492a grown in iron-plentiful and iron-depleted conditions the antigens described in Figure 3.30 were blotted with sera from rats infected with PA 492a. Figures 3.35a to 3.35d show the reaction of

FIGURE 3.33, a and b

STRIP IMMUNOBLOT OF THE OM ANTIGENS OF PA 492a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH SERIALLY DILUTED SERA COLLECTED FROM RATS SACRIFICED 7 (a) or 21 (b) DAYS AFTER INFECTION WITH PA 492a. THE REACTION OBTAINED WITH IgG IS SHOWN. SERUM DILUTIONS WERE:

LANE	1	1 in 50
	2	1 in 100
	3	1 in 200
	4	1 in 400
	5	1 in 800
	6	1 in 1,600
	7	1 in 3,200
	8	1 in 6,400
	9	1 in 12,800
	10	1 in 25,600
	11	1 in 51,200


a
IRMPs
Fla
H1
1
2
3
4
5
6
7
8
9
10

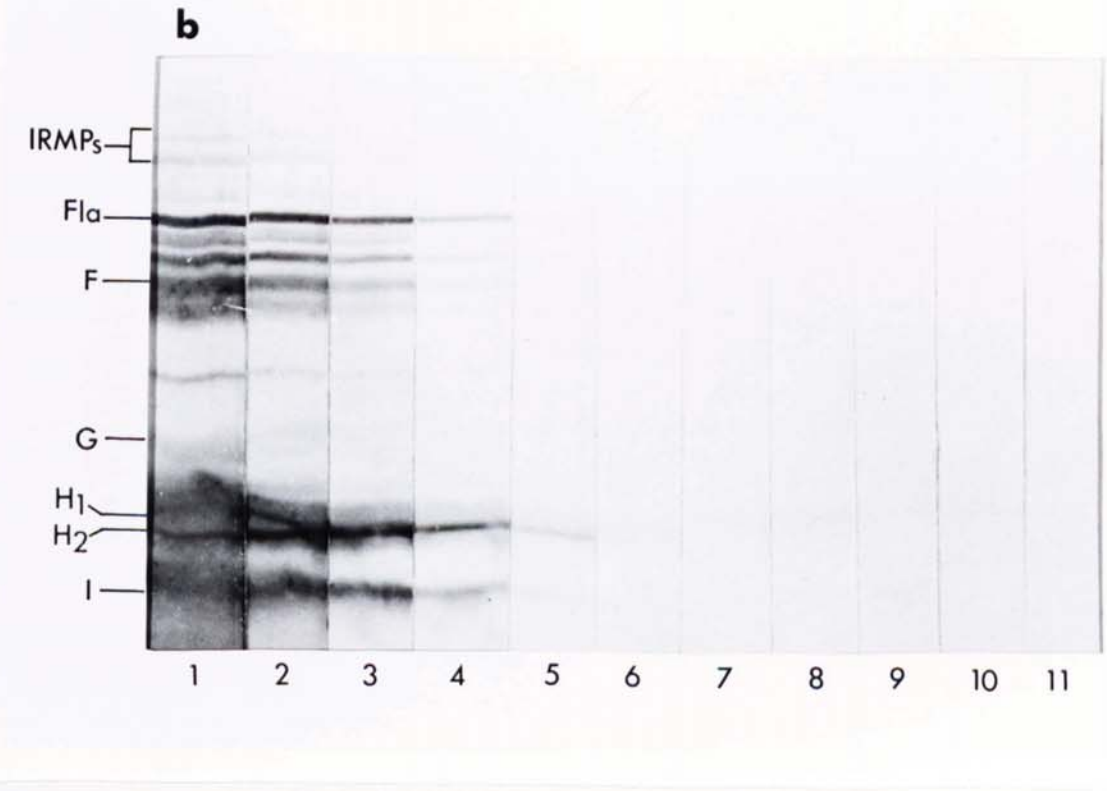
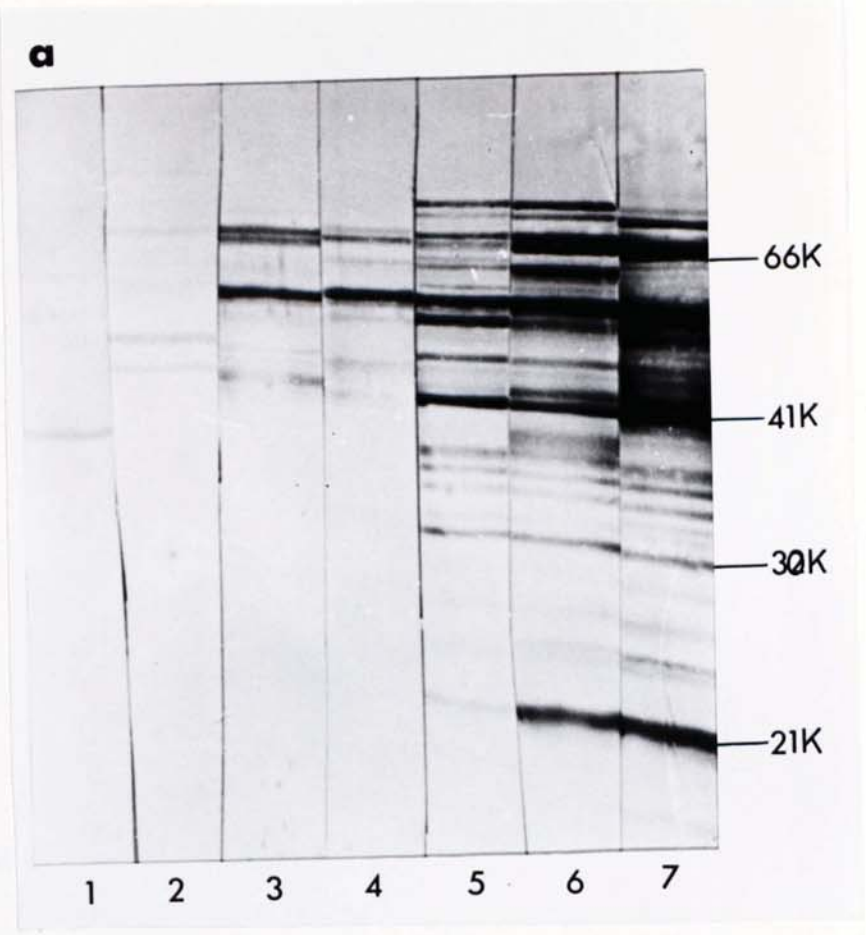

b
IRMPs
Fla
F
G
H1
H2
I
1
2
3
4
5
6
7
8
9
10
11

FIGURE 3.34, a and b

STRIP IMMUNOBLOTS OF WHOLE CELLS LYSATES OF PA 492a GROWN IN Fe- TSB ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERA FROM RATS INFECTED WITH PA 492a AND NON-INFECTED CONTROL RATS. REACTION OF IgG IN SERA COLLECTED 1 (LANE 2), 4 (LANE 3), 7 (LANE 4), 14 (LANE 5) 21 (LANE 6) AND 28 (LANE 7) DAYS AFTER INFECTION AND SERA FROM NON-INFECTED CONTROL RATS (LANE 1) ARE SHOWN IN (a).REACTION OF IgM IN SERA COLLECTED 1 (LANE 1), 4 (LANE 2), 7 (LANE 3), 14 (LANE 4), 21 (LANE 5) AND 28 (LANE 6) DAYS AFTER INFECTION ARE SHOWN IN (b).


a
66K
41K
30K
21K
1
2
3
4
5
6
7

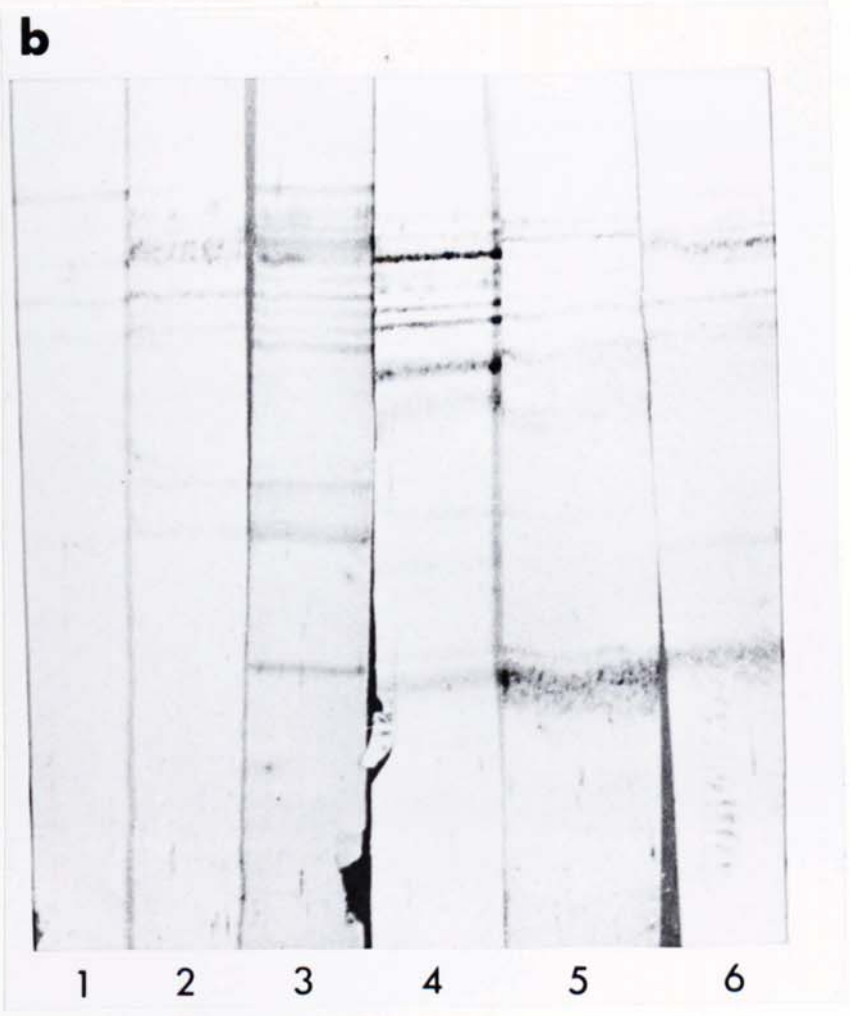
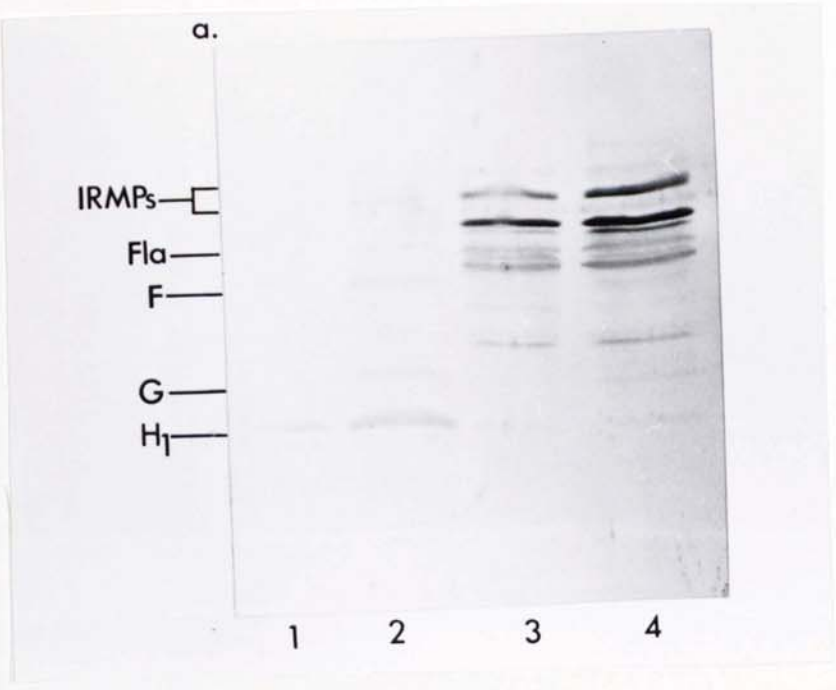
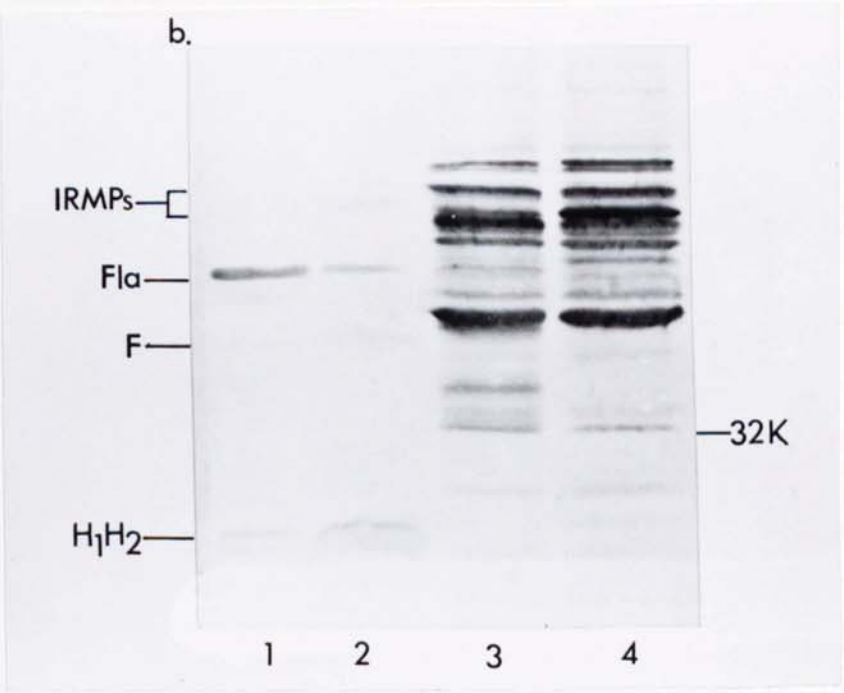
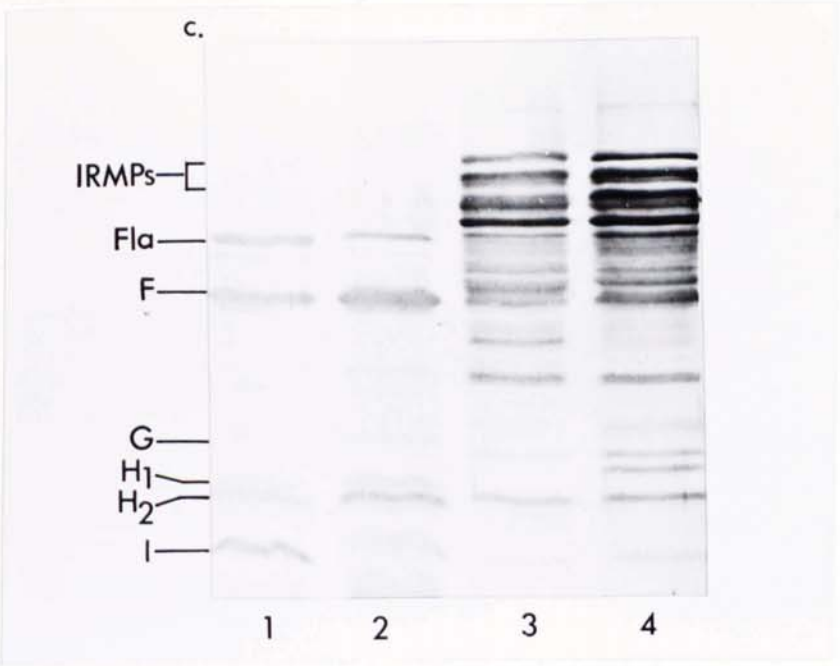

b
1
2
3
4
5
6

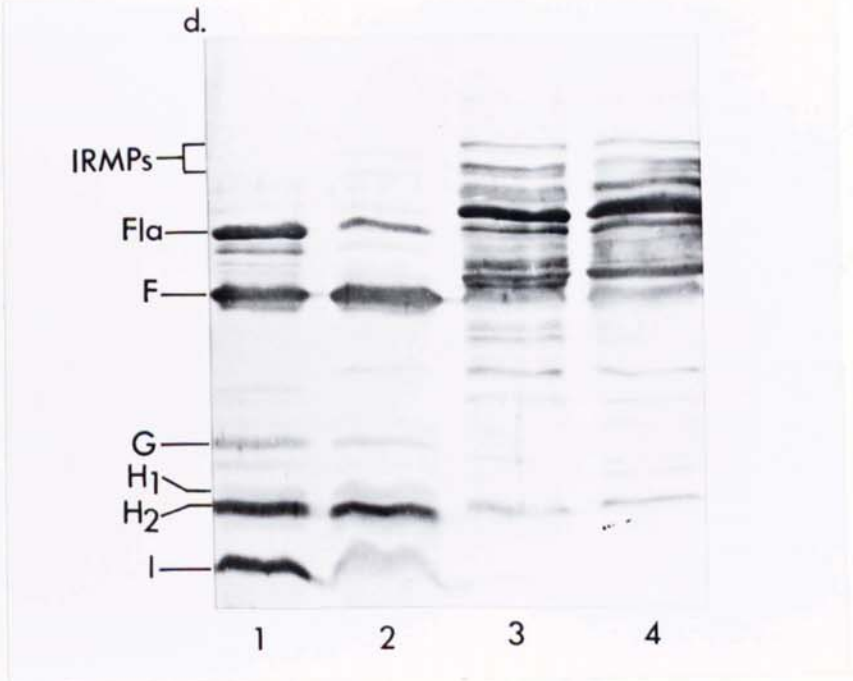
FIGURE 3.35, a - d

IMMUNOBLOTS OF OM ANTIGENS AND WHOLE CELL LYSATES OF PA 492a SHOWN IN FIG 3.30 ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERA COLLECTED FROM RATS 7 (a), 14 (b), 21 (c) AND 28 (d) DAY POST INFECTION. THE REACTION OBTAINED WITH IgG IS SHOWN.


a.
IRMPs
Fla
F
G
H1
1
2
3
4


b.
IRMPs
Fla
F
H1H2
32K
1
2
3
4


c.
IRMPs
Fla
F
G
H1
H2
I
1
2
3
4


d.
IRMPs
Fla
F
G
H1
H2
I
1
2
3
4

LPS-absorbed sera obtained 7, 14, 21 and 28 days post-infection. The results indicate that a number of whole cell antigens recognised on these immunoblots correspond to major OM proteins. Many antigens which were not major OM proteins were recognised by antibodies developed early in the immune response. In particular whole cell antigens with estimated molecular weights in the range 66-100K were strongly recognised. A 32K protein in solubilised whole cells of PA 492a was recognised by IgG in sera from rats. This 32K protein was present in the OMs prepared by sucrose density unltracentrifugation from PA 492a grown in Fe+ TSB and Fe- TSB (Fig 3.36).

The antigenic composition of PA 492a grown in Fe+ TSB or Fe- TSB appeared similar. However, an antigen with an apparent molecular weight of 64K was more strongly recognised in iron-depleted as compared to iron-plentiful grown cells.

The silver stained profile of SDS-PAGE resolved whole cell proteinase K digests of PA 492a indicated that this strain expressed only small amounts of 0-antigen. The LPS profile of proteinase K digests of whole cells of PA 492a revealed by reaction with sera from hyperimmune rats is shown in Figure 3.37 (lane 1). Figure 3.37 shows the reaction of IgG (3.37a) and IgM (3.37b) in sera from rats 1, 4, 7, 14, 21 an 28 days post-infection with proteinase K digests of PA 492a grown in Fe-TSB. LPS-specific antibodies of the IgG and IgM classes to the core and 0-antigen structures were detected as early as 4 days post-infection. The response to the antigens appeared to increase throughout the duration of the infection. Notably, IgG in 21 and 28 day sera reacted very strongly with PA 492a 0-antigen despite the small amount of LPS 0-side chains produced by *in vitro*-grown cells of this strain.

3.3.3.2 Lung

Figures 3.38a and 3.38b show the IgG and IgA responses respectively in the lung fluid from two rats sacrificed at each of the following intervals - days 1 (lane 1),

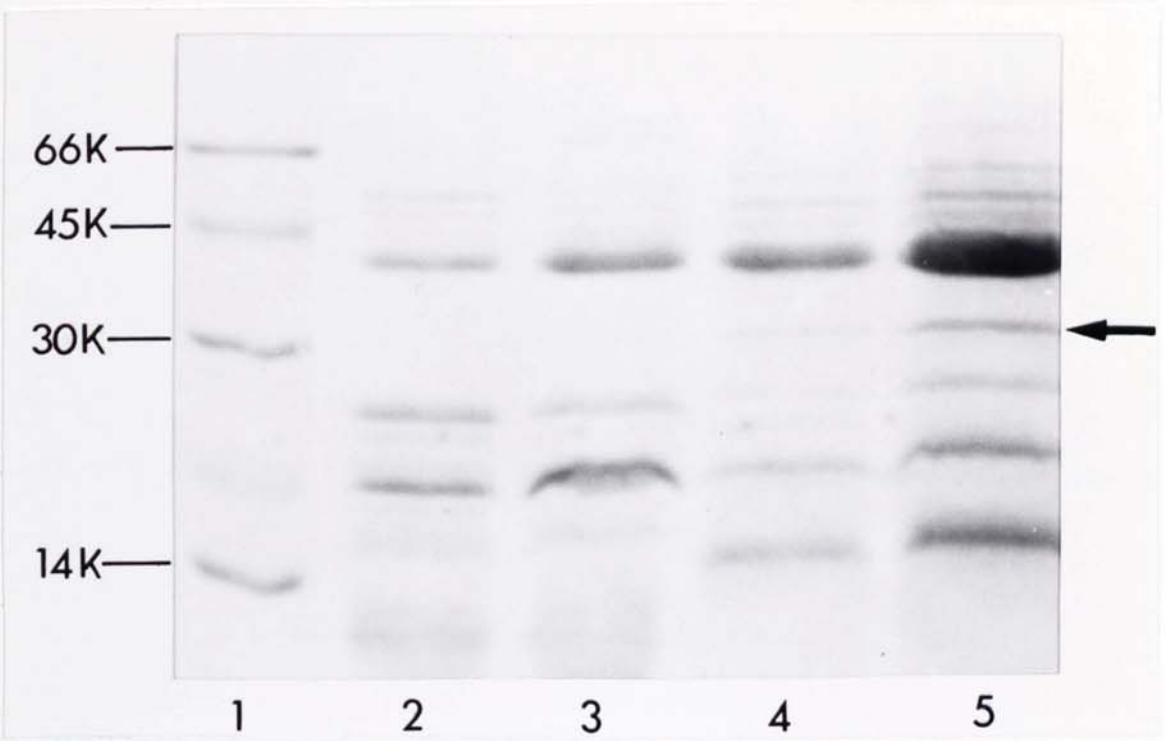
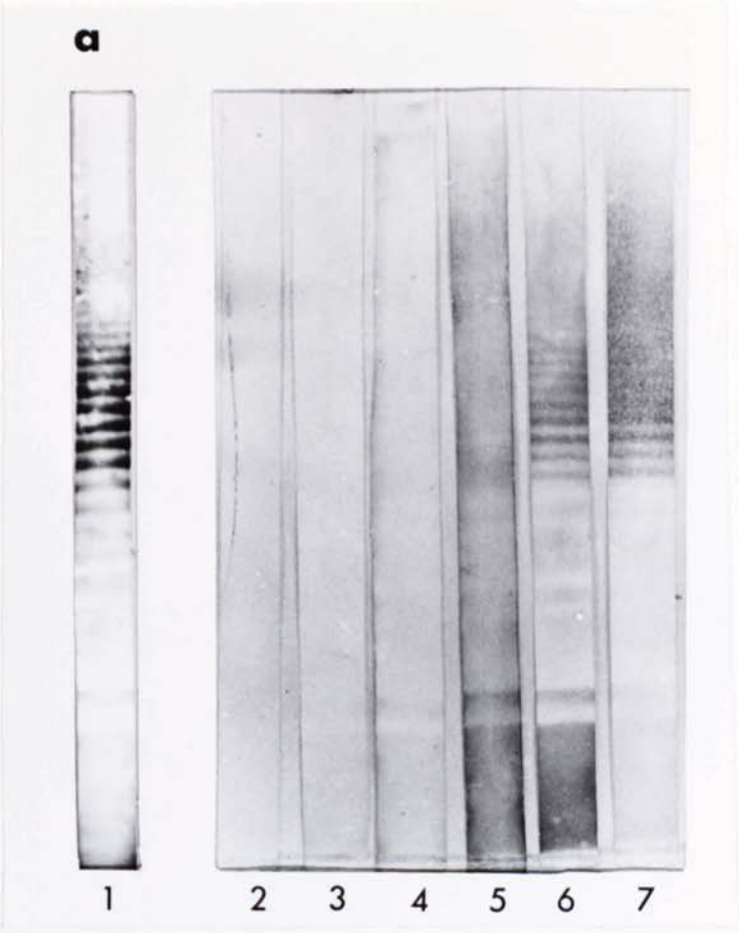


FIGURE 3.36

THE SDS-PAGE PROTEIN PROFILES OF OMs OF PA 492a PREPARED BY SARKSOYL EXTRACTION FROM CELLS GROWN IN Fe+ TSB (LANE 1) AND Fe- TSB (LANE 2) AND ALSO OF OMs PREPARED BY SUCROSE DENSITY ULTRACENTRIFUGATION FROM CELLS GROWN IN Fe+ TSB (LANE 3) AND Fe- TSB (LANE 4). PROTEINS WERE SEPARATED USING 14% w/v ACRYLAMIDE GELS AND VISUALISED WITH COOMASSIE BLUE STAIN.

FIGURE 3.37, a and b

IMMUNOBLOTS OBTAINED WITH PROTEINASE K DIGESTS OF WHOLE CELLS OF PA 492a GROWN IN FE- TSB ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH IgG IN WHOLE SERA FROM RATS INFECTED WITH PA 492a. SERA WERE COLLECTED 1 (LANE 2), 4 (LANE 3), 7 (LANE 4), 14 (LANE 5), 21 (LANE 6), AND 28 (LANE 7) DAYS POST-INFECTION. THE REACTION WITH IgG IN SERA FROM RATS IMMUNISED WITH PA 492a IS SHOWN IN LANE 1. REACTION OBTAINED WITH IgM IN SERA FROM RATS INFECTED WITH PA 492a COLLECTED 1 (LANE 1), 4 (LANE 2), 7 (LANE 3), 14 (LANE 4), 21 (LANE 5) AND 28 (LANE 6) DAYS POST-INFECTION.


a
1
2
3
4
5
6
7

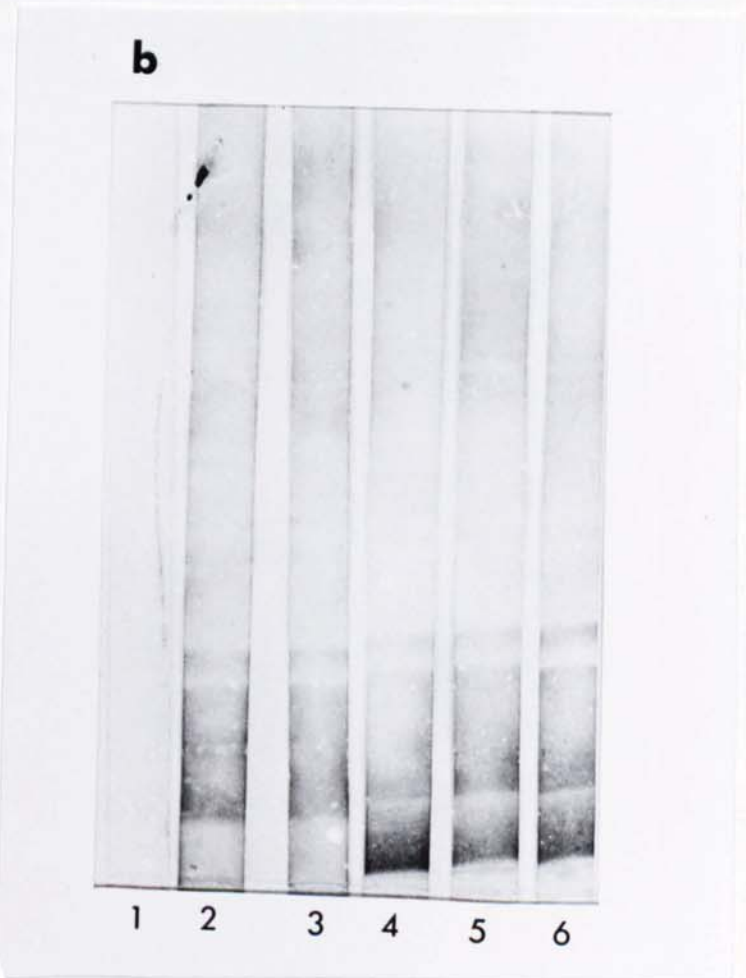
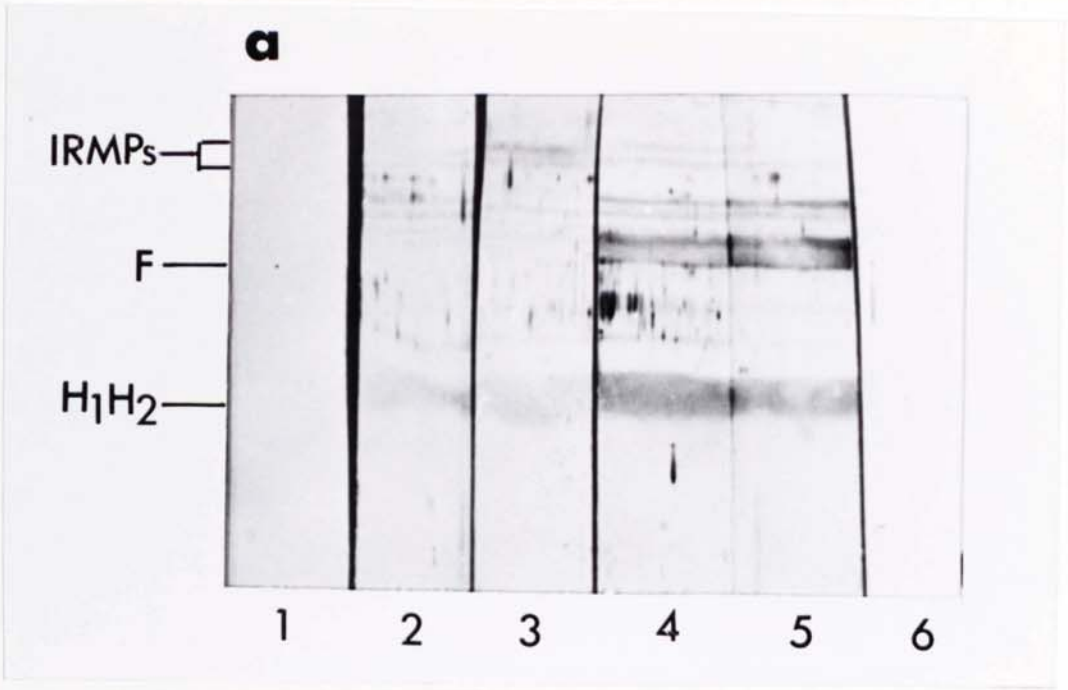
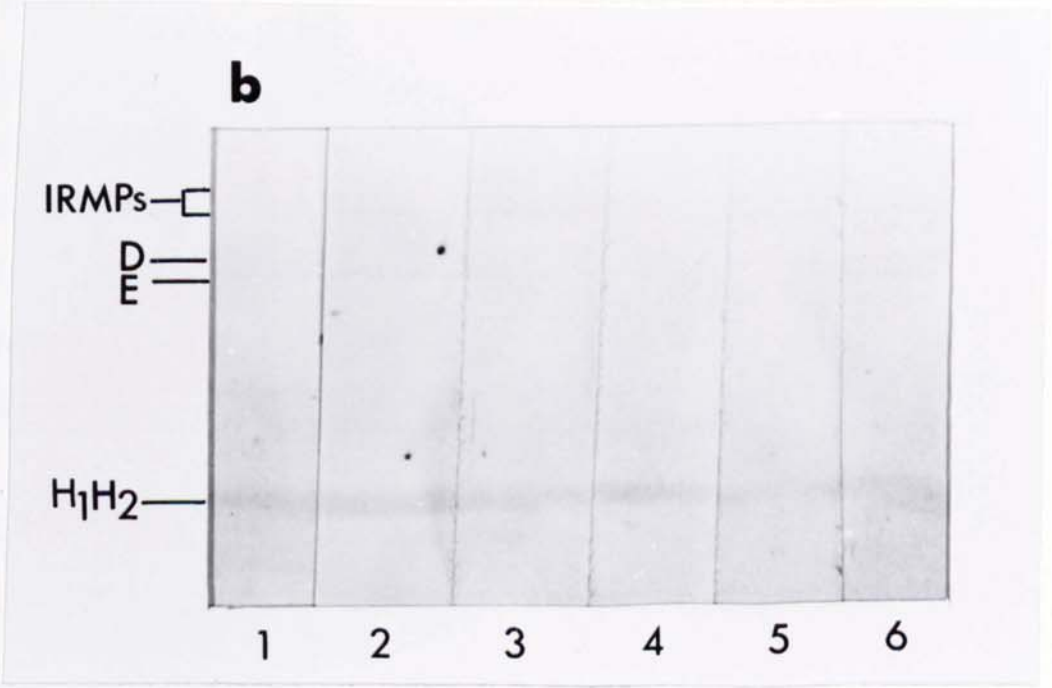

b
1
2
3
4
5
6

FIGURE 3.38, a and b

STRIP IMMUNOBLOTS OF THE OM ANTIGENS OF PA 492a GROWN IN FE- TSB ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LUNG FLUID FROM RATS INFECTED WITH PA 492a. PORTIONS OF NC WERE INCUBATED WITH LUNG FLUID FROM RATS SACRIFICED 1 (LANE 1), 4 (LANE 2), 7 (LANE 3), 14 (LANE 4), 21 (LANE 5) AND 28 (LANE 6) DAYS POST-INFECTION. THE REACTIONS OBTAINED WITH IgG AND IgM IN LUNG FLUID ARE SHOWN IN (a) and (b) RESPECTIVELY. THE LUNG FLUID USED TO INVESTIGATE THE IgG RESPONSE AT DAY 28 WAS OBTAINED FROM RATS FROM WHICH NO P. AERUGINOSA C.F.U. WERE ENUMERATED, THESE RATS PROBABLY WERE NOT INFECTED WITH P. AERUGINOSA.


a
IRMPs
F
H1H2
1
2
3
4
5
6


b
IRMPs
D
E
H1H2
1
2
3
4
5
6

4 (lane 2), 7 (lane 3), 14 (lane 4), 21 (lane 5) and 28 (lane 6) post-infection. The IgG response detected in the lung was similar to that obtained with serum except the response to protein F and H1/H2 appeared stronger in lung fluid relative to the other OM antigens. Also, an antibody response to the LPS 0-antigen was not detected in the lung fluid. Lung IgA reacted faintly with most of the major OM proteins including the IRMPs, but not protein F.

Immunoblotting was also carried out with lung lavage fluid obtained from one rat at each time of sacrifice. IgG in lung lavage reacted with the same OM proteins as seen with lung homogenate but the reaction was not as strong and could not be photographed.

Reaction of IgM with OM antigens was not detected on immunoblots probed with lung fluid.

3.3.4 Longitudinal antibody response in experimental rat lung infection examined using a whole cell ELISA

Figures 3.39 and 3.40 show the titration curves of IgG (3.39) and IgM (3.40a and b) in sequential sera from infected rats and also from non-infected control rats to whole cell surface antigens of PA 492a grown in Fe-TSB. Sera from control rats contained low titres of IgG and IgM to P. aeruginosa surface antigens. Figure 3.41 shows the end-point titres (reciprocal of the serum dilution giving an absorbance of 0.1 greater than blank wells at 450 nm) of IgG and IgM in infected rat sera at each time of sacrifice. The IgG titres increased throughout the infection whereas the IgM titres decreased after around day 14.

3.3.5 Effect of iron-depletion on the expression of P. aeruginosa OM protein H1 antigen

The results presented eariler in this section and in section 3.2 indicated that protein H1 was expressed in the OMs of mucoid P. aeruginosa PAO 579 and PA 492a grown *in vitro* in iron-depleted media. Protein H1 was also induced in the OM

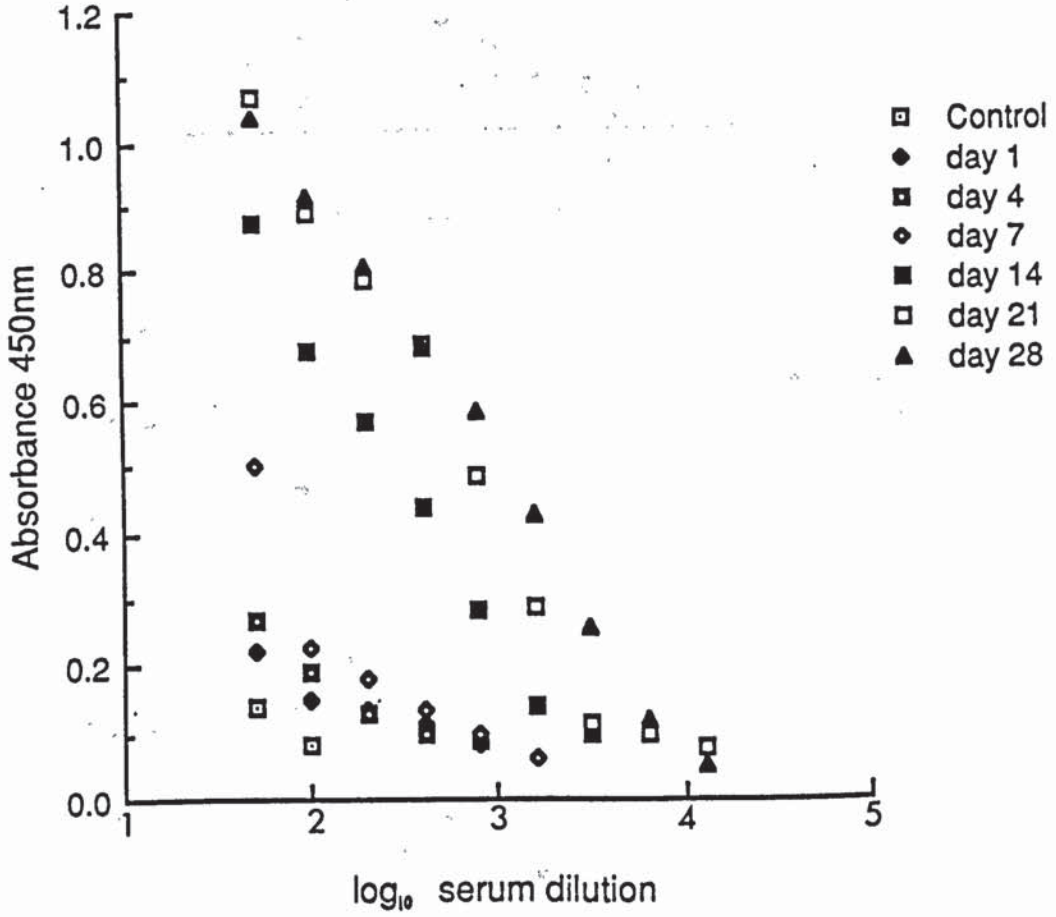


FIGURE 3.39

TITRATION OF IgG ANTIBODIES IN WHOLE SERA FROM RATS INFECTED WITH PA 492a AND FROM NON-INFECTED CONTROL RATS BY ELISA USING WHOLE CELLS OF PA 492a GROWN IN Fe-TSB AS ANTIGENS. SERA WERE COLLECTED 1, 4, 7, 14, 21 AND 28 DAYS POST-INFECTION. SERUM SAMPLES WERE INITIALLY DILUTED 50-FOLD FOLLOWED BY DOUBLING DILUTIONS.

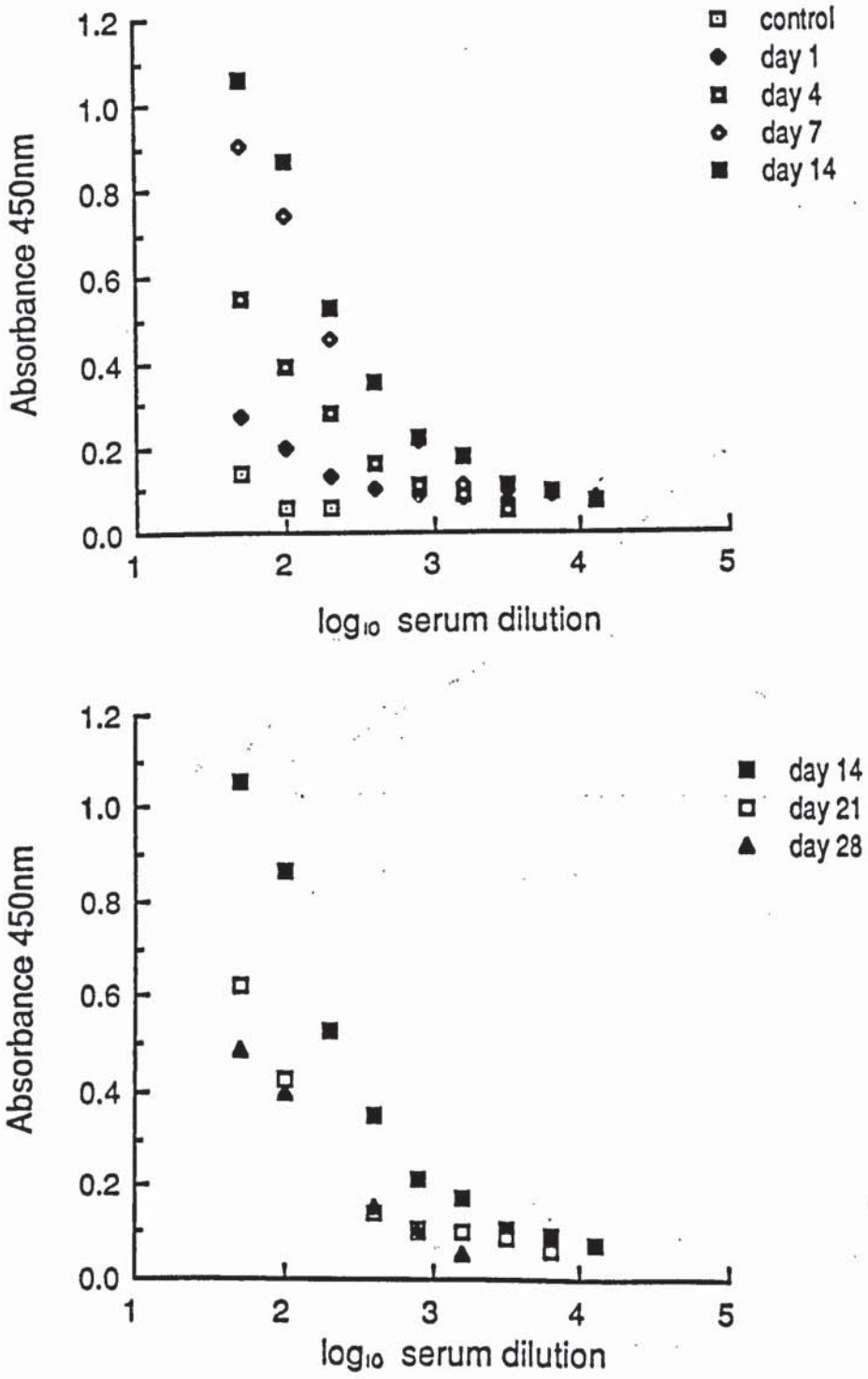


FIGURE 3.40, a and b

TITRATION OF IgM ANTIBODIES IN WHOLE SERA FROM RATS INFECTED WITH PA 492a AND NON-INFECTED CONTROL RATS BY ELISA USING WHOLE CELLS OF PA 492a GROWN IN Fe- TSB AS ANTIGENS. TITRES OF IgM IN CONTROL SERA AND IN SERA COLLECTED 1, 4, 7, AND 14 DAYS POST-INFECTION ARE SHOWN IN (a) AND TITRES OF IgM IN SERA COLLECTED 14, 21 AND 28 DAYS POST-INFECTION IN (b).

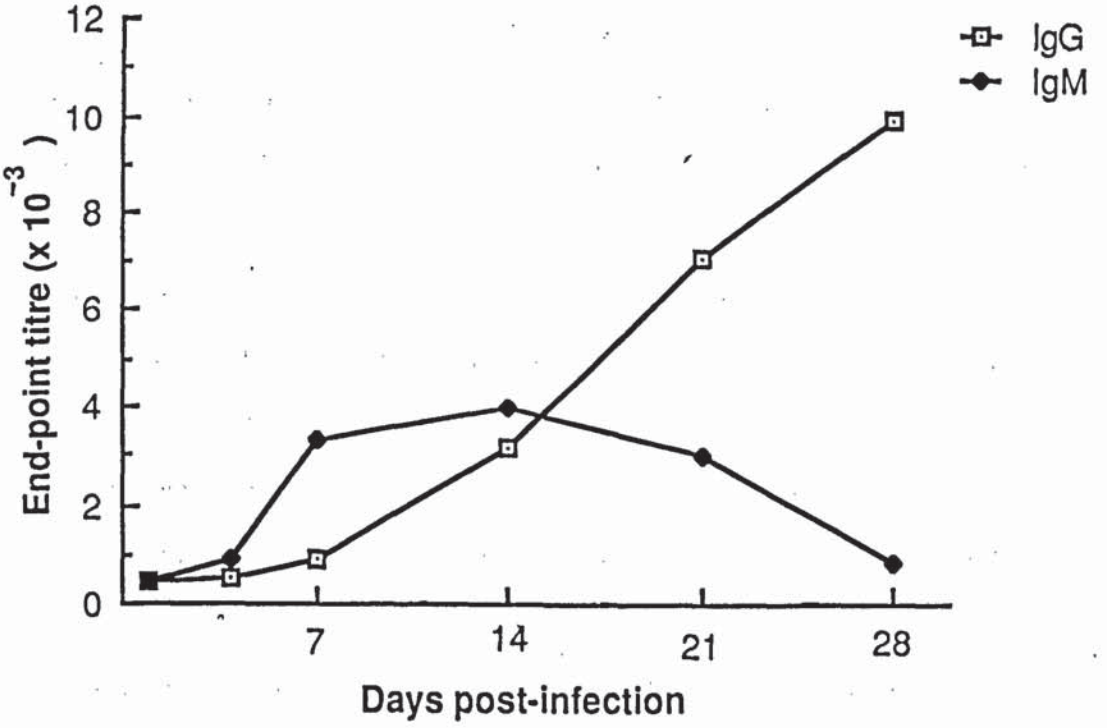


FIGURE 3.41

END-POINT ELISA TITRES OF IgG AND IgM IN SERA COLLECTED FROM RATS 1, 4, 7, 14, 21 AND 28 DAYS AFTER INFECTION WITH PA 492a TO CELL SURFACE ANTIGENS OF PA 492a. END-POINT TITRES WERE CALCULATED AS THE SERUM TITRATION WHICH GAVE AN ABSORBANCE AT 450 nm OF 0.1 UNITS GREATER THAN CONTROL WELLS.

of PAO 579 recovered directly and without subculture from the lungs of infected rats. Previous studies have indicated that protein H1 was induced following growth of P. aeruginosa in magnesium-depleted media (Anwar *et al*, 1983; Nicas and Hancock, 1980). Therefore, the effect of iron-, magnesium- and iron- and magnesium-depletion on the expression of protein H1 in the OM of non-mucoid and mucoid P. aeruginosa was investigated. Strains used were PAE1, an isolate from otitis externa; PAFF an isolate from urinary tract infection; PAJM, a mucoid isolate from CF lung infection and mucoid PAO 579. Each isolate was grown to early stationary phase in magnesium-depleted (Mg- CDM), iron-depleted (Fe- CDM) or iron- and magnesium-depleted (Fe- Mg- CDM) chemically defined media. For magnesium-depletion studies the concentration of added Mg^{2+} was 0.02mM. The growth rates during the exponential phase of each of the isolates was not affected by the nature of the limiting nutrient.

Figure 3.42a shows the OM protein profiles of PAE1 grown in Mg- CDM (lane 1), Fe- CDM (lane 2) and Fe- Mg- CDM (lane 3). Protein H1 was induced in the OMs prepared by sarkosyl extraction from cells grown under all these nutrient conditions, and magnesium- and iron-depletion appeared to have a synergistic effect on the expression of OM protein H1. Protein H1 is a heat modifiable protein (Hancock and Carey, 1979). The identity of the protein labelled H1 was confirmed by denaturing the OMs by heating at 37 °C for 10 mins (samples were routinely denatured by heating at 100 °C for 10 mins) when protein H1 migrated as a 19K protein (data not shown). These OM antigens were electrophoretically transferred to NC paper and reacted with the patient's own serum collected 10 days after onset of otitis externa due to P. aeruginosa (Fig 3.42c). IgG in the patient's sera reacted with the IRMPs proteins D, E, H1. There was also a faint reaction with protein G. Transfer of all the OM proteins to NC paper was confirmed by staining a replicate portion of the NC paper with amido black (Fig 3.42b).

Similarly, OMs prepared from PAFF grown in Mg- CDM, Fe- CDM and Fe-

FIGURE 3.42 a

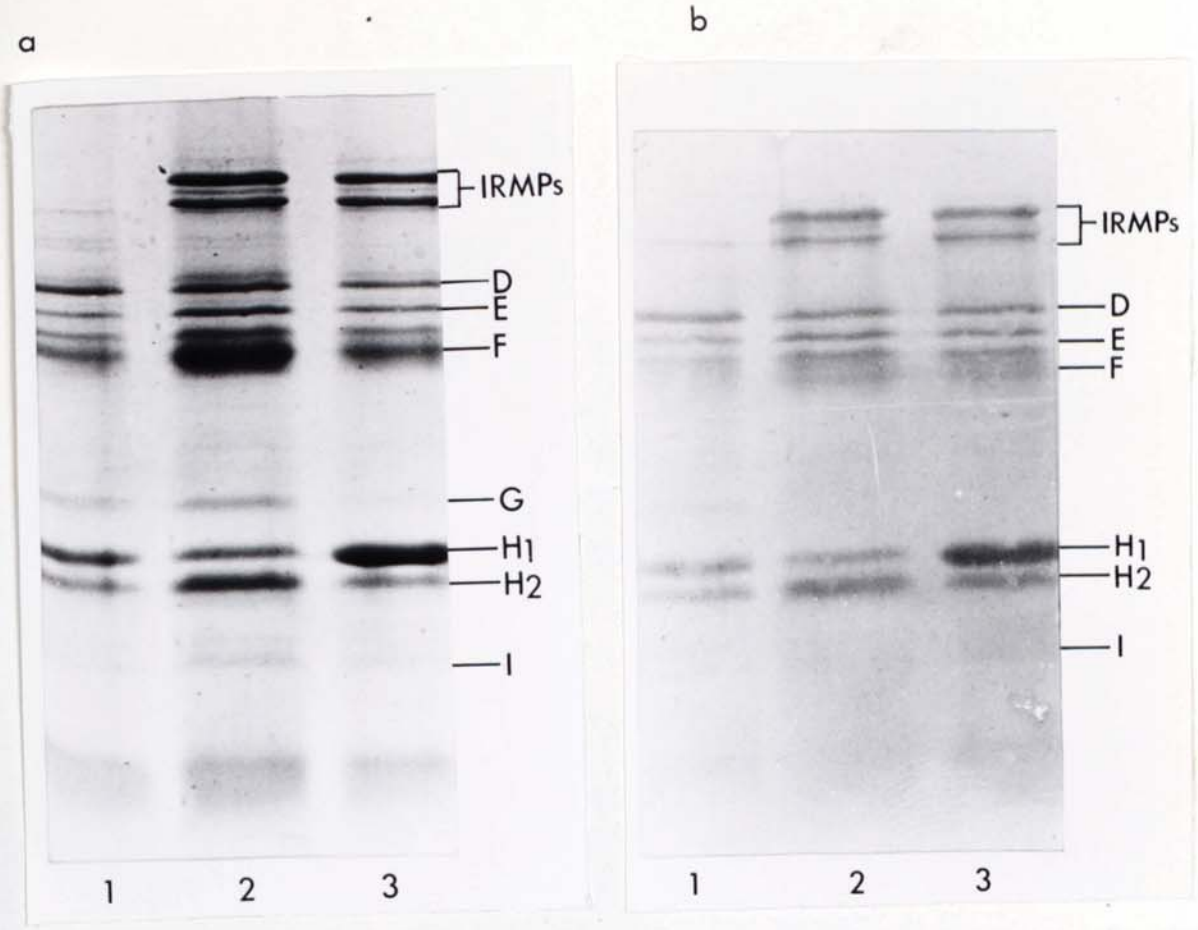
THE SDS-PAGE OM PROTEIN PROFILES OF P. AERUGINOSA E1, AN ISOLATE FROM OTITIS EXTERNA, GROWN IN Mg- CDM (LANE 1), Fe- CDM (LANE 2) AND Fe- Mg- CDM (LANE 3). PROTEINS WERE SEPARATED USING 14% w/v ACRYLAMIDE GELS AND WERE VISUALISED WITH COOMASSIE BLUE STAIN.

FIGURE 3.42 b

THE OM ANTIGENS SHOWN IN 3.42a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND VISUALISED WITH AMIDO BLACK STAIN.

FIGURE 3.42 c

THE OM ANTIGENS SHOWN IN 3.42a ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH LPS-ABSORBED SERUM FROM A DIVER WITH OTITIS EXTERNA DUE TO P. AERUGINOSA E1. THE REACTION WITH IgG IS SHOWN.


a
IRMPs
D
E
F
G
H1
H2
I
1
2
3
b
IRMPs
D
E
F
H1
H2
I
1
2
3

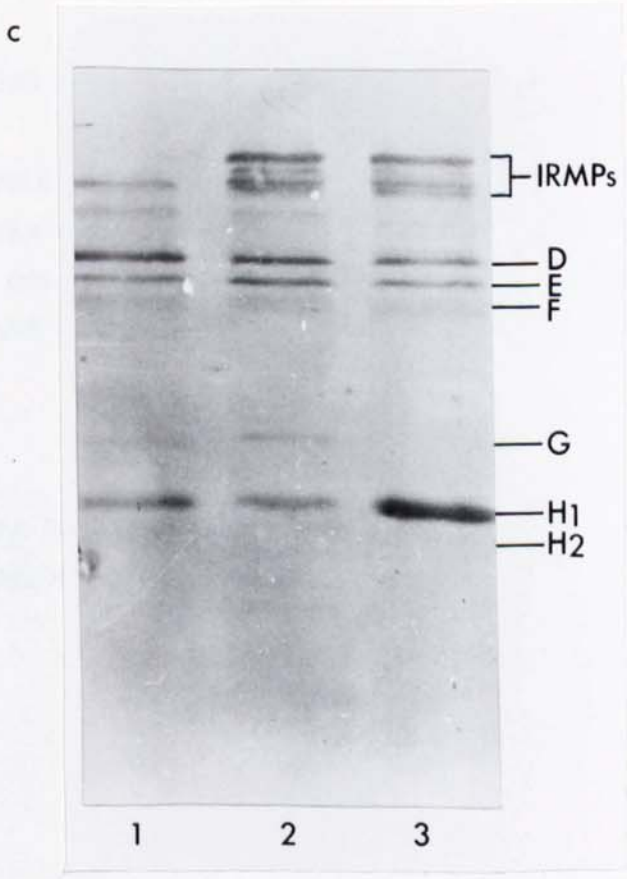

c
IRMPs
D
E
F
G
H1
H2
1
2
3

FIGURE 3.43a

IMMUNOBLOT OF THE OM ANTIGENS OF NON-MUCOID P. AERUGINOSA PAFF, AN ISOLATE FROM URINARY TRACT INFECTION, ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH THE PATIENTS OWN SERUM. OMs WERE PREPARED FROM PAFF GROWN IN Mg- CDM (LANE 1), Fe- CDM (LANE 2) AND Fe- Mg- CDM (LANE 3). THE REACTION OBTAINED WITH IgG IS SHOWN.

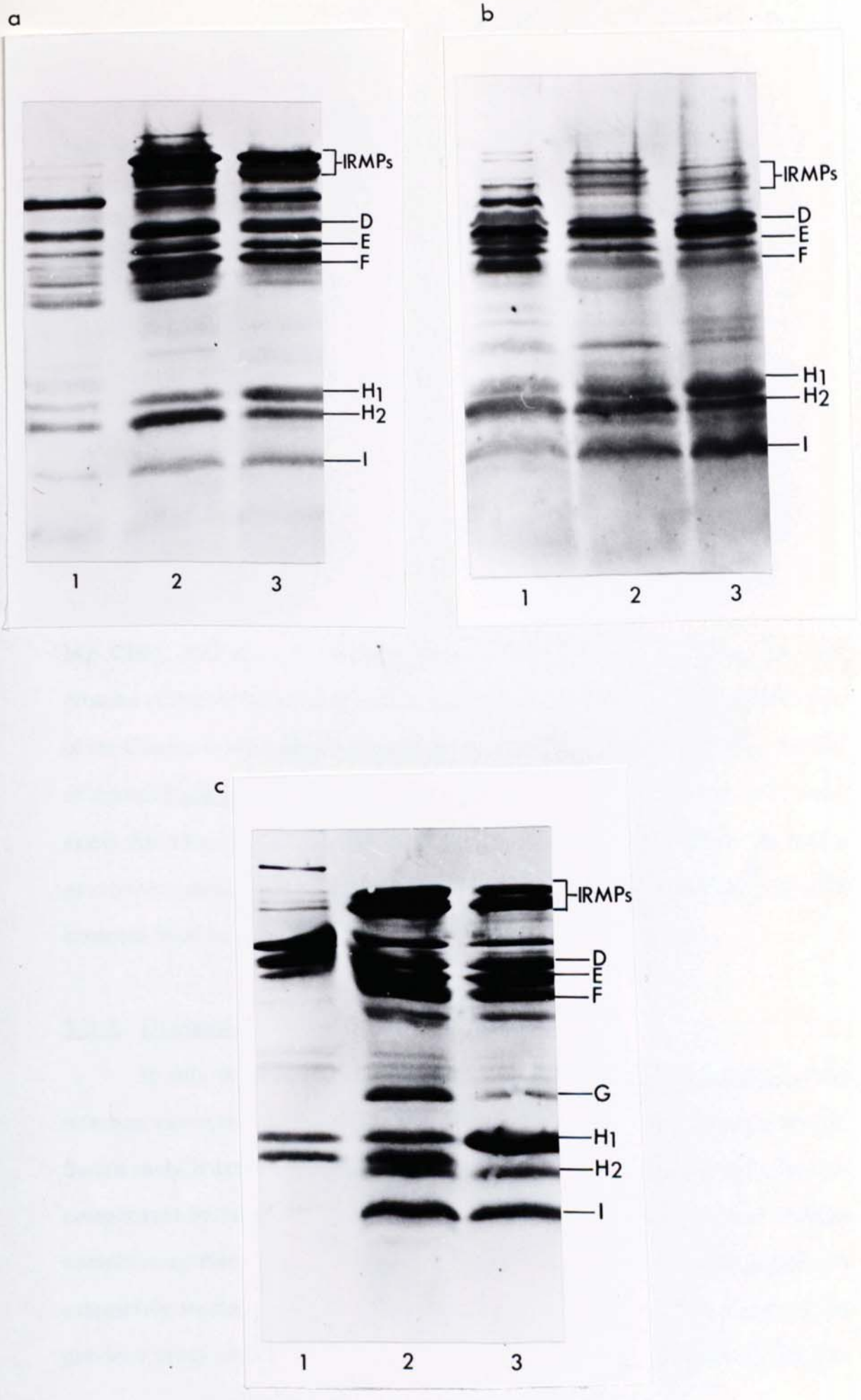
FIGURE 3.43b

IMMUNOBLOT OF THE OM ANTIGENS OF MUCOID P. AERUGINOSA PAJM, AN ISOLATE FROM CF LUNG INFECTION, ELECTROPHORETICALLY TRANSFERRED TO NC PAPER AND REACTED WITH THE PATIENTS OWN SERUM. OMs WERE PREPARED FROM PAJM GROWN IN Mg- CDM (LANE 1), Fe- CDM (LANE 2) AND Fe- Mg- CDM (LANE 3).

FIGURE 3.43 c

IMMUNOBLOT OF THE OM ANTIGENS OF MUCOID P. AERUGINOSA PAO 579 REACTED WITH SERA FROM RATS IMMUNISED WITH PAO 579 GROWN IN IRON-DEPLETED MEDIA. OMs WERE PREPARED FROM PAO 579 GROWN IN Mg- CDM (LANE 1), Fe- CDM (LANE 2) AND Fe- Mg- CDM (LANE 3). THE REACTION OBTAINED WITH IgG IS SHOWN.

ALL SERA WERE ABSORBED WITH LPS EXTRACTED FROM THE HOMOLOGOUS STRAIN PRIOR TO USE IN IMMUNOBLOTTING.


a
IRMPs
D
E
F
H1
H2
I
1
2
3
b
IRMPs
D
E
F
H1
H2
I
1
2
3
c
IRMPs
D
E
F
G
H1
H2
I
1
2
3

Mg- CDM were transferred to NC paper and reacted with serum collected four weeks after P. aeruginosa was first isolated from this patient's urinary tract. IgG in the patient's serum reacted strongly with all the major OM proteins including protein H1 and the IRMPs (Fig 3.43a). IgG in serum from a CF patient reacted strongly with all the major proteins, including protein H1, in OMs prepared from the patient own isolate grown in Fe- CDM, Mg- CDM and Fe- Mg- CDM (Fig 3.43b). The reaction with the IRMPs was not strong on this immunoblot; it was noted that the IRMPs were expressed less in the OM of PAJM as compared to the isolates PAE1, PAFF and PAO 579.

To confirm the immunogencity of protein H1 sera from rats immunised with whole cells of PAO 579 grown in iron-depleted media was used to probe immunoblots of OMs prepared from cells grown in Mg- CDM, Fe- CDM and Fe- Mg- CDM. IgG in sera from these immunised rats reacted with all the major OM proteins of PAO 579 including proteins H1 and the IRMPs (Fig 3.43c). Observation of the Coomassie blue stained gels indicated that protein H1 was induced in the OM of mucoid P. aeruginosa strains PAO 579 and PAJM grown in Fe- Mg- CDM to the extent that it became the predominant protein in the OM, however, this result was not examined quantitatively. All sera used in this study were absorbed with LPS extracted from the homologous strain prior to use in immunoblotting.

3.3.6 Discussion

In this investigation the antibody response during P. aeruginosa lung infection was examined longitudinally using the rat model of chronic lung infection. Such a study is extremely difficult in clinical infection as the response of patients is complicated by factors such as nutritional state, age, sex, the existence of other complicating diseases and therapy. The serum response to P. aeruginosa was extensively studied using a variety of immunological assays. The results of the previous study indicated some differences in the classes of immunoglobulins

involved in the local and systemic antibody responses. Nevertheless, time coursing the development of antibodies in serum, following infection with P. aeruginosa was considered a useful means of investigating the early antibody responses.

The results confirmed that the primary systemic immune response to P. aeruginosa involved predominatly IgG and IgM antibodies. IgM was the predominant antibody to P. aeruginosa present in serum between days 4 to 14 post-infection. The IgM response to P. aeruginosa, was maximal by day 14 and then declined whereas the IgG response to P. aeruginosa detected in both the lung and serum appeared to increase throughout the course of the infection.

This is the first study in whch immunoblotting has been used to investigate the sequential antibody response to bacterial antigens during infection. This technique allows the class of antibody involved in antigen-antibody reactions to be identified. Also, the antigens can be identified according to their molecular weights (Towbin and Gordon, 1984). The results indicate that an antibody response was elicited to the IRMPs and OM proteins D, E, G and H1 as early as 4 days after infection.

The factors which confer immunogencity or antigenicity on a molecule are complex but include its foreigness, its molecular size and its chemical complexity. Surface exposure and electrical charge are important factors involved in immunopotency of antigens (Goodman, 1980). Protein D, F and G seem to be exposed on the surface of P. aeruginosa as they were labelled by ^{125}I-lactoperoxidase (Lambert and Booth, 1982). Protein F was also labelled with a fluorescent monoclonal antibody to the porin (Mutharia and Hancock, 1983). The location of P. aeruginosa high molecular weight IRMPs in the OM has not been studied as yet. Surface exposed proteins would be expected to interact with the host's immune system and to stimulate an antibody response soon after infection. However, morphological and physiological studies have provided strong evidence for *in vivo* interactions of porin proteins or other OM proteins with LPS (van Alphen *et al*,

1979; 1978). Recently van de Ley *et al* (1986) reported that smooth LPS containing strains of Enterobacteriaceae do not bind antibodies to the porin phoE at their surface. Such interaction may account for the slower development of antibodies to protein F.

The protein portion of P. aeruginosa lipoproteins H2 and I are not exposed at the surface of the cell (Lambert and Booth, 1982) and protein H2 is largely associated with the peptidoglycan layer (Hancock and Carey, 1979). These proteins may become exposed only after a measure of phagocytic processing which may account for the delay in development of an antibody response to these antigens.

Also, the antigens released into the alginate surrounding cells within the microcolony may be very different from those exposed at the surface of cells grown *in vitro* in laboratory media. This will be an important factor in the interaction of P. aeruginosa with the host's immune system in chronic lung infection.

Results obtained using ELISA techniques suggest that sera collected from non-infected control rats contained very low titres of IgM and IgG antibodies to P. aeruginosa. These "natural" IgM antibodies may react with P. aeruginosa OM protein D, E, G, H1 and the IRMPs which would account for the early immune responses to these antigens.

P. aeruginosa core LPS was recognised on immunoblots by IgG and IgM classes of antibodies in sera obtained only 4 days after infection. The strain used in the infection was a nontypable isolate which expressed 0-antigen side chains only in very reduced amounts. Perez *et al* (1985) reported that hyperimmune sera to serum sensititive (0-antigen deficient) Campylobacter fetus strains reacted with core LPS structures of serum senstitive and serum resistant strains (which possess an 0-antigen) whereas hyperimmune sera to resistant strains contained no antibodies to core structures. The degree of exposure of core LPS epitopes appears to be related to the phase of bacterial growth (Fenwick *et al*, 1986). During periods of relatively rapid multiplication such as occurred during the first 4 days post-infection, fewer core epitopes may be covered with 0-antigen than during periods of slow growth. The

effect of growth phase on P. aeruginosa LPS is considered further in section 3.4. The 0-antigen was only faintly recognised by IgG in sera collected 4 days post-infection but was strongly recognised by IgG in sera obtained 28 days post-infection.

The porins and lipoproteins of Gram-negative bacteria were powerful mitogens and polyclonal activators of B-lymphocytes (Bessler and Ottenbreit, 1977; Melchers *et al*, 1975). Activation of B-lymphocytes by purified OM proteins F, H2 and I of P. aeruginosa has been demonstrated *in vitro* (Chen *et al*, 1980). LPS is known to be a thymus indepenent antigen that stimulates predominantly IgM and IgG3 responses in mice (Davie, 1982). This macromolecule in its purified form, is a potent mitogen that will stimulate non-specific clonal expansion of B cells in animals. A recent study provided evidence that polyclonal B cell activation by a synthetic analogue of bacterial lipoprotein was functionally different from activation by bacterial LPS (Kleine *et al*, 1987). Immunoblotting with serially diluted sera indicated that lipoproteins were the major protein antigens to which IgG was directed in the latter stages of the infection.

Irvin *et al* (1981) have shown that a carbenicillin hypersusceptible mucoid strain of P. aeruginosa possessed additional OM proteins of 25 and 32K and the32K protein was lost on reversion to normal carbenicillin sensitivity. Strain 492a, a carbenicillin resistant strain which was isolated from the same sputum sample as 492c, did not express this 32K protein. OM fractions were prepared by extraction with Triton X-100. In this study a 32K antigen was detected on immunoblots of sarkosyl-extracted OMs from 492a probed with sera from infected rats but this antigen was not visualised by Coomassie blue or amido black staining. A 32K protein was present in OM fractions of 492a prepared by centrifugation to equilibrium in sucrose density gradients. Chopra and Shales (1980) reported that the polypeptide profiles of OMs prepared from E. coli by sarkosyl extraction and sucrose density ultracentrifugation were different; sarkosyl extraction removed several minor proteins from the OM. Since the SDS-PAGE protocol used in this study was different from that in the study by Irvin *et al* (1981) the apparent molecular weights

assigned to OM proteins may be different. An antigen with a molecular weight of 32K was also detected on immunoblots of OMs prepared from mucoid PAO 579 probed with sera from CF patients (Fig 3.9 d and e). These data suggest that sucrose density gradient ultra-centrifugation techniques should be considered for preparation of P. aeruginosa OM fractions for future immunological studies.

Immunoblotting using solubilised whole cell lysates proved a very senstive technique for studying the host's immune response to P. aeruginosa whole cell antigens. The results indicated that a small number of antigens, some of which appear to correspond to OM proteins, were recognised soon after infection. The antigens were all of molecular weights greater than 41K. An antibody response was elicited to only a limited number of whole cell antigens during the first 28 days post-infection and most of these were recognised by day 14.

The intrinsic biological activity of individual polypeptides is usually destroyed by the denaturing effect of anionic detergents (Helenius and Simons, 1975). Whilst partial renaturation occurs following removal of detergent during immunoblotting, this process may not be complete. Crossed immunoelectrophoresis offers the advantage that antigens are usually solubilised, and allowed to react with antibody, in a non-denaturing system, thus allowing assessment of native antigenic properties. Another important advantage of CIE relates to its quantitative nature. The area (A) subtended by an immunoprecipitin arc is empirically found to be related to the amount of antigen X (C) analysed and to the amount of anti-x immunoglobulins (B) present in the serum preparation according to the equation

$$A = \frac{kC}{B}$$

where k is an area loading constant (Owen, 1985). As the volumes of antigen and serum used throughout this study were constant a decrease in the height of a precipitin peak indicates an increasing antibody titre to that antigen. Sequential sera obtained from rats with experimentally induced P. aeruginosa lung infection formed a small but increasing number of precipitins with P. aeruginosa antigens. This provides further evidence that there is a differential structural location and chemical nature of P. aeruginosa antigens. These results confirm and extend the results of Lam *et al* (1983) in which the immunogenicity of P. aeruginosa grown in common laboratory media was examined using sera from rats with chronic P. aeruginosa lung infection. Using purified OM proteins F, H2 and I Lam (1983; Lam *et al*, 1983) demonstrated that sera from infected rats (obtained 7 days post-infection) formed precipitins with these antigens.

Sera from several CF patients formed large numbers of precipitins with P. aeruginosa thus confirming results of earlier studies by Høiby and Axelsen (1973) and Høiby *et al* (1977). However 3/5 of the patients included in this present study formed less than 15 precipitins with P. aeruginosa antigens despite having harboured P. aeruginosa in their lungs for 5 years indicating that only some P. aeruginosa antigens were presented to the host at some stages in the infection. These data also indicated that there may be differences in the antigenicity of soluble P. aeruginosa antigens prepared from cells grown in iron-plentiful or iron-depleted media. Further work would be necessary to investigate the possibility that some of these precipitins may correspond to P. aeruginosa IRMPs.

Fewer antigens of P. aeruginosa were recognised in CIEs than on immunoblots probed with sera obtained at intervals following infection of rats with P. aeruginosa. It seems likely that this was due to the greater sensitivity of the immunoblotting technique as approximately the same amount of protein was present in the samples assayed by immunoblotting and by CIE. Alternatively, some of the precipitins may represent a protein-LPS complex and may contain several antigens (Owen, 1985). The use of monoclonal antibodies to specific P. aeruginosa antigens

included either in the intermediate gel, or in the second dimension, is one technique which could be used to identify the antigens involved in precipitin formation to be identified. The antigens recognised on immunoblots of whole cell lysates of P. aeruginosa could also be identified using these monoclonal antibodies.

The level of protein H1 in the OM of P. aeruginosa was found to be inversely proportional to cell envelope Mg^{2+} concentration (Nicas and Hancock, 1980). The results presented in this study suggest that this protein is also expressed by non-mucoid and mucoid strains of P. aeruginosa grown in iron-depleted media, and that magnesium and iron have a synergistic effect on the production of this OM protein. Protein H1 is thought to replace Mg^{2+} at sites on the LPS which can otherwise be attacked by EDTA or cationic antibiotics so that induction of protein H1 reduces the sensitivity of P. aeruginosa to these agents (Nicas and Hancock, 1980; Brown, 1975). Since the availability of iron in the body is restricted, and there is some evidence to indicate that magnesium may also be deprived in some infections, *in vivo*-grown P. aeruginosa may be more resistant to aminogylcoside antibiotics. Sera from patients with otitis externa, urinary tract infection and CF lung infection due to P. aeruginosa contained antibodies to OM protein H1 indicating that this protein is expressed *in vivo* in a variety of clinical infections. It may therefore be a suitable candidate for vaccine development.

Results obtained using this animal model of chronic lung infection indicate that extracellular products of P. aeruginosa including exotoxin A and elastase may be implicated in damage to the lung tissues (Cash *et al*, 1983; Woods *et al*, 1982). Yields of exotoxin A, elastase and alkaline protease in culture supernatants of P. aeruginosa were inversely proportional to the iron concentration of the medium (Bjorn *et al*, 1979). Sokol and Woods (1984) reported greater pathological alterations in the lungs of experimentally infected rats when the inoculum was grown in low-iron as compared to high-iron medium. Results of a recent study indicated that intrapulmonary challenge with E. coli was followed by a rapid increase in the

levels of lactoferrin in the lungs of mice (Laforce *et al*, 1986). The lactoferrin would contribute to the iron-restricted environment in the lung during infection. The results of this study indicated that rats infected with an inoculum grown in iron-plentiful media eventually produced antibodies to P. aeruginosa IRMPs thus providing evidence for an iron-restricted environment in the lung during infection. Differences in the lung pathology of rats infected with inoculum grown in iron-plentiful or iron-depleted media were not investigated.

Overall, the results of this study indicated that there was a progressive increase in the antibody response to P. aeruginosa protein and LPS antigens during chronic infection. The microcolony mode of growth may result in gradual release of bacterial antigens which may then interact with the host's immune system. Differences in the composition and surface localisation of antigens may also be important factors in determining the antibody response to these antigens *in vivo*.

3.4 ALTERATIONS IN THE LPS PROFILE AND FATTY ACID CONTENT OF P. AERUGINOSA PAAT AS A FUNCTION OF GROWTH PHASE AND GROWTH-LIMITING NUTRIENT

The results presented in section 3.3 indicated that iron and magnesium have a synergistic effect on the expression of protein H1 in the OM of P. aeruginosa. In this study the effect of iron- and magnesium-limitation, alone or in combination, on the LPS profile (revealed by SDS-PAGE) and fatty acid content of whole cell lysates of P. aeruginosa PAAT, an isolate from CF lung infection, was investigated. The growth kinetics of this isolate in complete (Fe+ CDM), iron-depleted (Fe- CDM), magnesium-depleted (Mg- CDM) and iron- and magnesium-depleted (Fe- Mg- CDM) chemically defined media were studied. The exponential doubling time of the isolate in each of these media was unaffected by the limiting nutrient however the onset of stationary phase occurred at an optical density which was characteristic of the growth-limiting nutrient.

Changes in envelope properties may take place several generations before the onset of stationary phase due to depletion of a specific nutrient (Williams *et al*, 1984). In this study logarithmic phase cells were defined as cells harvested three generations before the onset of stationary phase. This was achieved using a very small inoculum. Cells were harvested by centrifugation, rinsed twice with distilled water, and freeze-dried prior to analysis.

3.4.1 SDS-PAGE analysis of proteinase K digests of whole cells of PAAT

Figure 3.44 shows the LPS profiles of SDS-PAGE resolved proteinase K digests of logarithmic phase cells of PAAT grown in Fe+ CDM (lanes 1-3) and Fe- CDM (lanes 4-6). Consecutive lanes contained loadings equivalent to 50, 100 and 150 μg dry weight of whole cells. Each sample contained three low molecular weight, fast-migrating bands probably representing the core component and core substituted with an 0-polysaccharide containing one or two repeating units. Some

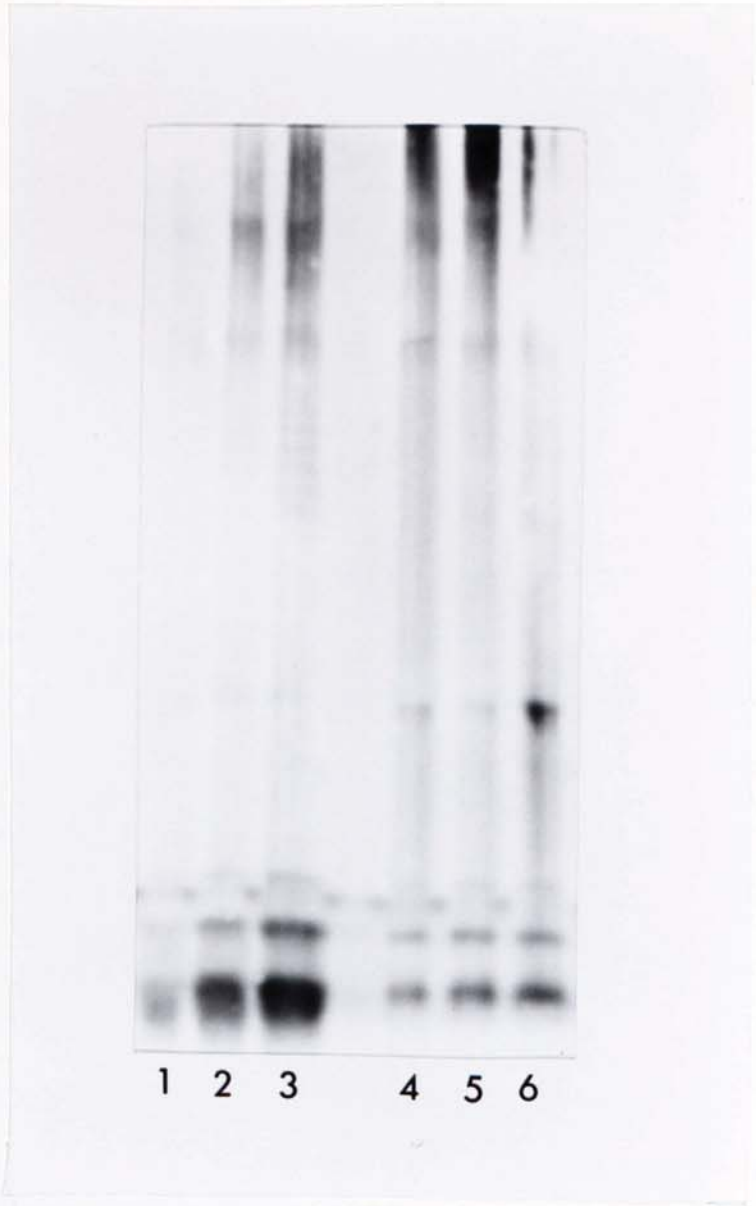


FIGURE 3.44

THE SDS-PAGE SILVER STAINED PROFILES OF PROTEINASE K DIGESTS OF LOGARITHMIC PHASE CELLS OF PAAT GROWN IN Fe+ CDM (LANES 1-3) AND Fe- CDM (LANES 4-6). LOADINGS WERE EQUIVALENT TO 50 µg (LANES 1 AND 4) 100 µg (LANES 2 AND 5) AND 150 µg (LANES 3 AND 6) DRY WEIGHT OF CELLS.

high molecular slow-migrating 0-polysaccharides which stained faintly were also present. Figure 3.45 shows the profiles obtained with cells of the same isolate grown to early stationary phase in Fe+ CDM (lanes 1-3), Fe- CDM (lanes 4-6), Mg- CDM (lanes 13-15) and Fe- Mg- CDM (lanes 16-18) and also to late stationary phase in Fe+ CDM (lanes 7-9) and Fe- CDM (lanes 10-12). Early stationary phase cells appeared to contain more low molecular weight LPS polysaccharides than logarithmic phase cells grown under the same nutrient conditions. The 0-antigen of early stationary phase cells appeared to contain three regions of polysaccharide material which stained with silver nitrate. Two of the high molecular weight regions consisted of finely divided bands which were closely spaced. The third region contained bands which reacted most strongly with silver nitrate. The results indicate that late stationary phase cells contained more LPS 0-antigen than early stationary phase cells and early stationary phase cells in turn contained much more 0-antigen than logarithmic phase cells.

Nutrient depletion also appeared to affect the amount of core and 0–polysaccharides present in cells of PAAT. Of the samples examined cells grown in Fe- Mg- CDM appeared to contain most, and cells grown in complete CDM least, LPS side chains. Differences were noted in the colour developed by the core region of cells grown in Mg- CDM and in Fe- Mg- CDM as compared to cells grown in Fe- CDM or Fe+ CDM. This region in the proteinase K digested whole cells grown in Mg- CDM and Fe- Mg- CDM stained a more intense bluish brown. This difference is not apparent in this photograph. Slightly greater heterogeneity in the number of 0–polysaccharide bands (in the region marked with an arrow) was obtained for nutrient-depleted cells than for cells grown in complete CDM.

Figure 3.46 shows the LPS profile of proteinase K digests of whole cell lysates of PAAT (lanes 1-3) and also LPS extracted from PAAT (lanes 4-6). In both cases cells were grown to early stationary phase in Fe- CDM prior to treatment with proteinase K or extraction with hot aqueous phenol. The loadings were equivalent to

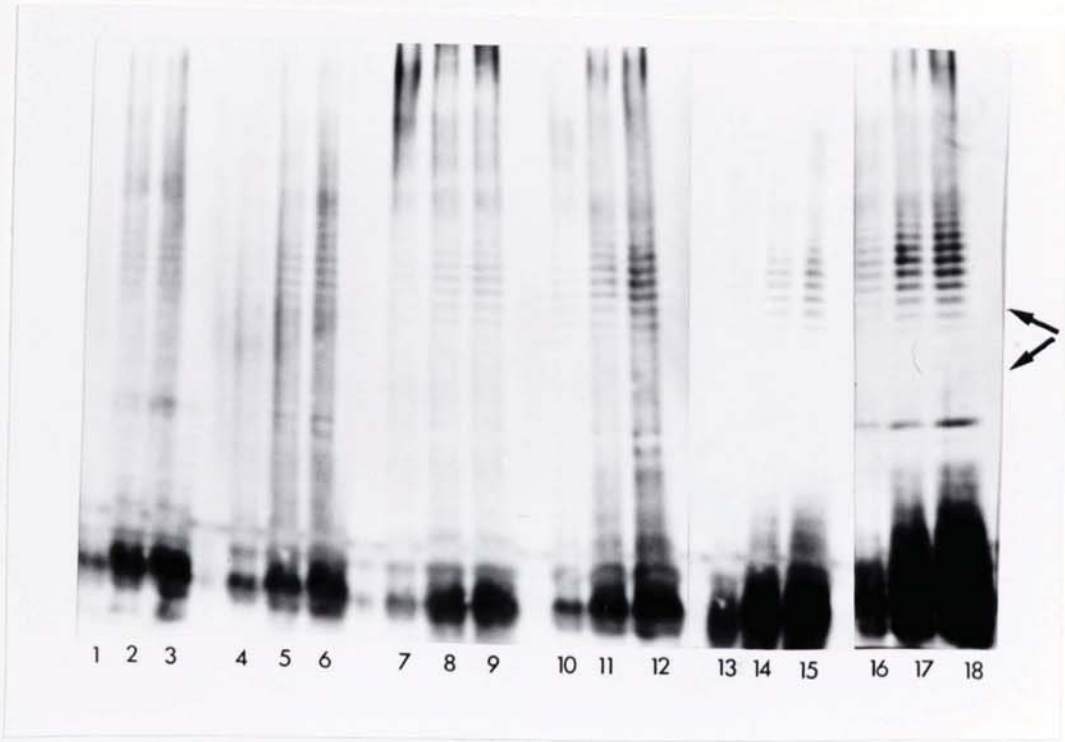


FIGURE 3.45

THE SDS-PAGE SILVER STAINED PROFILES OF PROTEINASE K DIGESTS OF WHOLE CELLS OF PAAT GROWN TO EARLY STATIONARY PHASE GROWN IN Fe+ CDM (LANES 1 - 3), Fe- CDM (LANES 4-6), Mg- CDM (LANES 13-15) AND Fe- Mg- CDM (LANES 16-18) AND ALSO TO LATE STATIONARY PHASE IN Fe+ CDM (LANES 7-9) AND Fe- CDM (LANES 10-12). CONSECUTIVE LANES REPRESENT INCREASING LOADINGS AS DESCRIBED IN FIGURE 3.44.

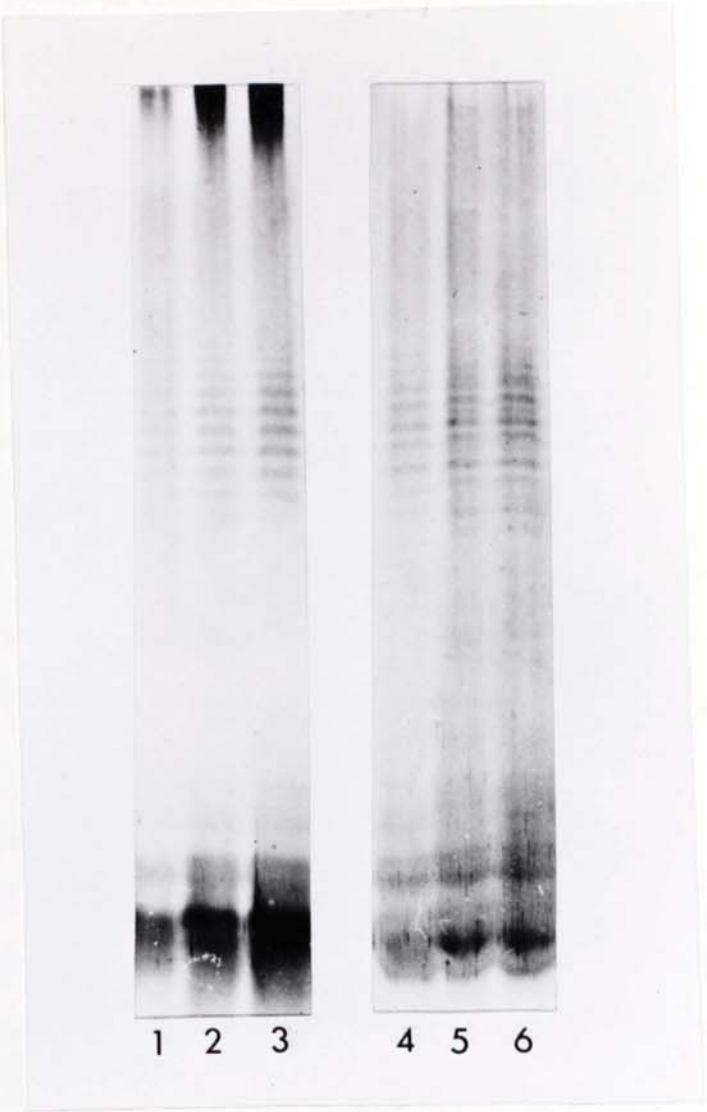


FIGURE 3.46

THE SDS-PAGE SILVER STAINED PROFILES OF EARLY STATIONARY PHASE CELLS OF PAAT GROWN IN Fe- CDM AND DIGESTED WITH PROTEINASE K AND OF LPS EXTRACTED FROM PAAT GROWN IN FE- CDM. THE LOADINGS WERE EQUIVALENT TO 50 µg (LANE 1), 100 µg (LANE 2), 150 µg (LANE 3) DRY WEIGHT OF WHOLE CELLS AND 5µg (LANE 4), 10 µg (LANE 5) AND 15 µg (LANE 6) DRY WEIGHT OF EXTRACTED LPS.

50, 100 and 150 µg of whole cells and 5, 10 and 15 µg of extracted LPS (aqueous phase). The results indicated that the SDS-PAGE resolved profiles of P. aeruginosa whole cell proteinase K digests and extracted LPS were similar.

This patients own sputum was collected over a two week period, freeze-dried and pooled. Samples of sputum were suspended in sample buffer and bacteria lysed. After digestion with proteinase K loadings equivalent to 2.5, 5 and 7.5 mg dry weight of sputum were resolved by SDS-PAGE and visualised by silver staining. The faint "ladderlike" banding pattern indicated that this isolate expressed an 0-antigen when grown *in vivo* in the patient's lungs (data not shown). However, the amount of LPS present in these samples was too low to allow comparison with the profile obtained for the isolate after *in vitro* growth. The pooled sputum was therefore treated with hot aqueous phenol to extract LPS. Figure 3.47 shows the silver stained SDS-PAGE profile of the material obtained from sputum (lanes 1-3 – 25, 50 and 75 µg of sample; lanes 4-6 – 5, 10 and 15 µg of samples) and the patient's isolate grown in Fe- CDM (lanes 7-9 – 5, 10 and 15 µg). The LPS 0-antigen profiles of *in vivo* and *in vitro* grown PAAT were qualitatively similar. However, a larger proportion of low molecular weight components were present in the samples from *in vivo*. Some of this material may be host cell debris or lung components.

Figure 3.48 shows the LPS profile of *in vitro* grown PAAT revealed by silver staining (Fig 3.48a) and immunoblotting with the patient's own serum (Fig 3.48b). Samples were grown in Fe+ CDM or Fe- CDM to logarithmic phase (lanes 1 and 2 respectively), early stationary phase (lanes 3 and 4 respectively), and late stationary phase (lanes 5 and 6 respectively) and were digested with proteinase K prior to analysis. Proteinase K digested cells grown in Mg- CDM (lane 7) and Fe-Mg- CDM (lane 8) and also extracted LPS from iron-plentiful (lane 9) and iron-depleted (lane 10) cultures were also included. The results indicated that antibodies in the patient's serum reacted with the LPS core and 0-antigen components expressed by

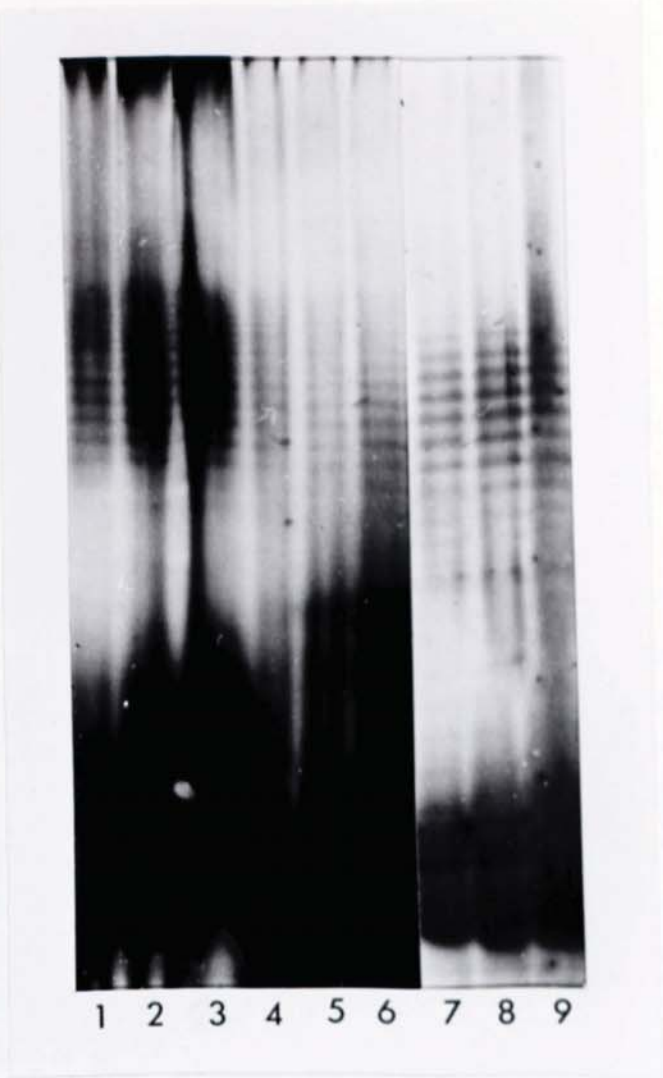
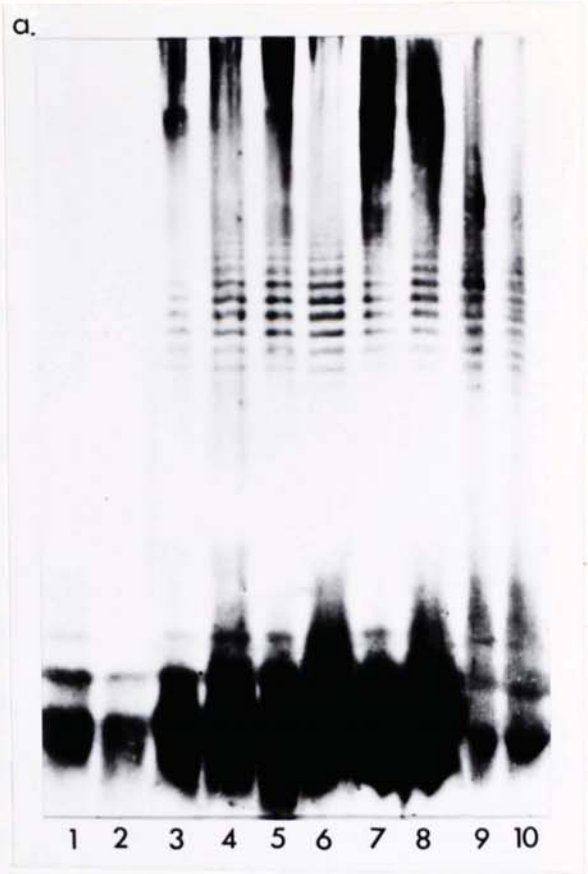


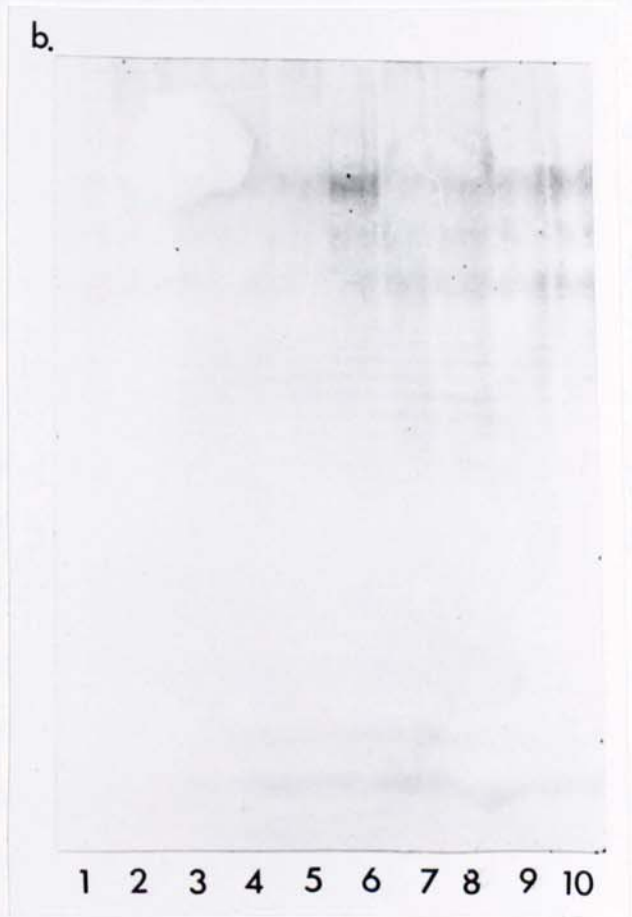
FIGURE 3.47

THE SDS-PAGE SILVER STAINED PROFILE OF AQUEOUS PHENOL EXTRACTED MATERIAL TAKEN DIRECTLY FROM THE SPUTUM OF A CF PATIENT AND ALSO LPS EXTRACTED FROM THE SAME ISOLATE GROWN *IN VITRO* IN Fe- CDM. THE LOADINGS WERE 25 µg (LANE 1), 50 µg (LANE 2), 75 µg (LANE 3), 5 µg (LANE 4), 10 µg(LANE 5), and 15 µg (LANE 6) OF THE MATERIAL OBTAINED DIRECTLY AND WITHOUT SUBCULTURE FROM *IN VIVO*. LPS LOADINGS WERE EQUIVALENT TO 5 µg (LANE 7), 10 µg (LANE 8) and 15 µg (LANE 9) DRY WEIGHT.

FIGURE 3.48, a and b

THE SDS-PAGE PROFILES OF PROTEINASE K DIGESTS OF PAAT LOGARITHMIC PHASE CELLS GROWN IN Fe+ CDM (LANE 1), Fe- CDM (LANE 2), AND EARLY STATIONARY PHASE CELLS GROWN IN Fe+ CDM (LANE 3), Fe- CDM (LANE 4), Mg- CDM (LANE 7), Fe- Mg- CDM (LANE 8) AND ALSO LATE STATIONARY PHASE CELLS GROWN IN Fe+ CDM (LANE 5) AND Fe- CDM (LANE 6). THE PROFILES OF AQUEOUS PHENOL EXTRACTED LPS FROM THE SAME ISOLATE GROWN IN Fe+ CDM (LANE 9) AND Fe- CDM (LANE 10) ARE ALSO SHOWN. THE LOADINGS WERE EQUIVALENT TO 150µg DRY WEIGHT OF WHOLE CELLS AND 15 µg DRY WEIGHT OF LPS. THE PROFILES WERE VISUALISED BY SILVER STAINING (a) AND BY REACTION WITH THE PATIENT'S OWN SERUM (b). THE REACTION OBTAINED WITH IgG IS SHOWN.


a.
1 2 3 4 5 6 7 8 9 10


b.
1 2 3 4 5 6 7 8 9 10

ion with logarithmic phase cells. The profiles revealed by silver staining and immunoblotting were similar.

3.4.2 Fatty acid analysis of extracted LPS and whole cells of PAAT

Extracted LPS and whole cell fractions were assayed for fatty acid methyl esters after mild alkaline hydrolysis and methylation. Although amide-linked hydroxy fatty acids are released more effectively by acid hydrolysis this method degrades the cyclopropane fatty acids, and produces a number of artefacts in the chromatograms (Moss, 1978).

Aqueous phase extracted LPS preparations from PAAT contained 3-hydroxydecanoic (3OH-$C_{10:0}$), dodecanoic ($C_{12:0}$), 2-hydroxydodecanoic (2OH-$C_{12:0}$), 3–hydroxy dodecanoic (3OH-$C_{12:0}$) acids and also small amounts of hexadecanoic ($C_{16:0}$) acid. The results of the GLC analysis of the fatty acids of LPS extracted from PAAT grown in Fe+ CDM or Fe- CDM are shown in Table 3.1. Fatty acids were identified on the basis of their retention times and were quantified by comparison with the calculated integration values of the $C_{17:0}$ methyl ester internal standard. The fatty acid composition of LPS extracted from Fe+ CDM and Fe- CDM cultures was similar except there was more $C_{16:0}$ present in LPS from Fe- CDM grown cells. $C_{16:0}$ is a major component of whole cell phospholipids (Hancock and Meadow, 1969). The increased level of $C_{16:0}$ in LPS from Fe- CDM cultures may be due to the presence of some phospholipid in the extracted material. The fatty acids were calculated to account for 9.9% and 10.1% by dry weight of the extracted LPS from Fe+ CDM and Fe- CDM grown cells respectively. Table 3.2 shows the KDO content of whole cells of PAAT grown in Fe+ CDM and Fe- CDM and LPS extracted from these cultures; the results suggest that iron-depleted cells contained more LPS than iron-plentiful cells.

The effect of growth phase and nutrient limitation on whole cell fatty acid composition of PAAT was investigated using GLC analysis. Figure 3.49 shows a

TABLE 3.1

GLC ANALYSIS OF FATTY ACID COMPOSITION OF LPS EXTRACTED FROM PAAT GROWN IN Fe+ CDM AND Fe- CDM

	GROWTH MEDIUM	
Fatty acid	Fe + CDM	Fe- CDM
$C_{12:0}$	22.9	19.9
$C_{16:0}$	3.1	10.0
$3OH\text{-}C_{10:0}$	28.3	29.5
$2OH\text{-}C_{12:0}$	32.9	33.6
$3OH\text{-}C_{12:0}$	5.3	4.4
Unknown	6.2	2.6
% (w/w) of LPS	9.9	10.1

TABLE 3.2

KDO CONTENT OF WHOLE CELLS OF PAAT GROWN IN Fe+ CDM AND Fe- CDM AND LPS EXTRACTED FROM THESE CULTURES

	GROWTH MEDIUM	
% (w/w) KDO	Fe+ CDM	Fe- CDM
LPS	5.0	4.4
Whole cells	7.0	11.5

FIGURE 3.49

FATTY ACID COMPOSITION OF P. AERUGINOSA PAAT. THE RELATIVE PERCENTAGE OF EACH OF THE FATTY ACIDS IS INDICATED. HYDROXY (LPS) FATTY ACIDS ARE INDICATED WITH AN ARROW. SAMPLES WERE PREPARED FROM CELLS GROWN IN:

a Fe+ CDM TO LOGARITHMIC PHASE
b Fe- CDM TO LOGARITHMIC PHASE
c Fe+ CDM TO EARLY STATIONARY PHASE
d Fe- CDM TO EARLY STATIONARY PHASE
e Fe+ CDM TO LATE STATIONARY PHASE
f Fe- CDM TO LATE STATIONARY PHASE
g Mg- CDM TO EARLY STATIONARY PHASE
h Fe- Mg- CDM TO EARLY STATIONARY PHASE

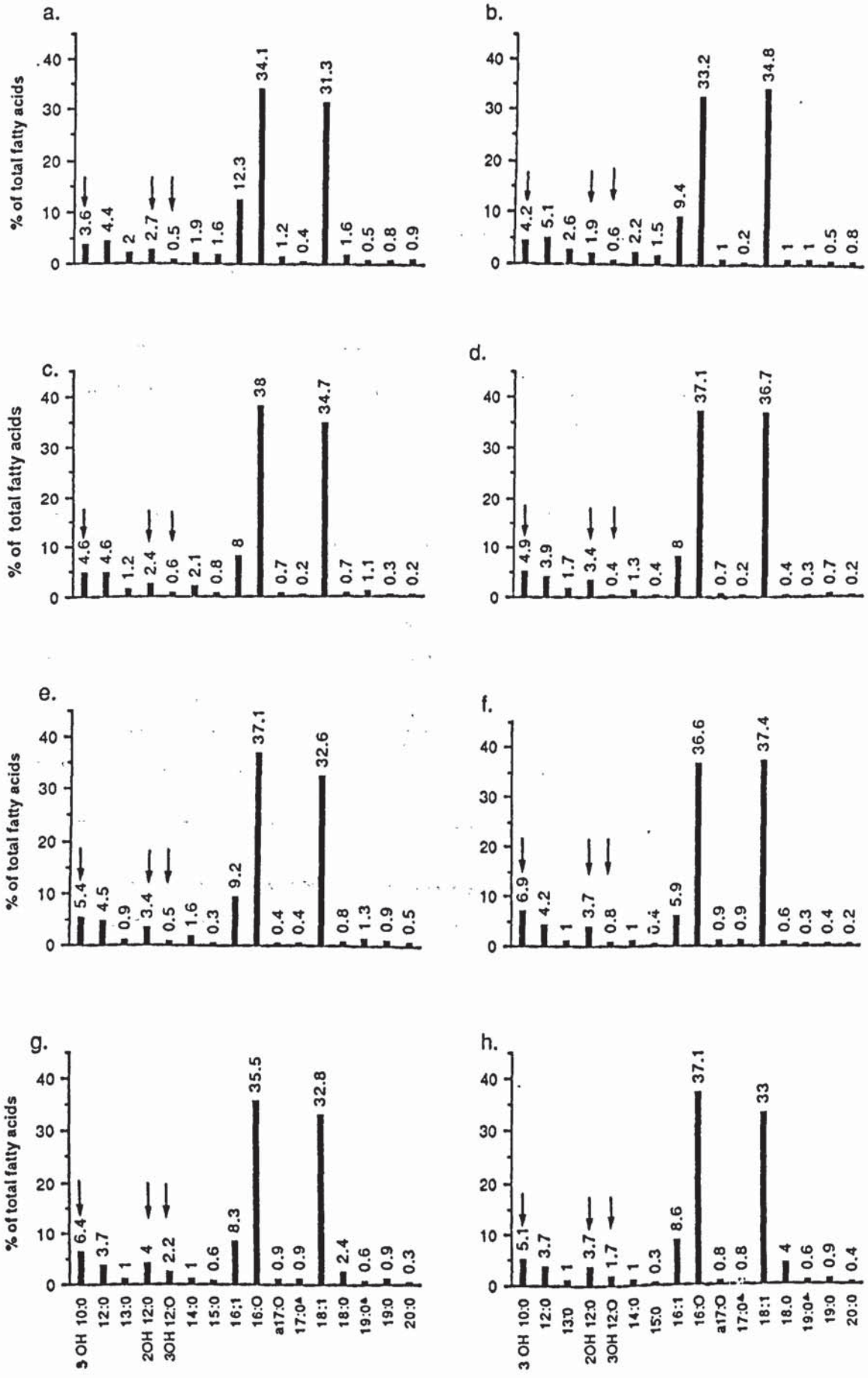

a.
b.
c.
d.
e.
f.
g.
h.
% of total fatty acids
3 OH 10:0
12:0
13:0
2OH 12:0
3OH 12:0
14:0
15:0
16:1
16:0
a17:0
17:0
18:1
18:0
19:0
20:0

TABLE 3.3

EFFECT OF GROWTH PHASE AND SPECIFIC NUTRIENT DEPLETION ON THE FATTY ACID AND PROTEIN CONTENT OF CELLS OF PAAT

Growth medium and phase	µg total fatty acid/ mg dry weight	µg protein#/ mg dry weight	µg hydroxy fatty acids/mg protein
Fe+ logarithmic	38.6 †(6.8)	347	7.6
Fe- logarithmic	27.3 (6.7)	341	5.4
Fe+ early stationary	51.2 (7.6)	384	10.1
Fe- early stationary	57.9 (8.7)	391	12.9
Fe+ late stationary	62.0 (9.3)	387	14.9
Fe- late stationary	64.9 (11.4)	401	18.5
Mg- early stationary	71.5 (12.6)	407	22.1
Fe-Mg- early stationary	86.8 (10.5)	442	20.6

† figures in brackets indicate percentage of hydroxy fatty acids

protein determinations were carried out after alkaline hydrolysis of whole cells

diagrammatic representation of the relative fatty acid composition of the various whole cell methanolysates. The results indicated that the relative percentage of the hydroxy (LPS) fatty acids increased along the growth curve and was dependent on the nature of the limiting nutrient (Table 3.3). It was also noted that the relative percentage of $C_{18:0}$ was increased in cells grown in Mg- CDM and Fe- Mg- CDM.

Quantitative comparisons show that growth phase and specific nutrient depletion had a marked effect on the total fatty acid content of cells (Table 3.3). Late stationary phase cells had an increased fatty acid content as compared to early stationary phase cells and early stationary phase cells in turn had an increased fatty acid content as compared to logarithmic phase cells. The increase in the level of fatty acids which occurred after onset of stationary phase may be related to the decreased rate of growth or lower oxygen tension but it also appears to be characteristic of the limiting nutrient. Cells grown in Mg- CDM and in Fe- Mg- CDM had a higher fatty acid content than cells grown in Fe- CDM A similar trend was found when these same samples were assayed for KDO content, though the presence of interfering substances may have affected these results.

The protein content of these samples was determined and used as a marker to allow comparison of the effect of growth phase and specific nutrient limitation on the hydroxy fatty acid LPS: protein ratio (Table 3.3). These factors had a less marked effect on the protein content as compared to the fatty acid content of whole cells. These results emphasise that the LPS content of this isolate varied markedly depending on the phase of growth and the specific limiting-nutrient. Magnesium-depletion appeared to have a greater effect on the LPS content of P. aeruginosa as compared to iron-depletion.

3.4.3 Discussion

The fatty acid composition of P. aeruginosa whole cells (Lambert and Moss, 1983; Moss, 1978; Ikemoto *et al*, 1978), extractable lipids (Wilkinson and

Galbraith, 1975; Hancock and Meadow, 1969) and LPS (Wilkinson, 1983; Wilkinson and Galbraith, 1973) have been investigated. However these studies did not consider the phenotypic variability of the bacterial cell. Growth rate, specific nutrient limitation and temperature have been shown to alter the phospholipid, fatty acid and LPS content of P. aeruginosa (Kropinski *et al*, 1987; Day and Marceau-Day, 1982; Gilbert and Brown, 1978). For example, chemostat grown cells of P. aeruginosa which were depleted of magnesium contained more LPS (quantified by changes in KDO content) than glucose-depleted cells (Gilbert and Brown, 1978). The purpose of this present study was then to investigate both qualitatively and quantitatively the effect of growth phase and specific nutrient limitation on the LPS profile and cellular fatty acid content of P. aeruginosa grown in batch culture. Results of SDS-PAGE and GLC analysis indicated that growth phase had a marked effect on the LPS profiles and fatty acid content of P. aeruginosa whole cells. Also, limitation of magnesium and/or iron lead to phenotypic variation in the total fatty acid content and the overall percentage of the lipid A (LPS) fatty acids expressed by this strain.

The silver stained SDS-polyacrylamide gels of proteinase K digests of whole cells of the isolate used in this study revealed small amounts of high molecular weight slow-migrating LPS which separated as fine closely spaced bands. Peterson and McGroarty (1985) reported that incomplete dissociation of E.coli LPS molecules gave rise to distinct anomalous slow-migrating bands in SDS-PAGE containing 0.1% w/v SDS. These multimers were dispersed by increasing the concentration of SDS in the gel. Increased concentrations of SDS did not affect the resolution of the high molecular weight bands obtained with the strain used in this study (data not shown). This material presumably represents LPS molecules with very long 0-antigen polysaccharides.

There are several steps involved in the biosynthesis of smooth LPS (Hammond *et al*, 1984; Luderitz *et al*, 1982). First, the 0-antigen repeat unit is

synthesised on a membrane-bound polyisoprenoid compound (acyl carrier lipid) followed by sequential addition of the component sugars. The repeating units are then polymerised on the lipid carrier by the action of 0-polymerase transferring the growing chain to a newly activated repeating unit. In the final steps, R-core-0-polysaccharide ligase transfers the 0-chains from the lipid carrier to the independently synthesised core lipid A to form the completed molecule. Results of a recent study which examined the effect of growth rate on the LPS profile of E. coli suggest that the activity of both these enzymes may be affected by growth rate, since high growth rates caused increases in the amounts of rough LPS and smooth-rough LPS (Dodds *et al*, 1987). Differences in the doubling times during the logarithmic and stationary phases of growth may account for the marked variation noted in the 0–polysaccharide content of logarithmic and stationary phase cells of the P. aeruginosa isolate used in this study. The core LPS of logarithmic phase cells appeared to be less highly substituted with 0-side chains than that of stationary phase cells. Also, the polysaccharide chains of stationary phase cells were more homogenous in length as compared to logarithmic phase cells. Specific nutrient limitation also appeared to affect the polysaccharide content of LPS. This effect would appear to be independent of growth rate. It is not known if growth conditions cause phenotypic alterations in the chemical composition of the sugars present in the LPS of this strain. It is possible that such alterations would affect the reaction of LPS with oxidising agents and therefore the profile revealed by silver staining. This is an unlikely explanation since the profile revealed by silver staining and immunoblotting were similar.

Growth phase and the nature of the limiting nutrient did not appear to affect the antigenic determinants present in LPS since IgG in the patient's own serum reacted with LPS regardless of the culture conditions. However, McCallus and Norcross (1987) reported differences in the reaction of antisera with the 0-antigen of LPS from 3 hour and 9 hour cultures of a heterologous strain of E. coli. Furthermore, LPS from E. coli grown for 19 hours was twice as lethal as LPS from cells grown for 3 hours. LPS is an important virulence factor of P. aeruginosa (Cryz

et al, 1984) so that phenotypically induced alterations in the content of LPS may alter the toxicity of P. aeruginosa *in vivo*.

The results of an earlier investigation indicated that sera from CF patients whose lungs were colonised with P. aeruginosa contained only low titres of antibody to P. aeruginosa LPS (Hancock *et al*, 1984). However, these investigators used an ELISA technique and the LPS antigen was prepared from a laboratory strain. The antibody combining sites of the laboratory strain may differ markedly from that expressed by P. aeruginosa isolates from CF lung infection. Results of another study in which an ELISA method was also used indicated that sera from infected patients contained high titres of IgG to P. aeruginosa LPS (Fick *et al*, 1986). The immunoblotting techniques used in this study indicate that serum from a patient whose lungs were colonised with P. aeruginosa reacted with LPS. Evidence is also presented for the continued expression of LPS 0-antigen during growth *in vivo* in the CF lung. Continued stimulation of the immune system by LPS components may lead to an ongoing immune response to this antigen; indeed LPS has been isolated from circulating immune complexes obtained from the sera of CF patients (Moss and Hsu, 1982; Berdischewsky *et al*, 1980).

KDO has been used to estimate P. aeruginosa core LPS (Miguez *et al*, 1986; Gilbert and Brown, 1978) as the number of KDO units per molecule of LPS is relatively constant. Disadvantages associated with this technique include its non-specificity and the likelihood of interference by other bacterial cell components. Recently, Tsai (1986) reported that SDS-PAGE can be used as a sensitive technique for quantifying endotoxin contamination of meningococcal polysaccharide vaccine preparation. This technique was not used in this present study as whole cell digests stained more intensely than extracted LPS. Instead the hydroxy fatty acids, which are unique to the lipid A component of Gram-negative bacteria, were used as an independent measure of LPS.

GLC analysis indicated that LPS prepared from the isolate used in this study

contained all the fatty acids which are commonly present in strains of P. aeruginosa namely 3OH–$C_{10:0}$, $C_{12:0}$, 2OH-$C_{10:0}$, 3OH-$C_{12:0}$ and $C_{16:0}$ (Wilkinson, 1983; Kropinski *et al*, 1982; Wilkinson and Galbraith, 1975). The relative concentrations of each of these fatty acids in the LPS from iron-plentiful and iron-depleted early stationary phase cultures were similar. Wilkinson and Galbraith (1975) reported that fatty acids contributed 12.6-24.9% by weight of acid hydrolysed LPS from a range of P. aeruginosa strains representing 7 different serotypes. The amide linked 3OH-$C_{12:0}$ may not have been completely released by the hydrolysis conditions used in this study which may account for the rather lower value (10% by weight) obtained. However, these values cannot be directly compared as different media were used in these studies and also Wilkinson and Galbraith (1975) extracted LPS from 16 hour cultures so that growth phase was not clearly defined.

Logarithmic phase cells had a much lower content of fatty acids as compared to stationary phase cells. The total fatty acid content and the relative concentration of individual fatty acids were also dependent on the nature of the limiting nutrient. Early stationary phase magnesium-depleted cells contained 80% more hydroxy fatty acids than iron-depleted cells. The latter in turn contained around 28% more hydroxy fatty acids than cells grown in iron-plentiful media. The ratio of hydroxy fatty acids:total cell protein demonstrated the marked effect of growth rate and specific nutrient limitation on this important surface antigen. An increase in the LPS content of the OM has been associated with an increased rigidity of the membrane (Nikado and Vaara, 1985) and may alter the permeability of the cell. Mg^{2+} ions play an important role in stabilising the OM of P. aeruginosa. It is suggested that an increase in LPS production by magnesium- (and possibly iron-) depleted cells may compensate in part for this change in the membrane properties.

The cell envelope plays a crucial role in the resistance of the bacterium to antibiotics (Brown and Williams, 1985b; Lambert, 1984; Brown, 1977; Brown, 1975). Changes in the envelope structure and cation content of magnesium-limited

cells from chemostat and batch cultures have been associated with increased resistance to polymyxin and EDTA (Boggis, *et al*, 1979b; Brown and Melling, 1969a; 1969b). It has been suggested that polymyxin acts initially by binding to components of lipid A in the OM (Vaara and Vaara, 1983). The results presented in this study would suggest that iron- and/or magnesium-depleted cells may contain more lipid A and therefore possibly more polymyxin binding sites. However, it has also been reported that low-level impermeability-type aminioglycoside resistance in some strains of P. aeruginosa may result from conversion of smooth LPS to superficial or deeper rough LPS phenotypes (Bryan *et al*, 1984). These workers suggested that the repeating side chains of LPS are necessary for direct binding of the aminoglycoside or for providing a suitable radius of hydrophilicity in relationship to OM pores to enhance access of aminogylcosides to that route through the OM. Magnesium- and iron-depleted P. aeruginosa would therefore be expected to be more sensitive to aminoglycosides than cells grown in complete media. Other alterations in the OM, including the induction of protein H1, which has been shown to occur in magnesium and/or iron-depleted cells of P. aeruginosa (section 3.3), may negate the effect of phenotypically induced alterations in P. aeruginosa LPS.

Phenotypically induced alterations in the cell membrane will also affect the susceptibility of the cell to phagocytosis and serum killing (Anwar *et al*, 1983c; Taylor *et al*, 1981; Finch and Brown, 1978). It is generally accepted that the polysaccharide portion of LPS is responsible for activation of complement by the alternative pathway whereas lipid A activates complement by the classical pathway in the absence or presence of antibody to LPS (Joiner *et al*, 1984; Bjornson and, Michael, 1974). Differences not only in the length but also in the structure of the 0-side chain of LPS affect complement activation (Grossman and Leive, 1984; Taylor, 1983). Strains of P. aeruginosa which express an 0-antigen generally resist serum killing whereas strains which lack 0-antigen are serum sensitive (Pitt *et al*, 1986; Hancock *et al*, 1983). Miguez *et al* (1986) reported that phenotypically induced alterations in P. aeruginosa surface protein and LPS lead to variations in the

hydrophobicity of the cells. Surface hydrophobicity plays a critical role in the interaction of cells with PMNs (van Oss, 1978; van Oss *et al*, 1975) and hence their ability to evade host defence mechanisms.

The results of this study clearly indicated that the LPS content of P. aeruginosa altered continuously throughout the period of growth and that specific nutrient limitation also lead to phenotypic alterations in LPS. This has some very important implications and possible applications. The environmental conditions in the infected host are very different from those in which pathogenic bacteria are grown in the laboratory (Brown and Williams, 1985a). The results obtained in this study with bacteria recovered directly from an experimental infection and also from other studies (Brown and Williams, 1985a) provide evidence that bacteria grow under iron-restricted conditions in the body. There is also some evidence to suggest that magnesium may also be limiting in some infections (Green *et al*, 1985; Broughton *et al*, 1968). The results of this study indicated that both iron- and magnesium-depletion have a marked effect on the LPS content of P. aeruginosa. Further work should be aimed at elucidation of the structure and chemical composition of LPS prepared from cells recovered directly and without subculture from *in vivo*. This would provide valuable information which would allow optimisation of culture conditions for growth of bacteria for vaccine production and antigen preparation. The effect not only of nutrient limitation but also growth rate must be considered.

4. CONCLUDING REMARKS

Lung infection with Pseudomonas aeruginosa remains the major cause of morbidity and mortality among patients with CF. Whilst the immune response in sera from CF patients to P. aeruginosa has been extensively studied only a few studies have considered the antibody response in the lung and in all these latter studies the antigens involved in antibody formation were not characterised. Furthermore, with only one exception (Anwar *et al*, 1984) the antigens used in these immunological studies were prepared from cells grown *in vitro* in common laboratory media, and therefore may have been incomplete with regard to antigenic determinants expressed *in vivo*.

The immune response to infection may be studied in animal models using conditions which mimic as far as possible the actual infection *in vivo* (Hambleton and Melling, 1983). The results of this study have provided direct biochemical evidence that the conditions of iron-restriction which occur in the lungs of CF patients are mimicked in this animal model of chronic lung infection; mucoid P. aeruginosa recovered directly and without subculture from the lungs of infected rats expressed IRMPs and the pattern of these IRMPs was similar to that expressed in the OM of another mucoid strain recovered directly and without subculture from the sputum of a CF patient. The need to acquire iron before proliferation can occur *in vivo* makes possession of IRMPs an important virulence factor. These IRMPs induced *in vivo* or *in vitro* under iron-depleted conditions were recognised by antibodies present in the lungs and sera from infected rats. Major differences were noted in the predominant classes of Igs present in the lungs and sera from infected rats and the results suggest that there is some local synthesis of antibody in the lung.

Evidence is also presented to suggest that growth *in vivo* in the lung affects the production of protein H1 in the OM of P. aeruginosa, and this protein was recognised by antibodies present in sera from a CF patient with P. aeruginosa lung infection and also from patients with otitis externa and urinary tract infection caused by P. aeruginosa. Induction of protein H1 in the OM of some strains of

P. aeruginosa has been correlated with their increased resistance to polymyxin and aminoglycoside antibiotics (Nicas and Hancock, 1980) so that *in vivo*-grown P. aeruginosa may be much more resistant to the action of these antimicrobial agents. Interestingly, an antibody response was detected in sera from experimentally infected rats to a 32K OM protein. Reversion of P. aeruginosa strain which was hypersusceptible to carbenicillin to a phenotype with normal sensitivity was associated with the loss of a 32K OM protein (Irvin *et al*, 1981). Evidence concerning the surface components of P. aeruginosa expressed during growth *in vivo* will be useful in designing *in vitro* test conditions for studying the antibiotic susceptibility of P. aeruginosa and other pathogenic bacteria.

Antibodies against P. aeruginosa IRMPs may play an important role in host defence (Griffiths *et al*, 1985; 1983). Antibodies to P. aeruginosa IRMPs and OM protein H1/H2 reacted with these antigens in the OM of other serotype strains of P. aeruginosa. These proteins seem obvious candidates for vaccine development. There was also some evidence to indicate that antibodies to the H proteins (probably protein H2) cross-react with an antigen of similar molecular weight in the OM of an index of strains of Enterobacteriaceae. It may be fruitful to explore the possibility that passive administration of antibodies to this antigen or active immunisation with purified antigens may afford protection against other Gram-negative bacteria. Escherichia coli, Klebsiella, Enterobacter and Serratia spp and P. aeruginosa are the organisms most frequently assoicated with mortality in bacteraemia arising from acute nosocomial pneumonia (Bryan and Reynolds, 1984). The results of studies in which rats were immunised intraperitoneally with P. aeruginosa indicate that transudation of IgG antibodies from the serum to the lung occurs, so that the intravenous route of vaccination might provide satisfactory titres of antibody in the lung. However, it ssems likely that immunisation schedules must be developed which induce the development of the appropriate classes of immunoglobulin at the alveolar surface.

LPS has probably received the most attention as a protective surface antigen

of P. aeruginosa and the results of this study have indicated that LPS was the major surface antigen recognised by antibodies in sera from infected rats.

The results presented in this thesis indicate that growth phase and specific nutrient limitation affect the LPS produced by P. aeruginosa. This might have important implications for antigen production *in vitro*. Growth conditions and media could be optimised to allow the most efficient production of the most appropriate LPS antigens. The effect of growth rate on LPS production might also be considered.

Overall the results of this study have indicated that the lung environment has a profound effect on the surface antigens of P. aeruginosa and these results may have some important implications and applications in the field of vaccine development and antibiotic sensitivity testing. The question remaining is what are the more immediate implications for the treatment of CF patients? Clearly, vaccination of infected CF patients would not be considered as increasing antibody titres might lead to further tissue damage due to immune complex formation. However, the immunoblotting and ELISA technique used in this study could be used in serodiagnosis of P. aeruginosa lung infection. A positive result could be taken as an indicator for treatment with anti-pseudomonal antibiotics. Also this animal model could be used to assess the efficacy of antibiotic therapy in chronic lung infection.

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