IRON-REGULATED SURFACE ANTIGENS OF *PSEUDOMONAS AERUGINOSA*

By

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SUMMARY

The iron uptake systems of pathogenic bacteria provide potential targets for immunological intervention. Essential components of the iron-uptake system of *Pseudomonas aeruginosa* and other Gram-negative bacteria are the high molecular weight iron-regulated membrane proteins (IRMPs) which are expressed in the outer membrane (OM), and which function as receptors for siderophore-iron complexes. Antibodies which bind to IRMPs in the intact bacterial cell will possibly block iron uptake and therefore exert a bacteriostatic effect upon the invading organism.

In order to study the potential of anti-IRMP antibodies as immunotherapeutic agents, the production of specific antibodies was required. Selection of antibodies specific for the protein components of Gram-negative bacteria has, however, been severely restricted due to the response to the immunodominant molecule lipopolysaccharide (LPS). To circumvent the response to LPS, a purification strategy was developed to minimise the LPS contamination in the preparation for immunisation. The strategy involved selection of a rough mutant of *P. aeruginosa*, selective solubilisation of IRMPs and gel-filtration or ion-exchange chromatographic separation. Additionally, a purification technique involving excision of these antigens from SDS-PAGE gels was applied. Polyclonal and monoclonal antibodies reacting with outer membranes and whole bacterial cells were generated. Monoclonal antibodies which reacted specifically with the IRMPs and protein D were selected, and these antibodies were used in the study of the complexity and expression of IRMPs. Investigation of the surface exposure of these antigens determined using immunofluorescence and immunogold labelling techniques indicated that the IRMPs and protein D were surface exposed immunogens, which were more accessible to antibody in a rough strain of *P. aeruginosa.* Polyclonal and monoclonal antibodies to these surface exposed antigens were found to be cross-reactive with OM antigens in other bacterial species and with antigenic determinants expressed by representative strains of the seventeen serotypes of *P. aeruginosa.* A selection of the monoclonal antibodies generated were effective at agglutinating whole bacterial cells.

IRMPs were confirmed in immunoblotting assays to be important immunogens, as they were expressed *in vivo* and recognised early by the host in both pleural cavity and *Otitis externa* infections.

The protective activities of polyclonal and monoclonal antibodies to IRMPs were evaluated in an experimental intraperitoneal challenge model. Polyclonal antibodies were found to provide significant protection against challenge with a homologous strain of *P. aeruginosa.*

Key words: *P. aeruginosa*, iron, outer membrane proteins, lipopolysaccharide, vaccines.

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Tables.

Abbreviations.

ATCC	American Type Culture Collection
CDM+Fe	chemically defined medium with added iron
CDM-Fe	chemically defined medium without added iron
cfu	colony forming unit
CM	cytoplasmic membrane
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FBP	ferri-pyochelin binding protein
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FPLC	Fast Protein/Polypeptide/Polynucleotide Liquid Chromatography
HRP	horse radish peroxidase
IATS	International Antigenic Typing Scheme
IRMP	iron-regulated membrane protein
KDO	3-deoxy-D-*manno*-2-octulonate
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
µl	microlitre
MOPS	morpholinopropane sulphonic acid
NCIB	National Collection of Industrial Bacteria
NCTC	National Collection of Type Cultures
OM	outer membrane
OMs Fe-	outer membranes extracted from bacteria grown in CDM-Fe.
PG	peptidoglycan
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	tris buffered saline
TBS/Tween	tris buffered saline with added Tween 20 (0.3%)
TEMED	N,N,N',N'- tetramethylethylene diamine
TMB	3,3',5,5',-tetramethylbenzidine
Tris	tris (hydroxymethyl) amino ethane
TSA	tryptone soy agar
TSB	tryptone soy broth
v/v	volume by volume
w/v	weight by volume

1 Introduction.

1.1 *P. aeruginosa* infections.

P. aeruginosa is an opportunist pathogen which generally persists and causes infection only when the defence mechanisms of the host are compromised or suppressed (Bodey, 1985; Bodey *et al*, 1983; Froland, 1981). Examples of *P. aeruginosa* infection include colonisation of the bladder after catheterisation, *Otitis externa* in deep-sea divers (Alcock, 1977), endocarditis in drug-addicts, infections of surgical wounds and burns (McManus *et al*, 1985; Pruitt *et al*, 1984) and pulmonary infections in patients with cystic fibrosis (Govan and Harris, 1986; Ramphal and Vishwanath, 1987). Additionally, patients undergoing intensive chemotherapy regimes which employ immuno-suppressive drugs are at high risk of becoming infected with *P. aeruginosa.* Infection is a serious problem due to the high patient mortality caused by the frequent occurrence of organisms which have an inherent resistance to the effect of many antimicrobial agents (Brown, 1977; Hancock, 1986).

1.2 Host responses to infection.

Protection against infection with this bacterial pathogen is largely dependent on phagocytosis and humoral immunity ie. the presence of circulating, specific antibodies (Bjornson and Michael, 1970). These antibodies can function either as opsonins (alone or in conjunction with complement) for the promotion of phagocytosis of the invading organism, or as bactericidal antibodies in the presence of complement (without phagocytes).

1.3 Immunological control of *P. aeruginosa* infections.

Therapy for *P. aeruginosa* infection is hindered by the antibiotic resistance intrinsic to the organism (Brown, 1977; Hancock, 1986). Recognition of the limitations of existing therapy against infection has stimulated urgent interest in both the molecular basis of the organisms pathogenicity, and in immunological approaches to prophylaxis and treatment. Active immunisation is thought to have potential in the control of certain *P. aeruginosa* infections. However it is limited by the time required to develop, and the host's ability to make specific protective antibodies. Passive immunisation with hyperimmune globulin may be critical in the management of certain patient populations (Cryz *et al*, 1983) as this could be used for high-risk patients, as well as for the treatment of established *P. aeruginosa* infection. Due to the multifactorial interaction between *P. aeruginosa* and its host, a number of experimental vaccines have been analysed for their abilities to elicit the production of protective antibodies (table 1.3.1).

Table 1.3.1. Experimental vaccines for *P. aeruginosa.*

LPS (Hanessian *et al*, 1971; Pennington, 1979).
Polysaccharide (Pier *et al*, 1983).
Polysaccharide-protein conjugates (Seid and Sadoff, 1981; Tsay and Collins, 1984).
Alginic acid (Pier *et al*, 1978; Woods and Bryan, 1983).
'Slime' polysaccharide (Alms and Bass, 1967).
'Slime' glycolipid (Sensakovic and Bartel, 1977).
Flagella (Drake and Montie, 1987; Holder *et al*, 1982).
Exotoxin A (Pavlovskis *et al*, 1977).
Elastase (Cryz *et al*, 1983).
Ribosomes (Lieberman and Ayala, 1983).
Lectin (Sudakevitz and Gilboa-Garber, 1987)
Polyvalent extract vaccine (Miler *et al*, 1977).
Aqueous cellular protein (Joo *et al*, 1982).
Temperature-sensitive mutants (Hooke *et al*, 1982).
Outer membrane proteins (Mutharia *et al*, 1982; Matthews-Greer and Gilleland, 1987; Gilleland *et al*, 1984).

Adapted from Lieberman (1985).

Studies indicating the protective activities of exotoxin A (Cryz *et al*, 1983; Pavlovskis *et al*, 1977), elastase (Cryz *et al*, 1983), ribosomes (Lieberman, 1983) and O-polysaccharide-toxin A conjugates (Cryz *et al*, 1987a; 1987b) have been reported. Certain of these experimental vaccines show potential. However, as the capsule, OM proteins and LPSs are the primary antigenic determinants of Gram-negative bacterial pathogens (Costerton *et al*, 1979), these cell envelope components have been investigated in detail for their potential to act as protective vaccines. Surface properties of bacteria are important in pathogenesis, being implicated in the host-bacteria interactions which determine the course of an infection. A pathogen is able to adapt it's surface in response to the *in vivo* situation of infection (Brown and Williams, 1985; Ellwood and Tempest, 1972; Tempest and Ellwood, 1969); the balance of competition between host and invader depends to a great extent on this ability to adapt. Many aspects of the environment can cause alterations in the cell surface of a pathogen and consequently modify the antigenicity of the organism (Brown and Williams, 1985; Dalhoff, 1985; Holme, 1972; Kadurugamura *et al*, 1988; Gilleland, 1988). Vaccines are developed to raise specific antibodies that will react with the pathogens concerned *in vivo*; it is therefore essential that the antigens involved are both expressed by the invader *in vivo*, and accessible to antibody. For these reasons, when designing vaccines it is important to investigate the cell surface of a pathogen, as it is in each infection situation. Brown *et al* (1988) reviewed the importance of antigens expressed and surface exposed *in vivo* in vaccine development, and the potential of such immunogens to act an effective vaccines.

The conditions found in host tissues are not only poorly defined but also likely to change as the infection proceeds. In general, little is known about the alterations that occur in pathogenic bacteria as they adapt to and multiply *in vivo,* however recent evidence has been acquired elucidating bacterial cell surface properties as they are expressed *in vivo*. A brief description of the cell envelope of Gram-negative bacteria follows, to form a basis of a discussion about the factors of the *in vivo* situation of infection that have dramatic

effects on the expression of bacterial OM antigens, and the potential of certain key antigens to act as protective immunogens. The description of the cell envelope is approached with emphasis on the antigenicity of OM components and, additionally, the factors affecting purification of OM proteins from bacterial cells, including LPS-OM protein interactions.

1.4 Composition of Gram-negative bacterial envelopes.

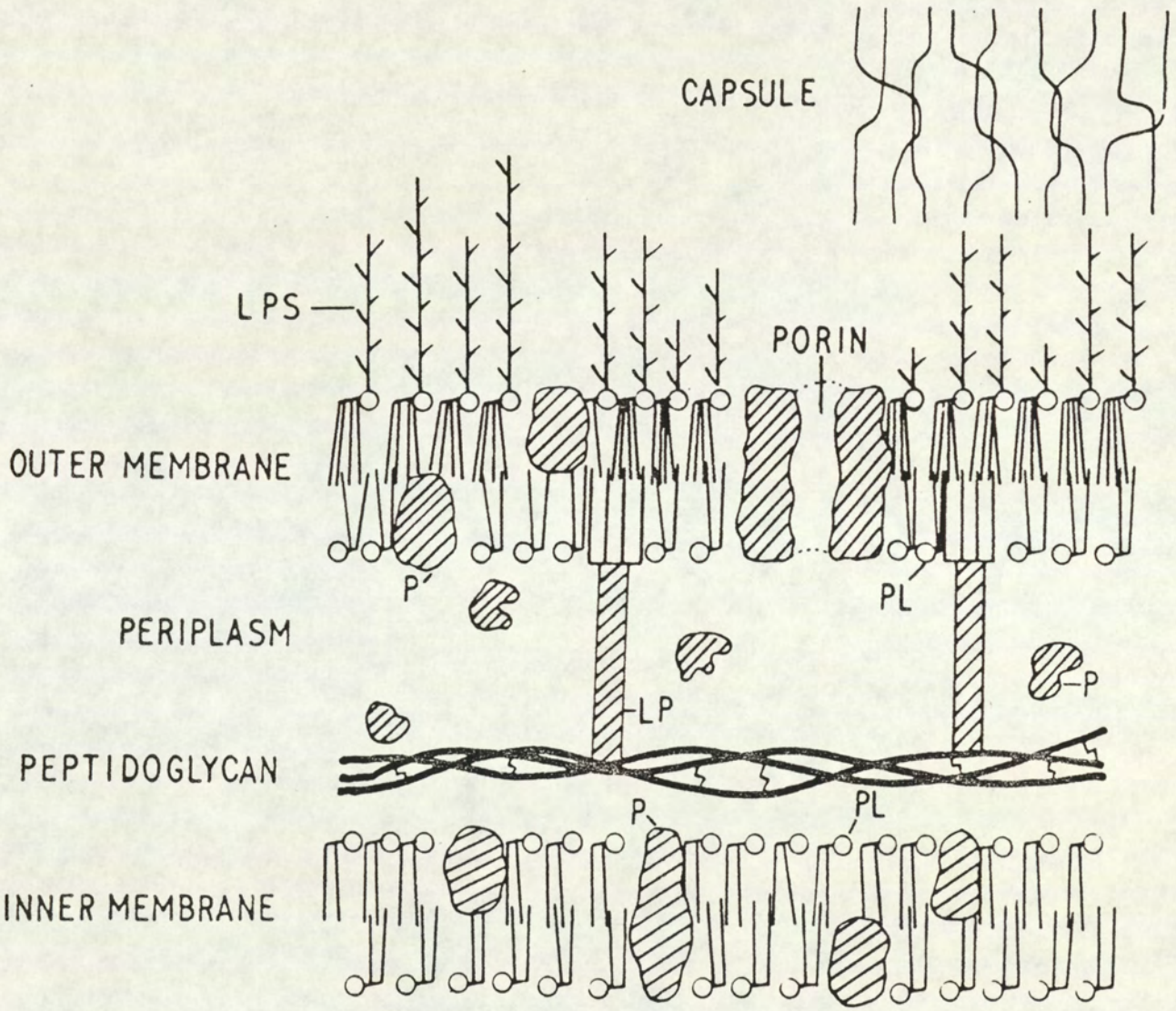
The OM consists of a mosaic of LPSs, OM proteins and phospholipids (PLs) and lies external to the peptidoglycan (PG) layer, to which it is covalently attached via a lipoprotein (Braun, 1975; Lugtenberg and van Alphen, 1983). Interior to the PG layer is the periplasmic space and the cytoplasmic membrane (CM); exterior to the OM lies the capsule. A schematic representation of the cell envelope is shown in fig. 1.4.1. The two OM components most relevant to this study are LPSs and OM proteins; these are discussed in more detail in sections 1.5.1 and 1.6.1.

1.5 Lipopolysaccharides as protective antigens.

1.5.1 Lipopolysaccharide structure.

LPSs are complex macromolecules, characteristic of the outer surface of Gram-negative bacteria, that comprise three genetically, biochemically and antigenically distinct domains referred as the O-antigen, core oligosaccharide and lipid A (fig. 1.5.1.1). The O-antigen and the core oligosaccharide form the hydrophilic portion of these amphipathic molecules. The O-antigenic moiety consists of repeating sugar units, the structure of the sub-units being extremely variable (Hitchcock and Brown, 1983); this property is the basis of the immunological typing of serotypes (Lanyi and Bergan, 1978). The O-specific side chains in *P. aeruginosa* are rich in rhamnose and the amino sugars hexosamine, fucosamine and quivosamine (Wilkinson, 1983). A morphological smooth to rough

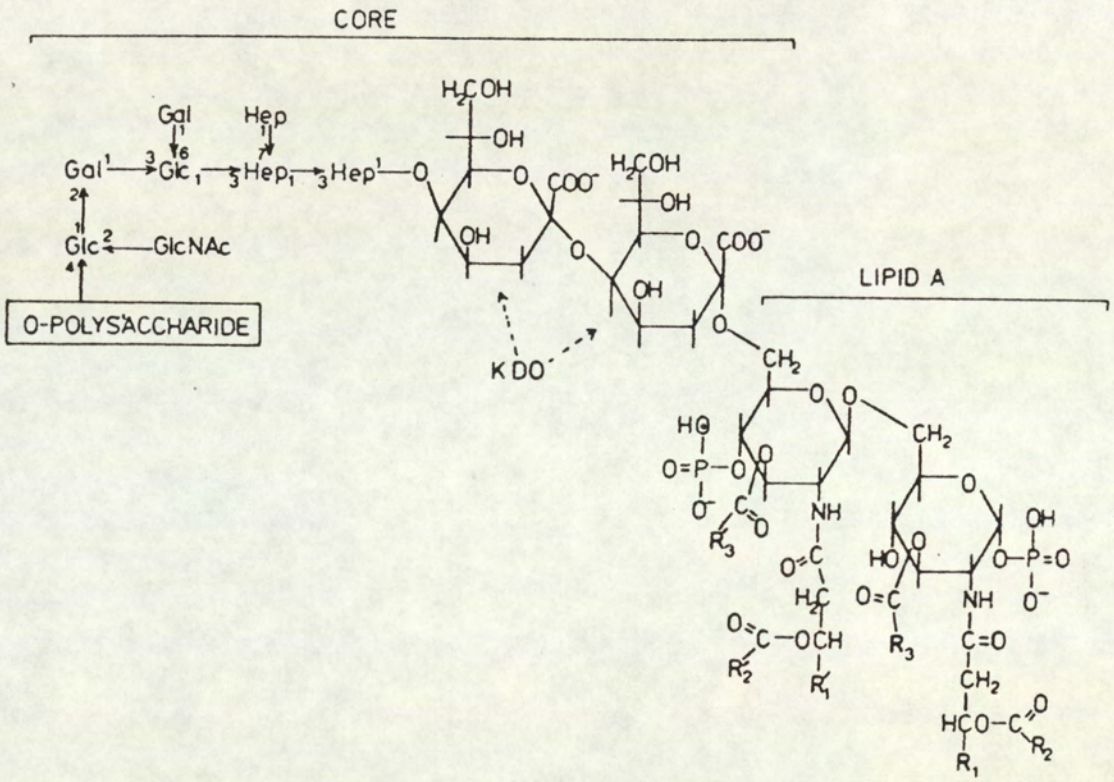
Fig. 1.4.1. Schematic representation of the OM of Gram-negative bacteria.



LPS	Lipopolysaccharide
P	Protein
PL	Phospholipid
LP	Lipoprotein

From Sleytr et al(1988).

Fig. 1.5.1. Schematic representation of bacterial lipopolysaccharide.



This is a composite structure showing features from several types of LPS.

KDO	3-deoxy-D-*manno*-2-octulosonate
Hep	D- or L-glycero-D-*manno*-heptose
Gal	D-galactose
Glc	D-glucose
GlcNac	N-acetyl--D-glucosamine

From Sleytr *et al* (1988).

mutation coincides with the disappearance of O-antigen leaving rough LPS consisting only of core oligosaccharide bound to lipid A (Westphal *et al*, 1983). Core polysaccharide has an inner and an outer region; the inner region consists of heptose and 3-deoxy-D-*manno*-2-octulonate (KDO) and the outer core is made up of glucose, galactose and in some cases N-acetyl glucosamine. KDO links the core polysaccharide to lipid A *via* an acid labile bond (Wicken and Knox, 1980; Wilkinson and Galbraith, 1975): the hydrophobic lipid A portion can easily be separated from isolated LPS by mild acid hydrolysis.

Lipid A regions of the LPSs expressed by different species of Gram-negative bacteria share a structural similarity: phosphatidyl glucosamine residues forming disaccharides which are attached by ester and amide bonds to fatty acids (Westphal *et al*, 1983). The absence of 3-hydroxytetradeanoic acid from lipid A, and the presence of 2-hydroxydodecanoic acid, 3-hydroxydecanoic and 3-hydroxydodecanoic are characteristic of *P. aeruginosa* (Wilkinson and Galbraith, 1975). The lipid A moiety is responsible for the endotoxic properties of LPS (Pennington, 1979; Wicken and Knox, 1980).

P. aeruginosa core LPS is highly phosphorylated (Wilkinson and Galbraith, 1975). Phosphate groups in the KDO-lipid A region of the LPS have a high affinity for divalent cations (Mg^{++} and Ca^{++}) which form cross-bridges that are important in the structural organisation of the OM. A substantial proportion of the LPS molecules in the OM form tight complexes with the OM proteins (van Alphen *et al*, 1978). The formation of these complexes has also been shown to require divalent cations, notably magnesium (Nakamura and Mizushima, 1976; van Alphen *et al*, 1978). Chelation of divalent cations by EDTA removes stabilising cross-bridges. OM constituents are tightly associated (probably without the benefit of covalent bonds) resulting in an impervious barrier capable of excluding many noxious substances.

1.5.2 Lipopolysaccharide-based vaccines.

LPS is an important, immunodominant antigen presented on the surface of *P. aeruginosa*. Investigators studying experimental vaccines for *P. aeruginosa* (as well as vaccines for other Gram-negative bacteria for human use) have been forced to contend with the LPS component of the cell envelope in either one of two ways:

1. To utilise LPS as a vaccine for its effective immunogenicity while attempting to control its relative toxicity.

2. To attempt to exclude LPS from the vaccine and demonstrate the efficacy of another antigen.

Due to the importance of LPS as a virulence factor (Cryz *et al*, 1984b) and as a major protective antigen in pseudomonal extract vaccine (MacIntyre *et al*, 1986a; 1986b), many studies have been carried out resulting in the production of antibodies specific for LPS. Hancock *et al*, (1982) produced monoclonal antibodies against both the O-antigen and rough core regions of LPS of strain PAO1, while Barclay *et al*, (1986) reported the production of eighteen monoclonal antibodies to the protective LPS antigens of IATS 01 strains. Sawada *et al*, (1984; 1985a; 1987) produced both mouse and human monoclonal antibodies against some serotypes of *P. aeruginosa*. Additionally, Sadoff *et al*, (1985) reported the production and characterisation of both type-specific monoclonal antibodies and a panel of eleven monoclonal antibodies which exhibited broad cross-reactivity with Fisher immunotype strains and IATS serotype strains.

The most widely used vaccines to date have relied on LPS components to act as protective antigens (Homma, 1982; Hanessian *et al*, 1971; Pennington, 1979). Antibodies directed to the LPS components of Gram-negative bacteria have been found to be protective

(Pollack and Young, 1979; Cryz *et al*, 1983; 1984a). Additionally, Stoll *et al* (1986) produced a monoclonal antibody that protected against challenge with live organisms in a murine burn infection model. This activity, in addition to the ability of the antibody to agglutinate and opsonise bacteria was found to be immunotype-specific. Correlation of the opsonic and protective properties of the monoclonal antibody with its recognition site on the LPS side chain confirmed that such immunotype-specific determinants are important targets for protective antibodies in *Pseudomonas* infection.

Other examples of the protective activities of anti-LPS antibodies include the antibodies described by Colwell *et al*, (1984) specific for *Salmonella typhimurium* O-polysaccharide, which protected mice against challenge with virulent organisms. In addition, Sawada *et al* (1985b) characterised a human monoclonal antibody directed to LPS of *P. aeruginosa* serotype 5, which demonstrated protective activity against infection with this organism and Zweerink *et al* (1988) demonstrated that monoclonal antibodies secreted by cell lines RM5, FDD7 11FD and 9H10 (anti-LPS antibodies) protected neutropenic mice prophylactically against challenge with *P. aeruginosa.*

Antibodies directed to LPS antigenic determinants are important mediators of protection, however the application in humans of vaccines containing LPS as a major component is not acceptable, because of the adverse reactions attributed to the toxic lipid A moiety (Pennington, 1979; Wicken and Knox, 1980), as well as the requirement for combining numerous monovalent serotype antigens into one multivalent vaccine to be effective against the various immunotype strains of *P. aeruginosa* (Homma, 1982; Lanyi and Bergan, 1978).

One approach to circumvent the toxicity problem is to isolate the high-molecular weight polysaccharide moiety of LPS (Pier, 1983). This has been shown to induce immunotype-specific antibodies (Pier *et al*, 1978). A further improvement of

polysaccharide vaccines can be achieved by conjugation of the O-polysaccharide to proteins in order to include helper T-lymphocytes in the activation process of B-lymphocytes (Seid and Sadoff, 1981). This will give rise to immunological memory that does not otherwise occur after immunisation with polysaccharide antigen alone.

A semi-synthetic vaccine against *P. aeruginosa* immunotype 3 was reported to induce high levels of IgG antibodies (van de Weil, 1987). Additionally, Tsay and Collins, (1984) prepared non-toxic polysaccharide-protein conjugates by acid treatment of LPS, oxidation of the low molecular weight polysaccharide fraction and covalent coupling to bovine serum albumin. The conjugate was 4,000-fold less active than native LPS in the *limulus* amoebocyte lysate assay, and was capable of producing active protection against a challenge of *P. aeruginosa* that killed 90% of control mice. In addition, immunisation with the conjugate induced humoral IgG that passively protected normal and burned mice. The study indicated that the conjugation of non-immunogenic polysaccharide antigen of *P. aeruginosa* restored immunogenicity similar to that of native LPS, without restoring the endotoxicity inherent in LPS.

Polysaccharide-based vaccines that do not exhibit endotoxic properties show potential to be used in the control of *P. aeruginosa* infections. It is thought, however, that a more effective cross-protective vaccine may be based on protein components of the cell envelope, possibly linked to the O-antigenic moiety of LPS which exhibits adjuvant activity. The potential of key OM proteins expressed *in vivo* to be included in immunising preparations is discussed in the following sections.

1.6 OM proteins as protective antigens.

1.6.1 OM proteins of *P. aeruginosa*.

Investigation of the protective potentials of antibodies specific for major OM proteins has been undertaken. OM proteins have been indicated as potential vaccine candidates as certain species are cross-reactive, important immunogens, abundant in the OM and located in a position where they are usually able to interact with antibodies. The OM proteins of *P. aeruginosa* have been characterised mainly according to their migration pattern following SDS-PAGE (see fig. 4.2.1). The nomenclature proposed by Mizuno and Kayeyama (1979) was modified by Hancock and Carey (1979) following improvements in the resolution of several proteins (particularly D and H). Five proteins (D1, D2, F, G and H1) are described as heat-modifiable since their electrophoretic mobilities differ depending on the temperature of denaturation (Hancock and Carey, 1979).

Protein F is the major porin of *P. aeruginosa* (Hancock and Carey, 1979) which is exposed on the cell surface (Lambert and Booth, 1982; Mutharia and Hancock, 1983; 1985a) and highly conserved in all serotype strains (Mutharia *et al*, 1982). Porins are trans-membrane proteins that form hydrophilic pores which allow passive non-specific diffusion of solutes across the membrane (Nakae, 1986; Nikaido and Vaara, 1985). They are characterised by their tight, but not covalent association with the PG layer and are resistant to denaturation by SDS at temperatures below 85-100^{o}C. They have a high content of ß-structure and exhibit an extremely strong self-association: *in vivo* they form trimers associated with LPS (Osborn and Wu, 1980; van Alphen *et al*, 1978). Protein I is a lipoprotein which has lipid substituted at the N-terminus; this protein is covalently bound at the C-terminus to PG. Protein D1 is a glucose-inducible pore-forming OM protein (Hancock and Carey, 1980). It is established that many OM proteins are only expressed under particular growth conditions; the expression of certain

iron-regulated outer membrane proteins (IRMPs) is discussed in section1.7.2.

1.6.2 OM protein-based vaccines.

OM proteins have been investigated for their potential to act as protective immunogens. For example, protection of mice against *P. aeruginosa* was obtained after administering a mixture of OM proteins (von Specht *et al*, 1987). The protective activities of individual proteins, for example, porin protein F (Gilleland *et al*, 1984), which was shown to be immunologically cross-reactive between the different serotype strains of *P. aeruginosa* (Mutharia *et al*, 1982) have also been investigated. Gilleland *et al* (1984) reported that a purified protein F preparation successfully protected mice after either passive or active immunisation in an acute infection model, from a subsequent challenge with a heterologous LPS immunotype strain of *P. aeruginosa.* These workers extended their studies to demonstrate that the protein F preparation afforded significant protection, above that provided by PAO1 LPS immunisation, against challenge with each of six heterologous LPS Fisher-Devlin immunotypes in a burned mouse model (Matthews-Greer and Gilleland, 1987). The immunotherapeutic potential of monoclonal antibodies to *P. aeruginosa* protein F has also been demonstrated (Hancock *et al*, 1985).

Recently, Duchene *et al*, (1989) reported the cloning and expression in *E. coli* of *P. aeruginosa* lipoprotein I. This protein is another potential vaccine candidate as it is also immunologically cross-reactive amongst the seventeen serotypes of *P. aeruginosa.* (Mutharia *et al*, 1982). Duchene *et al*, (1989) intend to use the recombinant product in studies investigating the immunogenic and protective capacities of this lipoprotein. Certain other OM proteins show potential to be included in immunising preparations-including the IRMPs which are induced in th OM of Gram-negative bacteria when they are grown in conditions of iron-depletion (Neilands, 1982). The potential of these important antigenic determinants to induce the production of protective antibodies is

discussed in the section 1.8.

1.7 The role of iron in infection and host defence.

1.7.1 The iron-acquisition system of *P. aeruginosa*.

As stated previously, the environment exerts a profound effect on the cell surface properties of Gram-negative bacteria (Ellwood and Tempest, 1972; Brown and Williams, 1985). Iron-limitation has been shown to be an important factor *in vivo* (Brown *et al*, 1984; Bullen *et al*, 1974; Finkelstein *et al*, 1983; Griffiths, 1983a; Weinberg, 1978): biological systems strive to maintain only an extremely low concentration of 'free' iron as ferric iron under physiological conditions tends to oxidise, hydrolyse and polymerise, forming essentially insoluble ferric hydroxide and oxyhydroxide polymers. The avidity of Fe^{3+} to polymerise is so great that the equilibrium concentration of 'free' ferric iron cannot exceed 10^{-18}M. The multiplicity of the mechanisms used by micro-organisms to obtain iron prove that this ion is important for their survival: it is an important element as it readily accepts or donates electrons and when complexed with various proteins, serves as a catalyst in biological processes requiring oxidation or reduction. It is predominantly located intracellularly within the body in ferritin, haemosiderin, haemoglobin or myoglobin; that which is extracellular in body fluids is attached to high affinity iron-binding proteins (Bezkorovainy, 1987). These proteins are transferrin in serum and lymph and lactoferrin in external secretions and milk (Aisen and Lieberman, 1972) and PMNLs. Transferrin is a glycoprotein with a single polypeptide chain bearing two iron-binding sites capable of reversibly binding one ferric ion each, with the simultaneous incorporation of two bicarbonate ions. Lactoferrin has a higher affinity for iron than transferrin and will bind at a lower pH (Ainsen and Lieberman, 1972).

These glycoproteins have association constants for iron of about 10^{36} and are normally

only partially saturated; eg. in humans the serum transferrin is usually only 30-40% saturated. This extremely effective biological chelating activity maintains the amount of free iron at approximately 10^{-18}M (Bullen *et al*, 1978; Weinberg, 1978; Griffiths, 1983a). Restricting the availability of iron to pathogens plays an important role in mammalian host defence strategies, a tactic termed nutritional immunity (Baynes *et al*, 1986; Bullen, 1981; Finkelstein *et al*, 1983; Griffiths, 1983a; Weinberg, 1978; 1984).

Pathogenic bacteria equipped with an iron-uptake system which sequesters iron more sucessfully than that of the host will have an advantage (Finkelstein *et al*, 1983), while host mechanisms designed to deprive bacteria of iron while retaining their own access to the element will convey an obvious advantage to the host. Since successful infection only occurs if a pathogen efficiently extracts and assimilates iron from the relatively guarded sources bound to the high affinity host proteins (Payne and Finklestein, 1978), pathogens must be able to adapt to the iron-restricted environment usually found *in vivo* and develop mechanisms for assimilating protein-bound iron, or for acquiring it from liberated haem. Under normal physiological conditions bacteria might be expected to assimilate glycoprotein-bound iron in several ways:

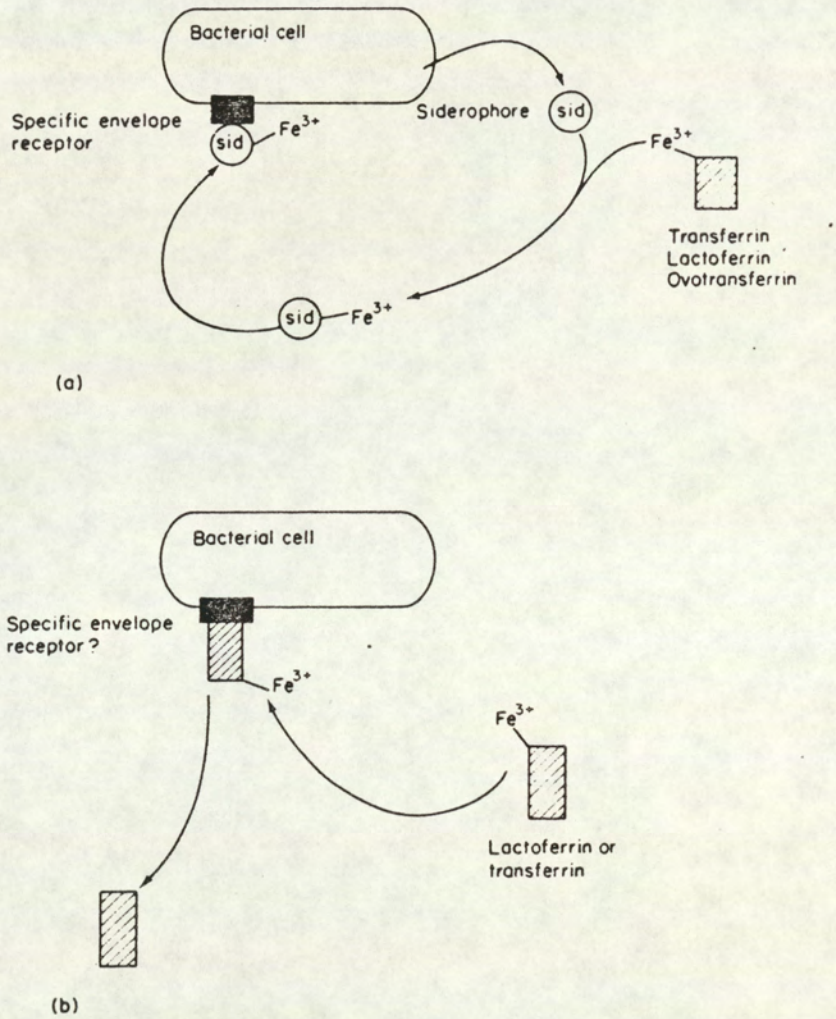
1. Through proteolytic cleavage of the iron-binding glycoproteins, disrupting the iron-binding sites to release iron.
2. By reduction of Fe^{3+} complexes and the consequent release of Fe^{2+} from the glycoproteins.
3. Through direct interaction between receptors on the bacterial cell surface and the Fe^{3+}-glycoprotein complexes.
4. By producing low molecular weight iron-chelating compounds, that are able to remove iron from Fe^{3+}-glycoprotein complexes and to deliver the iron to the bacterial cells.

Adapted from Griffiths (1987b).

Mechanisms 3 and 4 described above are represented in fig. 1.7.1.1. It has been demonstrated that one of the major ways by which *P. aeruginosa* reacts to iron starvation is the synthesis of iron-scavenging compounds, known as siderophores (Cox, 1985; Griffiths, 1983; Sriyosachati and Cox, 1986). These are iron-specific ligands whose biosynthesis is regulated by iron and which compete with host iron-binding proteins (Ankenbauer *et al*, 1985; Neilands, 1982). They scavenge for iron and convert the insoluble polymeric ferric oxyhydroxides into soluble chelates, or extract transferrin-bound iron (Sriyosachati and Cox, 1986): these soluble chelates are substrates for high-affinity bacterial transport mechanisms. A number of siderophores have been described, the majority of which are phenolic or hydroxamic acid compounds of molecular weights ranging between 5 and 10K.

Pseudomonas species are known to produce several siderophores: ferribactin, ferrioxamines, pyoverdin, pyochelin and pseudobactin (Cox, 1980; Cox and Graham, 1979; Liu and Shokrani, 1978; Yang and Leong, 1984). Of these, it is pyochelin and pyoverdin which are of most interest as regards infection. Pyoverdin has a binding constant for iron of 10^{32} (Wendenbaum *et al*, 1983), and has been shown to promote the growth of *Pseudomonas* in the presence of transferrin (Cox and Adams, 1985). Pyochelin has dramatic effects on the virulence of *Pseudomonas* (Cox, 1982): *P. aeruginosa* mutants deficient in the synthesis of pyochelin and pyoverdin, or both siderophores have been used to demonstrate that pyochelin is the siderophore responsible for the efficient capture of iron bound to transferrin enabling bacteria to grow in human serum (Aukenbauer *et al,* 1985). Pyochelin is a phenolate compound whose structure is known (Cox *et al* 1981). Pyoverdin contains a chromophore (2,3-diamino-6,7-dihydroxyquinoline) bound to a peptide chain (Wendenbaum *et al*, 1983). The chromophore contains the iron-binding site and has both hydroxamate and phenolate functions. Pyocyanin, the blue pigment characteristic of many *P. aeruginosa* isolates has been found to take part in the removal of transferrin-bound iron by a reduction mechanism. The structures of the siderophores

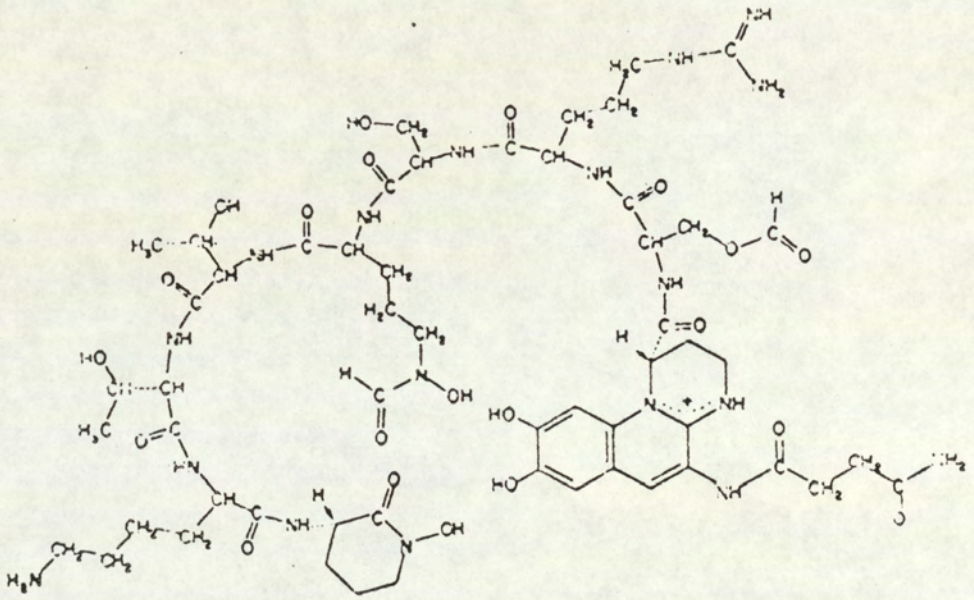
Fig. 1.7.1.1. Schematic representation of two ways by which pathogenic bacteria obtain iron from iron-binding proteins.



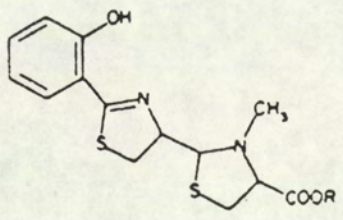
a siderophore-mediated iron uptake
b iron uptake by direct interaction with host iron-binding glycoproteins.

From Griffiths (1987).

Fig. 1.7.1.2. Structural formulae of two siderophores from *P. aeruginosa* .



1



2

1. Pyoverdin
2. Pyochelin

From Griffiths (1987)

pyochelin and pyoverdin are shown in fig. 1.7.1.2.

1.7.2 Production of iron-regulated membrane proteins by bacteria.

There have been several reports of the production of microbial envelope proteins related to iron (Anwar *et al*, 1984; 1985; Cochrane *et al*, 1987; 1988; Klebba *et al*, 1982; Mietzner *et al*, 1984; Neilands, 1982; Shand *et al*, 1985; Ward *et al*, 1988). These bacterial OM proteins have been implicated in acting as receptors for iron-siderophore complexes (Cody and Gross, 1987; Cornelis *et al*, 1989; Chart and Griffiths, 1985; Icihara and Mizushima, 1978; Pierce and Earhart, 1986; Sokol and Woods, 1983; 1984). For example, Sokol and Woods (1983; 1984), demonstrated the presence of an iron-siderophore-binding protein in the OM of *P. aeruginosa*. Autoradiographic analysis of SDS-polyacrylamide gel electrophretograms of OM preparations previously incubated with ^{59}Fe pyochelin indicated that iron-siderophore complexes bound to a low-molecular-weight protein of approximately 14K, termed ferripyochelin binding protein (FBP). Genetic evidence demonstrating the involvement of FBP in ferripyochelin uptake was obtained by Sokol (1987) who showed using Tn5 insertion mutants of *P. aeruginosa* PAO that these organisms were both deficient in the surface expression of FBP and also unable to take up ^{59}Fe-pyochelin. Evidence presented by Pierce and Earhart, (1986) demonstrated that *E. coli* K-12 envelope proteins were specifically required for ferrienterobactin uptake. Additionally, Cody and Gross (1987) demonstrated that a 74K IRMP from *P. syringae* pv. *syringae* was involved in iron uptake from pyoverdin pss., and Magazin *et al* (1986) genetically demonstrated that an 85K protein functioned as the receptor for the pyoverdin siderophore, called pseudobactin, from the fluorescent *Pseudomonas* strain B10.

1.8 Potential of IRMPs as vaccines.

Vaccines are developed to combat infectious diseases by raising specific antibody that will react with and neutralise the virulence determinants of the pathogens concerned. This implies that the virulence determinants must be expressed by the pathogen *in vivo* for the vaccines to be effective.

An analysis of the evidence indicating that IRMPs are surface-exposed, important antigens expressed *in vivo*, which might offer cross-protection against infection with *P. aeruginosa* is presented in the following sections.

1.8.1 Recognition of IRMPs *in vivo*.

Demonstrations indicating that IRMPs are recognised *in vivo* have been reported (Anwar *et al*, 1984; 1985; Black *et al*, 1986; Cochrane *et al*, 1987; 1988; Fernandez-Beros *et al*, 1989; Fohn *et al*, 1987; Griffiths *et al*, 1983; Mietzner *et al*, 1984; Neilands, 1982; Sciortino and Finkelstein, 1983; Shand *et al*, 1985; Ward *et al*, 1988). For example, it has been clearly demonstrated that both *E. coli* recovered directly from the peritoneum of lethally infected guinea pigs (Griffiths *et al*, 1983b) and four strains of *Vibrio cholerae* infecting the intestinal tracts of infant rabbits (Sciortino and Finkelstein, 1983) expressed high molecular weight IRMPs, providing strong evidence that these bacteria were growing under iron-depleted conditions *in vivo*. Iron-depleted growth of bacteria in human infection was first described by Brown *et al*, (1984), who observed IRMPs in the OMs of *P. aeruginosa* cells examined without sub-culture from the sputum of a patient with cystic fibrosis. Subsequently, Lam *et al*, (1984) recovered strains of *E. coli, Proteus mirabilis* and *Klebsiella pneumoniae* from the urines of patients with urinary tract infection and found that five out of twelve strains exhibited two or more IRMPs. IRMPs were also present in the OMs of *K. pneumoniae* and *P. mirabilis* isolated without

subculture from infected human urine by Shand *et al*, (1985).

Indirect evidence that IRMPs are expressed *in vivo* includes that presented by Fernandez-Beros *et al* (1989) who demonstrated using immunoblot analysis that the serum of patients with typhoid fever exhibited an immunoglobulin G response to IRMPs of molecular weights 83, 78 and 69K. Additionally, Fohn *et al*, (1987) showed that antibodies in acute-phase sera from patients with disseminated gonococcal infection reacted strongly with the 37K IRMP of *Neisseria gonorrhoeae.*

The conclusions drawn from the evidence reported are that IRMPs are important antigens as they are expressed *in vivo*. These antigens appear to be recognised early on during the course of acute infections (Ward, 1987; Fohn *et al*, 1987): this is an indication that they may have potential as protective antigens in the development of vaccines to combat *P. aeruginosa* infections.

1.8.2 Cross protection.

A vaccine based on OM protein components common to all strains of *P. aeruginosa* would offer an advantage over LPS-based immunoprophylaxis in that it would potentially confer protection against all serotypes. There have been several demonstrations of the cross-reactivity of IRMPs expressed by Gram-negative bacteria.

Evidence indicating the presence of common epitopes expressed by IRMPs includes that presented by Ikeda and Hirsh (1988), who investigated antigenic similarities between the iron-regulated 84K OMP of *Pasteurella multocida* P-1059 and proteins associated with isolates belonging to other somatic serotypes of *P. multocida.* These workers raised antibodies to the 84K IRMP and probed immunoblots of OMs prepared from isolates of *P. multocida* representing the sixteen different somatic serotypes. All of the isolates tested

expressed a protein antigenically related to the 84K protein of strain P-1059, when these organisms were grown in Brain Heart Infusion with dipyridyl. Other evidence indicating good cross reactivity was reported by Anwar *et al* (1985) who demonstrated that a burn patient's serum contained antibodies that reacted with the OMs of all serotypes of *P. aeruginosa.* In addition, Chart and Griffiths (1985), who investigated the antigenic and molecular homology of the ferric enterobactin receptor protein in *E. coli,* found that antibodies raised in rabbits to the 81K protein isolated from strain 0111 reacted with antigens in 17 pathogenic and laboratory strains tested. Chart and Griffiths (1985) concluded that at least some antigenic properties of this protein were conserved within the strains of *E. coli.*

Deneer and Potter (1989) demonstrated immunological conservation and expression *in vivo* of the IRMPs of *Haemophilus pleuropneumoniae.* These workers showed that convalscent-phase sera from pigs infected with *H. pleuropneumoniae* serotype 1 contained antibodies that reacted with the 105K and 76K IRMPs in six different serotypes examined. Additionally, Cornelis *et al* (1987) raised antibodies in a rabbit directed to OMs from *P. aeruginosa* PA1 grown in M9 medium with desferral (1mg/ml) and found that most of the proteins visible on Coomassie blue stained SDS-PAGE gels were also detected on immunoblots using the polyclonal antibodies. These workers looked at the cross-reactivity of these antibodies with proteins in a range of clinical isolates of *P. aeruginosa* and found that the IRMPs were detected in most of the clinical isolates examined, again pointing to the presence of common epitopes. Hybridisation and immunoblotting studies carried out by Fernandez-Beros *et al* (1989) suggested that the enterochelin system in *Salmonella typhi* and E. coli are functionally and structurally related.

Iron-aquiring systems are most likely to be conserved within a given bacterial species; if antibodies specific for these proteins are protective, as some recent evidence suggests (Bolin and Jensen, 1987; Sokol and Woods, 1986a), the epitopes involved would appear

to be ideal candidates for inclusion in immunising preparations, especially for those bacterial species that have multiple serotypes, such as *P. aeruginosa*.

1.8.3 Inhibition of iron-uptake by anti-IRMP antibodies.

IRMPs have been implicated as being directly involved in the uptake of iron (see section 1.7.2 and Cody and Gross, 1987; Cornelis *et al*, 1989; Chart and Griffiths, 1985; Icihara and Mizushima, 1978; Pierce and Earhart, 1986 Sokol and Woods, 1983; 1984), and therefore antibodies that bind to IRMPs will possibly interfere with siderophore-mediated iron uptake and exert a bacteriostatic effect on the pathogens concerned.

Examples of the inhibition of siderophore-mediated uptake include the results presented by Coulton (1982), who reported that specific antibodies raised in rabbits against the fhuA protein (M_r 78,000) of *E. coli* K-12, which is the outer membrane protein receptor for ferrichrome, partially inhibited ferrichrome-mediated iron uptake. Additionally, a purified polyclonal antiserum against the 80K major IRMP of *P. aeruginosa* PAO1 was able to block uptake of ^{59}Fe pyoverdine in PAO1 (Cornelis *et al*, 1989). Evidence acquired by Sokol and Woods (1986a; 1986b) demonstrated that IgG monoclonal antibodies specific for ferri-pyochelin binding protein (FBP) and polyclonal rabbit antiserum raised to purified FBP were effective at blocking the binding of ^{59}Fe-labelled pyochelin to isolated OMs of *P. aeruginosa*. Polyclonal serum to FBP was also shown to inhibit the uptake of ferripyochelin by whole cells.

The evidence suggests that binding of antibody to certain IRMPs inhibits the uptake of iron. As iron is an essential nutrient, this blocking activity will exert a bacteriostatic effect on the bacteria involved.

1.8.4 Surface exposure of IRMPs.

If antibodies specific for the IRMPs are able to bind to intact cells, they will demonstrate a greater potential to be effective *in vivo.* Evidence indicating the surface location of IRMPs has been reported (Sokol and Woods, 1986b; 1987a; Griffiths *et al*, 1983a). Sokol and Woods (1986b) used indirect immunofluorescence and I^{125} surface labelling techniques to show that FBP was present on the cell surface of *P. aeruginosa* grown in iron-restricted conditions, but not iron-replete conditions. Additionally, Griffiths *et al* (1983a), showed by I^{125} labelling with lactoperoxidase that siderophore receptors are exposed on the surface of at least some pathogenic strains of *E. coli* and they showed that the receptors could interact with large proteins. Evidence indicating the lack of surface exposure of IRMPs of the *Yersinia* spp. was demonstrated, however, using iodination and proteolysis experiments by Carniel *et al* (1989a). These workers found that the majority of the high molecular weight IRMPs were not surface exposed.

Other proteins in the OMs of Gram-negative bacteria have been investigated with respect to their surface exposure. Lambert and Booth (1982) using radiolabelled lactoperoxidase demonstrated the surface location of protein F of *P. aeruginosa.* This observation was confirmed by Mutharia and Hancock (1983;1985a). Mutharia and Hancock (1983) isolated hybridomas secreting highly specific monoclonal antibodies against porin protein F of *P. aeruginosa.* The cell surface localisation of antigenic sites on protein F was shown by indirect immunofluorescence techniques with these antibodies. Surface exposed protein antigens of *H. influenzae* type b (Hansen *et al*, 1981) and gonococci (Swanson, 1981) have also been demonstrated in studies utilising antiserum directed against the intact organism.

1.8.5 IRMPs related to pathogenicity.

Each bacterium responds individually to iron starvation: synthesis of siderophores, expression of novel proteins involved in the binding, transport and utilisation of iron, and production of toxins. Iron-limitation may be important in triggering the expression of virulence determinants by bacteria (Crosa, 1984; Miller *et al*, 1989); the ability to acquire iron has been linked to pathogenic strains (Carniel *et al*, 1989b)

Carniel *et al* (1989b) used mouse antibodies specific for the iron-regulated high molecular weight proteins to screen a genomic library (constructed in bacteriophage lambda gt11 expression vector) containing DNA fragments (0.5-2kb pairs in length) from *Yersinnia entercolitica* serovar 0:8. Two positive clones were detected and isolated-these coded for ß-galactosidase fusion proteins of 151 and 138K. The 2 cloned DNA fragments were used to construct the recombinant amplification plasmid pVC13 and Southern hybridisations performed with the inserts used as probes revealed that the gene coding for the high molecular weight protein is conserved among all of the highly pathogenic *Yersinia* strains that Carniel *et al* (1989) studied. The gene was missing from the low-virulence and nonvirulent strains. Carniel *et al* (1989b) demonstrated that transcription of the high molecular weight proteins was induced by iron-starvation and showed that two high-molecular weight proteins synthesised were expressed only by the highly pathogenic strains. The less pathogenic and non-pathogenic strains did not express antigens with epitopes cross-reacting with the two high molecular weight IRMPs of other pathogenic strains, suggesting that these strains do not synthesise even an altered form of IRMP.

Meitzner *et al* (1986) suggested that as there was high antigenic conservation of IRMPs among the pathogenic members of the genus *Neisseria*, these proteins may serve a common function in pathogenicity.

Additionally, Gross (1985) demonstrated that iron tightly regulates the synthesis of the necrosis-inducing phytotoxin, syringomycin, and Poole and Braun (1988) showed that iron regulates hemolysin gene expression of *Serratia marcescens.*

Brener *et al* (1981), investigated the relative virulence of *N. meningitidis* grown in iron-limited conditions at pH6.6 to the same cells cultivated in the same medium but at pH7.2 and with sufficient iron. The iron-limited cells had an increased relative virulence 1,200-fold greater than the iron-sufficient cells. This study indicated that pH and iron can be important factors in determination of meningococcal virulence. Other examples include demonstrations that surface exposure of FBP in the OM is required for virulence of *P. aeruginosa* (Sokol and Woods, 1987b).

Immunoblotting techniques were used by Anwar *et al* (1985) to study the antibody response of a burn patient during the acute phase of a *P. aeruginosa* infection. The results showed the presence in the patient's serum of antibodies directed towards OM proteins including IRMPs of the infecting strain of *P. aeruginosa.* Various other indications of the involvement of iron in virulence have been reported (Miller *et al*, 1989). It is suggested that the blocking of iron uptake will also inhibit the expression of certain virulence factors of *P. aeruginosa.*

1.8.6 Protective activity of anti-IRMPs antibodies.

The degree to which antibodies reacting with IRMPs might be involved in host defence is relatively unknown. Some recent evidence has been acquired by Bolin and Jensen (1987), who evaluated the effect of passive immunisation of turkeys with antibody against *E. coli* IRMPs on the severity of disease produced after challenge with virulent *E. coli.* These workers cultivated *E. coli* under iron-limited conditions, prepared OM-enriched fractions and separated the proteins by SDS-PAGE. The region containing the IRMPs was excised

from the gel and emulsified in buffer. Hyperimmune rabbit serum was raised to the antigens, and the antibodies to LPS were removed by absorption to LPS-sensitised rabbit erythrocytes. The antiserum was further absorbed to remove antibodies directed against OM proteins other than IRMPs, by incubation with Fe+ whole cells. Turkeys were administered the antiserum and challenged two hours later with a virulent strain of *E. coli.* Ninety six hours after challenge, the turkeys were killed and gross legions were recorded. Bolin and Jensen (1987) concluded that antibodies to IRMPs provided protection against challenge.

Evidence also alluding to the potential of IRMPs as protective antigens was acquired by Sokol and Woods (1986a) who showed that antibodies to FBP significantly enhanced phagocytosis of *P. aeruginosa* by human polymorphonuclear leukocytes. Additionally, passive transfer of anti-FBP antibodies was shown to protect against infection by some *P. aeruginosa* strains in a mouse burn infection model. The protection provided by the antibodies was strain dependent but LPS-serotype independent (Sokol and Woods, 1986a).

1.9 Other factors in the environment affecting bacteria.

It must be noted that iron is not the only factor in the environment that can have an effect on bacterial antigenicity. Sub-minimum inhibitory concentrations (sub-MICs) of antibiotics have been demonstrated to have effects on the cell envelope (Atkinson and Amaral, 1982). For example, Morris and Brown (1988) demonstrated that sub-MICs of various aminoglycosides inhibited the excretion of alginate and the production of the iron-chelating siderophores. Additionally, Kadurugamuwa *et al* (1985;1988) found that at concentrations of cephalosporins which did not affect growth rate, there was a un-masking of surface protein antigens of *Klebsiella pneumoniae.* Sub-MICs of antibiotics have effects *in vivo*, for example Gemmell (1984) demonstrated the potentiation of phagocytosis of pathogenic bacteria by exposure to low concentrations of antibiotics.

Other factors of the growth environment include pressure effects (discussed in chapter 5) and additionally, the biofilm mode of growth, both of which have been shown to effect many aspects of bacterial physiology, including susceptibility to antimicrobial agents (Kenward *et al*, 1984; Costerton *et al*, 1983a; 1983b; 1987).

1.10 Generation of antibodies specific for OM components.

In order to produce antibodies specific for individual components of the bacterial OM, cell components have to be isolated. The major factors affecting the purification of cell envelope components are the interactions between lipopolysaccharides (LPSs) and OM proteins. Even after organic solvent extraction, detergent lysis, heat treatment, separation by SDS-PAGE or chromatographic separation, it has been suggested that OM proteins may still be contaminated by LPS (Hitchcock, 1984; Lam *et al*, 1987a; Poxton *et al*, 1985). In *E. coli* it has been shown that LPS is critical for the macromolecular association of porins, (Yamanda and Mizushima, 1980). It has not been established, however, to what degree LPS association with OM proteins occurs in the intact cell.

In some reports the contamination of 'purified' OM proteins with LPS has not been of concern. Gabay *et al* (1985) reported the purification of the major OM protein of *Legionella pneumophila* found that after selective extraction, ion-exchange and gel-filtration chromatography that the preparation contained approximately 20µg of LPS per mg of OM protein. Due to the recognised antigenicity of LPS, this would greatly affect the results of any immunological studies carried out using LPS-contaminated proteins.

For the purposes of this study, it was necessary to obtain preparations of IRMPs which had a minimal amount of contaminating LPS: these preparations were subsequently utilised to produce specific antibodies to the IRMPs. Previously, there have been reports of the generation of antibodies specific for IRMPs, for example, Carniel *et al* (1989a)

reported the production of antibodies specific for IRMPs of *Y. enterocolitica* serovar 0:8. These workers found that purification of the proteins was necessary to obtain specific antibodies and demonstrated on immunoblots that the antibodies specifically recognised the two high molecular weight IRMPs.

Additionally, Kelly *et al* (1989) attempted to raise polyclonal antibodies to purified IRMPs, however the serum these workers produced demonstrated only a weak response to one of the IRMPs. This serum was used to investigate whether bacteria recovered from a peritoneal implant in rats and mice were expressing IRMPs. No IRMPs were detected, however, due to the variability of expression of IRMPs, it is suggested that IRMPs were immunologically recognised *in vivo*, however they may not have been the same protein species as the IRMP detected by the polyclonal serum. These workers also used high titre cystic fibrosis serum to probe immunoblots of OMs extracted from the bacteria recovered from the chamber and it can be determined from the photographs that this serum reacted with one of the IRMPs.

Selection of antibodies specific for the protein components of Gram-negative bacteria is hindered due to the association of these antigens with immunodominant LPS molecules. A detailed discussion of the factors affecting the purification of IRMPs of *P. aeruginosa* and methods for selection of antibodies specific for these proteins is presented in chapters 4 and 6.

1.11 Delivery of vaccines.

Highly purified proteins may lack effective immunogenicity, and therefore may require to be delivered or presented to the host's immune system in a particular way, or administered by a particular route. Liposomes have been proposed by several workers as vehicles for vaccines (Alving, 1980, 1986; Gregoriadis, 1980) and additionally for

carriers of antimicrobial agents (Lopez-Berenstein, 1987) and drugs. These are a potentially promising vaccine delivery system as they:

1. are biodegradable.
2. demonstrate great structural versatility.
3. are relatively non-toxic.
4. are relatively non-immunogenic.
5. are able to be targetted.
6. act as a depot for the vaccine.
7. protect the antigen.
8. are able to retain the native antigen configuration.

Liposomes considerably improve the immune response to a diverse range of antigens; examples including Hepatitis B surface antigen (Gregoriadis *et al*, 1980), *Streptococcus mutans* cell wall antigens and tetanus toxoid. It is proposed that in addition to acting as an antigen depot, immunoadjuvant activity may result from the presentation of the antigen in a hydrophobic micro-environment (Tom, 1980). The charge, composition, method of preparation and number of lipid bilayers all influence the adjuvant activity of liposomes.

1.12 Aims of the study.

OM proteins have been proposed as vaccine candidates as they are important antigens, abundant in the cell envelope of the pathogen and located in a position where they are surface accessible (Gilleland and Matthews-Greer, 1987). The essential problem is to determine which antigens should be incorporated into a potential vaccine to trigger the protective immune response. IRMPs are considered to be key OM proteins in the infection process, as they are involved in the uptake of iron which is essential to bacterial growth (Cody and Gross, 1987; Cornelis *et al*, 1989; Chart and Griffiths, 1985; Icihara and Mizushima, 1978; Pierce and Earhart, 1986; Sokol and Woods, 1983; 1984), and have

been shown to be recognised early during the course of acute infections such as infections of burn wounds (Ward *et al*, 1988).

It is suggested that the iron-uptake system is a potential target for immunological intervention. The aim of this study was to investigate the potential of IRMPs to act as effective, cross-protective antigens. In order to exploit this approach in the immunological control of *P. aeruginosa* infections, the production of antibodies specific for the IRMPs was required so analysis of the expression, and investigation of the antigenic roles played by these important cell envelope components in immune protection against *P. aeruginosa* infection could be undertaken. The project was therefore designed to generate specific antibodies to IRMPs. Purification techniques were used in an attempt to isolate IRMPs away from other components of the cell envelope and IRMP preparations were subsequently utilised to produce polyclonal and monoclonal antibodies. Preliminary formulation studies were undertaken to establish experimental vaccination strategies and to investigate the effect of presenting antigens incorporated in liposomes on the level and specificity of immune response initiated.

In order for IRMPs to be considered as potential vaccine candidates, various facts concerning their expression and conservation had to be elucidated, including cross-reactivity and surface expression. In addition, aspects of the project were designed to gain additional evidence regarding the recognition of IRMPs by the host's immune system in different clinical infection situations.

2 Materials.

2.1 Bacterial strains.

Table 2.1. *P. aeruginosa* strains used in the study.

Strain	Source	Rough/Smooth phenotype
PA01 (ATCC 15692)	Aston collection	S
18r	Colindale	S
7	Colindale	S
AK 957.	A.M. Kropinski	S
AK 1282	A.M. Kropinski	R
AK 1012.	A.M. Kropinski	R
7/1	Colindale	R
18s	Colindale	R
CF003	Colindale	R
NCTC 6750	Aston collection	S
NCIB 8295	Aston collection	S
NCTC 10662	Aston collection	S
P1	Pleural infection	S
E1	*Otitis externa* isolate	S
PaSA	Cystic fibrosis isolate	R
PaWH	Cystic fibrosis isolate	R

Representative strains of the seventeen serotypes of *P. aeruginosa* described by the IATS, were obtained from the Aston culture collection. In addition, the *Klebsiella pneumonia and Escherichia coli* strains used were clinical isolates from leukaemic patients. All strains were maintained on nutrient agar slopes at 4°C and subcultured at approximately 3 monthly intervals.

2.2 Clinical material, serum and conjugates.

Patients' sera were separated from blood obtained by venipuncture during the course of clinical investigation. Polyclonal antisera were produced by immunising mice or New Zealand White rabbits, ip with antigen preparations. The primary injection on day 1 was emulsified 1:1 in Freund's complete adjuvant and subsequent injections on days 14 and 40 were emulsified 1:1 in Freunds incomplete adjuvant (Freund *et al*, 1937). Rabbit blood was obtained by ear vein bleeding or cardiac puncture and mouse blood by either tail vein bleeding or cardiac puncture, 3 days after the final injection. Serum was separated by centrifugation at 2,000 x g for 10min after incubation for 2 hrs at 37°C and 16 hrs at 4°C. Samples were stored at -20°C.

For immunodetection of antigenic sites during immunoblotting (section 3.3.1) and ELISA (section 3.3.2) studies, various anti-immunoglobulin antibodies were used, conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase (AP). Immunochemicals used included protein A-HRP, protein A-AP, anti-mouse/rabbit/human-immunoglobulin subclass-HRP and anti-mouse $F(ab')_2$ fragments-HRP (Sigma). For determination of the isotypes of monoclonal antibodies, various Ig-subclass antibody conjugates were used in an ELISA class typing assay. Biotinylated anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM conjugates (Amersham) were used in addition to streptavidin-HRP. Protein A (Goding, 1978) conjugates were used for $IgG_{2a/2b/3}$ isotype monoclonal antibodies in the later stages of characterisation. Sections 3.3.1 and 3.3.2 detail the colour development reactions.

Immunogold conjugates were obtained from BioCell Ltd (Cardiff). Goat anti-mouse IgG, particle size 15nm, was used in the immunogold staining of *P. aeruginosa* strains PAO1 and AK1282.

A clinical isolate of *P. aeruginosa* (P1), samples of pre- and post-infection serum and a sample of infected pleural fluid were obtained from an 81 year old patient suffering from a pleural cavity infection. The patient was originally admitted to hospital with faecal peritonitis and a perforated carcinoma of the sigmoid colon was found at surgery. Colostomy was performed (pre-immune serum was taken at this point) and the patient recovered initially, whilst being administered cefuroxime and metronidazole. Eighteen days after surgery, the patient developed a fever and suffered rapidly worsening respiratory distress. A left sided pleural effusion was found and fluid was aspirated for bacteriological examination. The fluid yielded a profuse growth of *P. aeruginosa*, susceptible to gentamicin and ceftazidime. The patient was treated with these two antibiotics and the empyema fluid was drained from the pleural cavity. There was an initial improvement for a week, but the patient gradually deteriorated, developed multiorgan failure and died fifteen days after starting the antibiotics. Attempts were made to recover bacteria directly from the pleural fluid by sequential centrifugation and solubilisation stages, following the method of Ward (1987).

P. aeruginosa strain E1 was isolated from a diver with *Otitis externa.* A corresponding sample of serum was obtained 14 days after the infection occurred.

2.3 Chemicals and media.

2.3.1 Chemically defined medium (CDM).

The CDM (table 2.3.1) designed by Noy (1982) for growth of *P. aeruginosa* was used with an increased concentration of NaCl (from 0.5mM to 5mM) so the medium was iso-osmotic with body fluids (Shand, 1985).

Table 2.3.1. CDM for growth of *P. aeruginosa.*

Nutrient	Final concentration (mM)
NaCl	5.0
KCl	0.62
$MgSO_4.7H_2O$	0.4
$K_2HPO_4.3H_2O$	3.2
$(NH_4)_2SO_4$	40.0
$FeSO_4.7H_2O$	0.062
MOPS	50.0
Glucose	40.0

Iron-restriction of *P. aeruginosa* was achieved by growing this organism in CDM to which no iron had been added (CDM-Fe). Alternatively, media designed to limit the iron supply of the bacteria was prepared by passing tryptone soya broth through a column of ion-exchange resin, Chelex 100 (Bio-Rad). Chelex 100 removes all metal cations, including iron, and so other essential ions are replaced to a final concentration of:

$CaCl_2$	5×10^{-7} M
H_3PO_4	5×10^{-7} M
$COCl_2$	5×10^{-8} M
$CuSO_4$	1×10^{-8} M
$ZnSO_4$	1×10^{-8} M
$MnSO_4$	1×10^{-7} M
$(NH_4)_6Mo_7O_{24}$	5×10^{-9} M
$MgSO_4$	4×10^{-4} M

The pH of the medium after this treatment was adjusted to 7.4. The preparation of chelexed nutrient broth +Fe could be achieved by the addition of the above essential ions, and also $FeSO_4$ at a final concentration of 0.02 mM.

2.3.2 Complex media.

Nutrient agar and typtone soya broth were obtained from Oxoid (London) and *Pseudomonas* isolation agar (PIA) from Difco (West Molesey, Surrey). Both were prepared and sterilised according to the manufacturer's instructions.

Reagents not specified in the text were supplied by BDH Chemicals Ltd., (Poole, Dorset), Sigma Chemical Company (Poole, Dorset) and Fisons (Loughborough, Leics.) and were of Analar grade or equivalent.

2.4 Preparation of glassware.

All glassware was rinsed in tap water, fully immersed in 5% v/v Extran and allowed to stand overnight at room temperature. It was then rinsed in distilled water, once in 1% v/v hydrochloric acid, 6 times in single distilled water and 3 times in double distilled water, before drying at 60°C. Glassware was sterilised by dry heat at 160°C for 3 hrs.

2.5 Culture of bacteria and measurement of bacterial cell growth.

Cells were grown at 37°C, shaking at 200 rpm. Changes in cell concentration during bacterial growth were followed using a spectrophotometric method based on 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 bacterial 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. This relationship holds up to an OD of approximately 0.3 (Kenward, 1975). Measurements of absorbance were made at a wavelength of 470nm (A_{470nm}) to minimise absorption by metabolic products of the bacteria, such as pyocyanin. An aliquot of growth medium was retained as a blank.

Calibration curves determining the A_{470} to viable count relationship for *P. aeruginosa* serotypes 1, 2, 3, 4, 5, and strain AK1282 were constructed for the LD_{50} studies (section 3.3.7.1). Two hundred ml of pre-warmed CDM-Fe was inoculated with 2ml of an overnight CDM-Fe culture *P. aeruginosa*, and shaken for 5 hrs at 37°C. The cells were harvested by centrifugation at 5000 x g, washed once in sterile 0.85% NaCl and resuspended to various A_{470} values, ranging between 0.02 and 0.3. Viable count determinations were performed by plating out 100µl of serially diluted samples onto nutrient agar plates in triplicate, and counting after overnight incubation at 37°C.

2.6 Equipment.

Automatic pipettes: Gilson pipetman, P-20, P-200 and P-1000 (Anachem Ltd., Luton, Beds.)

Balances: Sartorius 1702 balance (Sartorius Intruments Ltd., Belmont, Surrey), Oertling HC22 (Oertling, Orpington, Kent).

Centrifuges: MSE high speed 18, MSE superspeed 50 (Measuring and Scientific Equipment Ltd., Crawley, Sussex), Beckman J2-21, Beckman L8-60M Ultracentrifuge (Beckman Intruments Ltd., High Wycombe, Bucks.) and Eppendorf 5412 bench centrifuge (Baird and

Tatlock Ltd., Atherstone, Leics.).

Fast Protein/Polypeptide/Polynucleotide Liquid Chromotography (FPLC) System: High precision pumps P-500, pH monitor, pH flow through electrode, automatic V-7 injection valve, Superose 12 HR 10/30 gel-filtration column, Mono Q anion-exchange column, (Pharmacia, Uppsala, Sweden)

Filtration equipment: Clamped glass funnel with 47mm diameter, 0.2μm cellulose acetate filters (Millipore, Harrow, Middx.)

Freeze dryer: Edwards Modylo freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex).

French Press: Amicon Corp., High Wycombe, Bucks.

Gel electrophoresis equipment: Mini-Protean system (Bio-Rad Laboratories Ltd., Watford, Herts.). Preparative gel apparatus was obtained from Aston Glass. Additionally, a Paragon Electrophoresis system (Beckman Instuments Ltd., High Wycombe, Bucks.) was used for separation of serum proteins.

Gel Drier: Model 224, (Bio-Rad, Watford, Herts.)

Immunoblotting apparatus: TransBlot Cell. (Bio-Rad, Watford, Herts.)

Incubators: Mickel Reciprocating water bath (Cam Lab Ltd., Cambridge), Gallenkamp orbital shaking incubator (Gallenkamp, London).

Laser Densitometer: Ultroscan Laser Densitometer, LKB 2202 run by an Apple II Europlus computer with Gelscan software (LKB, Croydon, Surrey).

Photography equipment: Nikon camera FG (Nippon Kogaku KK, Tokyo, Japan), with Kodak Technical Pan film 2415.

Rotary evaporator: Buchi Rotorvapor-R (Fisons Scientific Apparatus, Loughborough, Leics.).

Sonicator: MSE Soniprep 150 (Measuring and Scientific Equipment Ltd., Crawley, Sussex).

Spectrophotometers: LKB ultrospec 4050, Cecil CE292 and Unicam SP8000 scanning UV spectrophotometer (Pye Unicam Instruments Ltd., Cambridge).

3 Experimental methods.

3.1 Preparative techniques.

3.1.1 Preparation of bacterial OMs.

Outer membranes were prepared by a modification of the method of Filip *et al* (1973). Cells were cultivated under various conditions and harvested by centrifugation at 5000 x g for 10min at 4°C. The pellet of cells was washed once in 0.85% NaCl, then resuspended in 10-25ml distilled water (depending on the volume of cell culture). Cells were broken by 10 x 30s pulses of sonication in an ice bath with 30s intervals for cooling. Alternatively, cells were disrupted by passing through a French Pressure Cell 2-3 times. Any remaining unbroken cells were removed by centrifugation at 5000 x g for 10min. The supernatant was collected and Sarkosyl (N-lauryl sarcosine) was added to give a final concentration of 2% v/v to solubilise the cytoplasmic membrane and leave the outer membrane intact (Lambert and Booth, 1982). Ten mM EDTA was included at this stage of membrane preparation. After incubation for 1 hr at room temperature, the preparation was centrifuged at 100,000 x g for 40min at 4°C. The final pellet was washed in distilled water, resuspended in 0.5ml of distilled water and stored at -20°C.

3.1.2 Preparation of solubilised OM extracts.

OMs prepared as described in section 3.1.1 were extracted using a wide range of detergents in order to determine which detergents would be most effective at selectively solubilising IRMPs. Aliquots of OM preparations were suspended in the detergent solution for 1 hr at room temperature then centrifuged at 100,000 x g for 40 min. The supernatant was removed and the pellet was resuspended in distilled water to an equal volume. Both soluble and insoluble fractions were analysed by SDS-PAGE. The detergents

selected were:

1% Deoxycholic acid
1% Sodium dodecyl sulphate
1% Cetyl trimethyl ammonium bromide
1% CHAPS 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
0.3% Empigen BB
1% Polyoxyethylene ether W-1
1% Triton X 100
1% Triton X 114
1% Tween 20
1% Tween 80
1% Nonidet NP40

All detergents were made up in a solution containing 0.02% sodium azide, 0.001M PMSF, 20mM triethanolamine, pH 7.5. Duplicate buffers were prepared and 10mM EDTA was added to one set.

3.1.3 Extraction and purification of bacterial LPS

LPS was extracted by an adaptation of the hot phenol technique of Westphal and Jann (1965). A 2L stationary-phase cell culture was harvested by centrifugation at 5000 x g for 10min. The cells were washed once in 0.85% saline, resuspended in 20ml 30mM Tris-HCl, pH 8.0 and broken by 10 x 30s pulses of sonication in an ice bath, with 30s intervals for cooling. Deoxyribonuclease (Bovine pancreas type III), ribonuclease (Bovine pancreas type 1-AS) and lysozyme (to digest the DNA, RNA and peptidoglycan respectively) was added to a final concentration of 0.1mg/ml, and the preparation was incubated for 2hrs at 37°C. Five ml tetrasodium EDTA (0.5M) (to remove cations binding the LPS together), and protease (*Strep. griseus* type XIV; final concentration 1mg/ml) were added. The preparation was incubated overnight at 37°C with constant shaking. The protease was destroyed by heating the mixture to 80°C for 20min. The digested cell

suspension was mixed with an equal volume of 90% w/v phenol, pre-heated to 80°C. The preparation was stirred vigorously for 5min and then centrifuged at 5000 x g for 25min to permit phase separation. The upper aqueous layer (containing LPS) was removed, care being taken not to disturb any of the proteinaceous material at the interface. Two further phenol-water extractions were performed by reheating the phenol layer to 80°C and adding a further 50ml water at the same temperature. The pooled aqueous fractions were dialysed for 48hrs against tap water to remove phenol.

Following dialysis, magnesium chloride was added to a final concentration of 50mM. The LPS was pelleted by centrifugation at 100,000 x g for 4hrs, washed in double distilled water, re-centrifuged and lyophilysed.

An alternative method (Darveau and Hancock, 1983) was also used for the isolation of LPS from rough and smooth strains of *P. aeruginosa.*

3.1.4 Removal of anti-LPS antibodies from antiserum.

In order to determine the contribution of antibodies directed against LPS to the activity of a sample of antiserum, it was necessary to absorb out anti-LPS antibodies and compare immunoblotting and ELISA results obtained with absorbed and non-absorbed sera. LPS-absorption of serum was also required in the preparation of serum for the passive protection studies. LPS was extracted from *P. aeruginosa* whole cells following the method of Westphal and Jann, (1965), as described in section 3.1.3.

Two mg of purified LPS was added to 1ml of serum and shaken, at 37°C for 4hrs and then at 4°C for 24hrs. The serum was centrifuged in an Eppendorf microcentrifuge for 30min to pellet immune complexes. The procedure was repeated and the supernatant was recovered and stored at -20°C.

3.1.5 Preparation of liposomes.

Liposomes were prepared using 50mg of L-distearoyl-phosphtidylcholine (DSPC) dissolved in 7ml of cholesterol in chloroform (1mg/ml). The solution was placed in a 250ml round bottomed flask and rotary evaporated to dryness in a water bath at 58°C, the transition temperature of DSPC. The lipid film was dried under vacuum for a further 10min and 5ml of a solubilised extract of OMs extracted from *P. aeruginosa* strain PAO1 was added to the flask which was shaken in the water bath until the lipid film dissolved. The liposomes were left to hydrate for 60min at 4°C. After centrifugation of the preparation at 40,000 x g for 15min at 4°C, a solid lipid pellet was obtained. The supernatant was decanted and retained, and the pellet washed and resuspended in water.

In order to assay the amount of OMPs incorporated in the liposomes, protein assays on liposome washings and disrupted liposomes were performed. Liposomes were disrupted by the addition of sodium taurocholate to a final concentration of 37mM, for 60min at 60°C. The protein assay standards contained Empigen BB and sodium taurocholate where this was necessary.

3.1.6 Excision of antigens from polyacrylamide gels and nitrocellulose paper.

3.1.6.1 Excision from polyacrylamide gels.

OM components separated by SDS-PAGE (section 3.2.1) were excised from the gels following the method of Knudsen (1984). A 10% SDS-PAGE gel was prepared and loaded with denatured OMs extracted from *P. aeruginosa* strains PAO1 or AK1282 and pre-stained molecular weight markers. The gel was electrophoresed for 30min and stained with Coomassie brilliant blue. In this manner the positions of the IRMPs were

determined relative to the standard markers. Subsequently, a large number of similar gels were prepared and each one was loaded with 75µl of one of the 1µg/ml preparations of denatured OM samples (using a one-well comb), 5µl of the pre-stained molecular weight markers being loaded in a separate well at the edge of the gel. On completion of electrophoresis, the proteins of interest were excised from the gels, without staining, and gel strips were sealed in dialysis tubing with 5ml of double distilled water. The proteins were then eluted from the gel by dialysing the gel strips for 2-3 days in running water, and the eluted proteins were obtained by lyophilising the dialysate.

3.1.6.2 Excision from nitrocellulose blots.

Twenty mg dry weight of OM preparation (derived from *P. aeruginosa* cells grown in CDM-Fe) was separated by SDS-PAGE, using a preparative gel apparatus. The gel was blotted (section 3.3.1) overnight at 50V to transfer the OM components from the gel to a sheet of nitrocellulose paper. A section of the latter was stained with amido black to locate the position of the proteins on the paper and the area of nitrocellulose paper containing the IRMPs was excised and digested in 1ml of dimethylsulphoxide (DMSO).

3.1.7 Detoxi-Gel.

Detoxi-Gel is an immobilised matrix which has a specificity to bind and remove pyrogens from solution. A column format was used and 300µl 0.5% Nonidet NP40 solubilised *P. aeruginosa* PAO1 OM samples were applied to the column. Three columns were used:

1 0.5% Nonidet NP40 in 0.1M NaCl/10mM Tris, pH7.8

2 0.5% Nonidet NP40 in 0.1M NaCl/10mM Tris, pH7.8 with 10mM EDTA.

3 0.5% Deoxycholate, 0.5% Nonidet NP40 in 10mM Tris, pH7.8 in 0.1M NaCl.

Elution buffers contained 0.3M NaCl, and eluted samples were analysed by SDS-PAGE (section 3.2.1) to determine their protein and LPS compositions.

3.2 Analytical techniques.

3.2.1 SDS-Polyacrylamide gel electrophoresis.

3.2.1.1 Gel electrophoresis of OM proteins.

Separation of OM proteins was carried out using either a Mini-Protean or a preparative gel electrophoresis apparatus and the gel systems described by Laemmli (1970) and Lugtenberg *et al* (1975). The running gel was prepared as detailed in table 3.2.1, and polymerisation was initiated by the addition of N,N,N',N'-tetramethylethylene diamine (TEMED). The gel solution was poured between glass plates, separated by 0.25mm plastic spacers, to within 1cm of the top and allowed to set for 10min. A spray of electrode buffer was used on top of the gel to ensure that the surface would be level. After polymerisation, the excess electrode buffer was removed and the stacking gel was cast. A teflon comb was inserted into the stacking gel to create wells for sample application. Samples were denatured by heating at 100°C for 10min with an equal volume of sample buffer (table 3.2.1) and 5µl/lane was loaded onto the mini-gels. The electrode buffer contained 0.025M Tris, 0.19M glycine and 0.1% SDS. In the Mini-Protean system, a constant voltage of 200V was applied through the gel and electrophoresis was allowed to continue until the tracking dye had migrated to within 0.5cm of the bottom of the gel. Preparative gels were electrophoresed at a constant current of 30mA. Gels were either used directly for immunoblotting (section 3.3.1) or stained for protein (section 3.2.4).

3.2.1.2 Gel Electrophoresis of LPS.

LPS was separated by SDS-PAGE using 12% gels and the buffer system described for gel electrophoresis of OMPs (3.2.1.1). Two types of LPS sample were analysed: proteinase-K digests of bacterial OM preparations and whole cells following the method of Hitchcock and

Brown (1983). One hundred µl of whole cells, OMs or fractions from a chromatography column (equivalent to 1mg/ml of protein) were denatured for 10min at 100°C with 60µl sample buffer (table 3.2.1). After cooling, 40µl sample buffer containing 2.5mg/ml proteinase-K was added and the preparation incubated at 60°C for 60min. Five µl samples were loaded onto the gels. Electrophoresis was carried out at a constant voltage of 100V until the tracking dye had migrated to within 0.5cm of the bottom of the gel. *E. coli* LPS was used as a standard. Gels were either used directly for immunoblotting (section 3.3.1) or stained with silver (section 3.2.4).

Table 3.2.1. Composition of separating gel, stacking gel and sample buffer for SDS-PAGE.

	Separation gel	Stacking gel	Sample buffer
Stock 1	6.0ml	-	-
Stock 2	-	2.0ml	-
10 % SDS	600µl	120µl	5.0ml
1.5M Tris[1] pH 8.8	7.5ml	-	-
0.5M Tris pH 6.8	-	3.0ml	2.5ml
Distilled water	9.5ml	6.4ml	5.0ml
TEMED[2]	56µl	32µl	-
10 % AMPs[3]	80µl	40µl	-
2-mercaptoethanol	-	-	0.3ml
5 % bromophenol blue	-	-	0.2ml
Glycerol	-	-	2.5ml

Stock 1 = 44 % w/v acrylamide and 0.8 % w/v N,N'-methylene-bis acrylamide (Bis)

Stock 2 = 30 % w/v acrylamide and 0.8 % w/v (Bis)

[1]Tris (hydroxymethyl) amino ethane

[2]N,N,N',N'- tetramethylethylene diamine

[3]Ammonium persulphate (freshly prepared)

3.2.2 Determination of the molecular weights of OM proteins of *P. aeruginosa* separated by SDS-PAGE.

Denatured polypeptides bind SDS in a constant weight ratio and have essentially identical charge densities and therefore migrate in polyacrylamide gels according to size. A linear

relationship exists between $\log_{10}$ of the polypeptide molecular weight and the relative mobility (R_f) values, where:

$$R_f = \frac{\text{Distance migrated by polypeptide}}{\text{Distance migrated by dye front}}$$

The apparent molecular weights of OM proteins were determined on a 12% gel by comparing their R_f values with those of known protein markers. Pre-stained molecular weight markers were obtained from Bio-Rad. These consisted of :

	RMM(K)	Log_{10}RMM	R_f
Phosphorylase b	130	2.11	0.125
Bovine serum albumin	75	1.88	0.264
Ovalbumin	50	1.70	0.380
Carbonic anhydrase	39	1.60	0.500
Soyabean trypsin inhibitor	27	1.43	0.638
Lysozyme	17	1.23	0.805

3.2.3 Coomassie brilliant blue stain of protein and silver stain of LPS.

Detection of protein was achieved by staining the gel with 0.1% Coomassie brilliant blue R-250 in 50% methanol/10% acetic acid for 60 min. Gels were subsequently destained in 10% methanol/10% acetic acid and either photographed using diffuse transmitted light or dried onto filter paper.

LPS separated by SDS-PAGE was visualised by silver staining following the method of Tsai and Frasch (1982). Immediately after electrophoresis, the gel was immersed for 60min in 40% ethanol/5% acetic acid. This fixing solution was then replaced with fresh solution

containing 1% periodic acid and the LPS was oxidised for 30min. After 3 x 10min washes with double distilled water to remove unreacted periodic acid, staining reagent was poured over the gel. The stain was freshly prepared by slowly adding 2.5ml 20% silver nitrate solution to a mixture of 1ml concentrated ammonium hydroxide and 14ml 0.1M NaOH. The solution was made up to 75ml with double distilled water and the gel was shaken in the reagent for 30min. Further washing was performed for 30min with 3 changes of water and then developing solution containing 50mg citric acid and 0.5ml (37 %) formaldehyde in 500ml of double distilled water was placed on the gel. When the LPS was stained to the desired intensity (2-10min), colour development was terminated by replacing the developer with fixing solution. Gels were photographed immediately.

3.2.4 Isoelectricfocussing and titration curves using detergent/urea soaked gels.

The transparent film from a IEF 3-9 PhastGel was removed and the gel was washed twice for 5min in 80ml of distilled water to remove the ampholytes from the gel so that a different pH interval could be used. The gel was soaked in 80ml of 10% glycerol for 10min and then dried by placing on the separation bed of the Phast system which was held at 70°C. The gel was then rehydrated for 10min in a solution of 8M urea, 0.5% Nonidet NP40, and 7.5% v/v of Pharmalyte 3-10. Following this, the surface moisture was removed by blotting and the gel was used for isoelectrofocussing (IEF) or titration curve analysis. For IEF, the gel was placed on the separation bed and samples were applied using the PhastGel sample applicator 8/1. In one lane, a mixture of IEF pI standards (Pharmacia) were applied (ß-lactoglobulin A (pI 5.13), Methyl red (pI 3.75), Trypsinogen (pI 9.3) and Carbonic anhydrase (pI 6.57)). To produce a titration curve the gel was pre-focussed then rotated through 90°; the sample was applied as a continuous line across the width of the gel with a PhastGel sample applicator TC.

3.2.5 Gel-filtration chromatography.

A Pharmacia Fast Protein/Polypeptide/Polynucleotide Liquid Chromatography system (FPLC) was used in an attempt to separate IRMPs from other OM components. The method developed is potentially applicable to the purification of other proteins from the cell envelopes of a variety of Gram-negative bacteria. Gel-filtration using Empigen BB, SDS, and Nonidet NP40 was applied. Additionally, a deoxycholate system was investigated for use as part of the purification strategy as deoxycholate has been reported to be effective at disrupting protein-LPS complexes in *Neisseria gonorrhoeae* (Heckels, 1977).

A Superose 12 Gel-filtration column was used and the running buffer contained 10mM EDTA, 0.2% sodium azide, 0.001M PMSF and 20mM triethanolamine; the pH was adjusted to 9.5 for deoxycholate systems and 7.5 for the other detergents. A 500µl sample size of solubilised OMs was injected onto the column and 1ml fractions were collected from 12min after injection until the end of the program. Protein elution was monitored in-line by absorbance at 280nm. Fractions were analysed as described in section 3.2.7.

3.2.6 Ion-exchange chromatography.

A Mono Q (Pharmacia) column was used in the FPLC system. Mono Q is a strong anion exchanger based on quaternary amine groups supported on a monobead matrix. A 0.3% Empigen BB buffer system was used and a linear 0-1M salt gradient formed after an Empigen BB-solubilised extract had been applied to the column and all the unbound material had been washed from the column. A flow rate of 1ml/min was used and 1ml fractions were collected immediately after the salt gradient began. The A_{280nm} of the fractions was monitored by an in-line spectrophotometer and samples analysed as described in section 3.2.7.

3.2.7. Analysis of fractions after ion-exchange and gel-filtration chromatography.

In most of the chromatography experiments, the fractions obtained were too dilute to be analysed directly by SDS-PAGE and therefore had to be lyophilised and resuspended to a volume smaller than that of the original. However, the detergent concentration of these concentrated fractions was too high to run on polyacrylamide gels and so the fractions were dialysed overnight before freeze-drying. Dialysis was carried out against either 50mM Tris, pH8, or 20mM triethanolamine, pH 7.5 or 9.5, depending on which chromatography system had been used. Fractions were analysed by SDS-PAGE (section 3.2.1) and either the OMP or the LPS components were visualised (section 3.2.4). Fractions were also analysed by immunoblotting (section 3.3.1). Quantitative analysis was performed using the protein and KDO assays described in sections 3.2.9 and 3.2.10 respectively.

3.2.8 Determination of antibiotic sensitivities and analysis of OMs of bacteria grown under hyperbaric oxy-helium conditions.

A series of experiments were performed to examine the effects of growth in a hyperbaric oxy-helium environment on the antigenicity and antibiotic resistance of *P. aeruginosa* strains NCIB 8295, NCTC 10662 and E1. An antibiotic-sensitivity screen was developed to compare the antibiotic resistances of *P. aeruginosa* strains grown under normal pressure oxy-helium (5% oxygen) and hyperbaric (90psi) oxy-helium. Oxoid multodiscs were placed on the plates which were incubated in either isobaric conditions in a closed system, or in hyperbaric conditions. To check that the hyperbaric oxy-helium was not altering the diffusion of the antibiotics through the agar, a pre-incubation of agar and multodiscs was prepared. Eighteen hrs later, an overlay of *P. aeruginosa* dispersed in molten agar was added to the plates. The plates were then incubated in either isobaric or

hyperbaric conditions. In experiments investigating the effect of hyperbaric oxy-helium on the surface of *P. aeruginosa*, solid-phase media were used, prepared by the addition of 1.5% agar to CDM-Fe. Growth in a hyperbaric oxy-helium environment was achieved by incubation of seeded plates in a pressure chamber containing 5% oxygen and 95% helium. OMs of bacteria grown in this way were prepared as described previously (section 3.1.1) and analysed by SDS-PAGE (section 3.2.1) for protein and LPS profiles (section 3.2.4). In order to analyse the antigenic profile of bacteria grown under hyperbaric oxy-helium conditions, immunoblotting (section 3.3.1) was performed using serum taken from the diver from whom the clinical strain had been isolated.

3.2.9 Lowry protein assay.

The protein concentrations of OM preparations or fractions eluted from a chromatography column were determined using the assay developed by Lowry *et al* (1951). In some cases, the method of Peterson (1977) was employed; the latter is a simplification of the Lowry method and is more applicable to membrane proteins. Bovine serum albumin (BSA) standards (0-200μg) or samples for analysis were made up in 350μl volumes of distilled water. Each sample was mixed with an equal volume of 1M NaOH and heated at 100°C for 10 min. On cooling, 3ml of a solution containing 1ml 1% (w/v) $CuSO_4.7H_2O$ and 1ml 2% (w/v) NaK tartrate solution in 100ml 2% (w/v) sodium carbonate in 0.1M NaOH was added. After leaving for 10 mins at room temp, 0.3ml Folin Ciocalteau reagent diluted 1:1 with water was added to each tube and the contents were mixed by vortexing. After 30mins the A_{750nm} was recorded and a calibration curve was constructed and used to estimate the protein concentration of the samples.

3.2.10 KDO assay.

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

3.2.11 Analysis of serum proteins.

Purity of monoclonal antibody in ascitic fluid or end supernatant was monitored by analysis on a Paragon gel (Bio-Rad). The agarose gel was loaded with samples and electrophoresed for 25min at 100V. Gels were fixed, dried and destained, according to the manufacturers instructions. Normal mouse serum was run to standardise the gels.

3.3 Immunological techniques.

3.3.1 Immunoblotting.

Transfer of OM components separated by SDS-PAGE to a nitrocellulose membrane and investigation of antigenic sites was performed following the method of Towbin *et al* (1979), as modified by Anwar *et al* (1984). Following electrophoresis, the gel and nitrocellulose (Trans Blot Membrane, Bio-Rad) pore size 0.45μm were sandwiched between chromatography paper, scotch-brite pads and perforated plastic support grids. Electroblotting was performed in a Trans-blot cell, at 100V for 60min in ice-cold blot buffer (192mM glycine, 25mMTris, 20% methanol, pH 8.3). If the LPS antigenic sites were to be investigated the transfer was performed at 50V for 60min. After transfer, the nitrocellulose was removed from the cell, and soaked in Tris-buffered saline (TBS), containing 0.3% Tween 20 and 1% BSA, for 60min to saturate the non-specific binding sites on the paper (Batteiger *et al,* 1982). It was found not to be necessary to bake the nitrocellulose blots, as recommended by Sturm *et al* (1984), for detection of high-molecular weight polysaccharide. Transference of OMPs was confirmed by staining with 1% amido black in 10% methanol/7% acetic acid. Complete quantitative transfer of protein or LPS was confirmed by staining the gel after blotting with Coomassie blue or silver, respectively (section 3.2.4).

Specific immunological detection of antigenic sites was achieved by incubating the immunoblot with the serum under investigation, adjusted to an appropriate dilution (usually 1 in 1000), for 3hrs at 37°C, with shaking. Following this the blot was washed in TBS-Tween and incubated for a further 2hrs with either protein-A-HRP, protein-A-AP or a conjugated secondary antibody (see section 2.2).

HRP conjugates were visualised using a solution containing 25μg/ml 4-chloro-1-napthol

and 0.01% (v/v) hydrogen peroxide in 10mM Tris, pH7.4. The colour was allowed to reach an optimum intensity and then the reaction was terminated by placing the immunoblot in water. The colour reaction for AP conjugated antibodies was initiated by incubating the blot in developing solution, containing 33µl NBT substrate (50mg Nitro blue tetrazolium/1ml 70% dimethyl formamide) and 16.5µl BCIP (50mg 5-bromo-4-chloro-3-indoyl phosphate/1ml 70% dimethyl formamide) / 5ml of alkaline phosphatase buffer (100mM Tris-HCL, 100mM NaCl, 5mM $MgCl_2$, pH 9.5) (Blake *et al*, 1984). The colour was allowed to develop and then the reaction terminated by transferring the immunoblot into a solution containing 20mM Tris-HCl and 5mM EDTA, pH 8. The immunoblots could either be stored in this stop solution or stored dry.

To investigate the reaction of several antisera or monoclonal antibodies with the antigenic determinants in one preparation (or screen with a variety of conjugates) strip immunoblotting was performed. A comb with one large well was placed in the stacking gel of a polyacrylamide gel. After transfer of the electrophoretically separated antigens to the solid phase, the nitrocellulose paper was divided into 6mm strips. One strip was stained with amido black or developed using a serum sample hyper-immune to LPS. The other strips were then placed in plastic trays divided into individual compartments (9cm x 1cm; holding a volume of 5-10ml). Sealed plastic bags were used when the supply of serum was limited. The remaining stages of the immunoblotting protocol were performed as described above.

3.3.2 Enzyme-linked immunosorbent assay.

A whole-cell ELISA method was developed based on the method of Borowski *et al* (1984) for detection of IgG, IgA and IgM antibodies to *P. aeruginosa* antigens. Cells were harvested by centrifugation at 10,000 x g for 10min at 4°C and the pellet was resuspended in PBS,

pH 7.4. A coating cell suspension of 10^8 cfu/ml was found to give optimal sensitivity and low background due to non-specific absorbance.

Two hundred and fifty µl cell suspension was added to each well of a 96 well microtitre plate and the plate shaken for 30min then left overnight at 4°C so that the antigen would adhere to the plastic. Plates were washed 3 times with PBS, pH7.4 containing 0.5% Tween 20. Non-specific binding sites were blocked with a solution containing 1% bovine serum albumin in PBS containing 10% foetal calf serum (Gibco Ltd.) by incubating for 30min with shaking and then at 37°C for 2hrs. The wells were washed as above and then 50µl of the serum under investigation, serially diluted in gelatin phosphate buffer (0.07M sodium phosphate buffer pH 6.5 containing 0.2% gelatin), was added to each well. The plates were incubated for a further 2hrs with shaking at room temperature. The plates were then washed again as above and incubated for 2hrs with 100µl 1:1000 dilution of conjugated antibody per well. The plates were washed as above and 100µl of a substrate solution was added. The substrate solution was prepared by dissolving 10mg 3,3',5,5', tetramethylbenzidine (TMB) in 1ml 70% dimethylsulphoxide and adding this to 100ml 0.1M sodium acetate/citrate buffer pH6. Immediately before use 8µl H_2O_2 was added to the preparation. The plates were shaken at room temperature until a blue colour developed (approximately 10min). The reaction was terminated by the addition of 30µl of 2N H_2SO_4 to each well. Absorption at 450nm was measured using a Dynatech plate reader. Control wells to which gelatin phosphate buffer and conjugate were added consistantly produced absorbance values of <0.2. The mean absorbance of the control wells was subtracted from the test wells. Corrected absorbance readings > 0.1 optical density units at 450nm were considered positive and results were expressed as the reciprocal of the serum dilution giving a corrected A_{450nm} of 0.2. All ELISA determinations were performed in duplicate and standard serum samples were included to standardise the plates. ELISA techniques (Engvall and Perlmann, 1971) using OM preparations as the coating antigen were also

performed.

ELISA techniques were used to determine the isotypes of antibodies in end supernatants and ascites produced during monoclonal antibody generation. Purified LPS (2μg/ml) or OM preparations (diluted 1:500) extracted from iron-limited cultures of the strain used to produced the antibodies were used to coat 96-well microtitre plates, 100μl/well. (Preparations were diluted or dispersed in coupling buffer, containing 1.59g/L sodium carbonate, 2.93g/L sodium bicarbonate and 0.2g/L sodium azide, pH 9.6). End supernatants or ascitic fluids (the latter diluted 1:500) were incubated in the plates for 60min, after a washing procedure had been performed. After washing, 100μl of various biotinylated anti-Ig isotypes (section 2.2, diluted 1:1000 in PBS-Tween containing 1% sheep serum) were added to the plates and incubated for 60min. The plates were then washed and incubated with 100μl streptavidin-HRP for 20min. The plates were washed and developed using 100μl of substrate containing 99ml 0.1M sodium acetate, pH 5.2, 5.8mg TMB in 1ml DMSO and 10μl H_2O_2 . After colour development, the reaction was stopped by the addition of 50μl 2M H_2SO_4. Absorption at 450nm was measured and the isotype of the antibodies determined.

3.3.3 Production of monoclonal antibodies.

3.3.3.1 Preparation of plasmacytoma cells for fusion.

Five to 10 days prior to fusion, a vial of each of the plasmacytoma cell lines was removed from liquid nitrogen storage. Two myeloma lines were used: P3-NS1-Ag4-1 (NS1) (Kohler and Milstein, 1976) and NS0 (Galfre and Milstein, 1981). Both these cell lines originated in Balb/c mice. In addition, an anti-OKT3 hybridoma was used as the fusion partner in fusion A. The cells were rapidly thawed by placing the vials in sterile water at

37°C, then aseptically transferred to 20ml of RPMI 1640 medium containing 10% FCS, and supplemented with pyruvate, glutamine and antibiotic: RPMI medium was made up of 10ml x10 concentrated RPMI 1640 (purchased sterile), 86ml sterile, double distilled, deionised water, 1.1ml 7.5% w/v sodium bicarbonate (filter sterilised and stored at 4°C), 1ml 200mM glutamine (filter sterilised and stored at -20°C), 1ml 10mg/ml sodium pyruvate (filter sterilised and stored at -20°C) and 40µl 250mg/ml kanamycin (Winthorp Ltd.).

The cell suspension was centrifuged at 150 x g for 10min, the supernatant discarded and the cells resuspended in 10ml of fresh medium with 10% FCS. This cell suspension was cultured in a $25cm^3$ tissue culture flask and incubated overnight at 37°C. The cells were then diluted to 10^5 cells/ml and cultured in RPMI 1640 with 10% FCS, in a 5% CO_2 atmosphere. The cell viability is very density-dependent and was maintained at 1 x 10^5-8 x 10^5 cells/ml.

Three days prior to the fusion, the cells were seeded at 1 x 10^5 cells/ml into a Spinner culture vessel (Techne Ltd.) containing a total of 200ml of medium. Twenty four hrs before the fusion, the cells were diluted to 1-2 x 10^5cells/ml so that they were in the logarithmic phase of growth required for optimal fusion efficiency. On the day of fusion, the plasmacytoma cells were harvested by centrifugation (150 x g, 10min), washed 3 times in serum-free medium and counted.

3.3.3.2 Preparation of macrophage feeder layer.

Twenty four hrs prior to fusion, a mature mouse was killed (using chloroform) and soaked in 70% alcohol. The skin was dissected away from the abdomen and held back, exposing the peritoneum. The peritoneal membrane was swabbed with 70% alcohol and

7ml of cold hypoxanthine and thymidine (HT) medium containing 20% FCS was injected into the peritoneal cavity. The abdomen was massaged gently for 1-2min then, using a syringe and a 21G needle, as much fluid as possible was recovered. The cell suspension was made up to 60ml with the same medium. One hundred µl per well of the cell suspension was dispersed into 6 x 96-well tissue culture plates.

3.3.3.3 Immunisation schedule for mice and preparation of spleen cells.

Three F_1 (CBA x BALB/c) mice were immunised with purified IRMPs from *P. aeruginosa* strain PAO1 or strain AK1282. Antigens were prepared by either selective solubilisation (section 3.1.2) and 1% deoxycholate gel filtration chromatography (section 3.2.5), or excision from SDS-PAGE gels (section 3.1.7.1). Fifty-100µg of protein was emulsified in CFA and injected into 3 sites subcutaneously (SC) in the peritoneal wall (100µl/site), using a 23G needle. Four weeks later, 50µg of antigen emulsified in IFA was injected ip, and after a further 4 weeks, a final boost of 10-20µg was given intravenously (iv)(3 days prior to fusion).

During the immunisation schedule, mice were bled either by cardiac puncture or from the tail, and ELISA (section 3.3.2) and immunoblotting (section 3.3.1) screens were used to determine the level and specificity of circulating antibody.

The hyperimmune mouse was anaesthetized with chloroform vapour, then bled by cardiac puncture using a 1ml syringe fitted with a 23G x 5/8" needle. The mouse was swabbed with 70% alcohol in a laminar flow cabinet, and the skin was dissected away from the peritoneum. The peritoneal membrane was swabbed with 70% alcohol and then the peritoneal cavity was opened, exposing the spleen. The spleen was aseptically removed and any fatty or connective tissue was dissected away. It was was placed in 20ml of

serum-free RPMI 1640 medium. The spleen cells were dispersed in the medium, and then the cell suspension was placed in a 50ml tube; the large tissue fragments were allowed to settle out for 5min. The upper cell suspension was recovered and 100µl sample was removed and counted. The cell suspension was washed once by centrifugation in 50ml of medium, resuspended and divided into two halves.

3.3.3.4 Fusion procedure.

The washed spleen cells were split between the two plasmacytoma cell suspensions and mixed at a ratio of 5:1) in a 50ml centrifuge tube. The mixed cell suspension was centrifuged at 150 x g for 10min. After removal of the supernatant, 1ml of a 40% (v/v) polyethylene glycol (PEG) 4000 (Merck Ltd.) in serum-free medium was added to the cells over a period of 1min, while stirring with the pipette tip. The cell suspension was allowed to stand for 1min, then 45ml serum-free medium was added over a 5min period. The cell suspension was centrifuged a 150 x g for 10min and the cell pellet crudely resuspended in 10ml of RPMI with 20% FCS and a x2 concentration of H and T. The cell suspensions were then incubated at 37°C for 2hrs, then centrifuged at 150 x g for 10min. The supernatant was removed and the cell pellet disaggregated by gently tapping the tube. The cells were resuspended very gently in 35ml HT medium containing 20% FCS and a x2 concentration aminopterin (A). One hundred µl of this cell suspension was pipetted into the 3 x 96 well flat-bottomed tissue culture plates containing the macrophages in 100µl of medium with 20% FCS containing H and T. The plates were incubated at 37°C in small chambers gassed with 5% CO_2 in air.

3.3.3.5 Maintenance of hybridomas.

The cells were fed every 2-3 days after fusion by removing 100µl of spent medium and

gently replacing this with 100µl of fresh medium containing HAT and 20% FCS. Care was taken not to disturb the colonies. After about 10 days in culture, the cells were re-fed with medium containing 20% FCS with H and T. The colonies were maintained in this medium until cloning was completed. Clonal growth was monitored with an inverted microscope and wells were screened after about 12-14 days in culture.

3.3.3.6 Screening of cell supernatants.

Initial screening of supernatants for specific antibody production was performed using the ELISA technique (section 3.3.2). After this, immunoblotting (section 3.3.1), using the cell supernatants to probe blots of OMs, was carried out.

3.3.3.7 Cell cloning.

Ninety µl of medium containing 20% FCS + HT was placed in each of the first wells (12 per plate) of 7 plates containing 100µl/well of macrophages (section 3.3.3.2). Ten µl of cells from the clone was aspirated and then the cells were placed in these first wells (3 wells per positive well). The cells were doubly diluted down the plate and then 100µl of medium with 20% FCS + HT was added to each well. The plates were incubated as before and clonal growth was assessed. The plates were assayed (ELISA, section 3.3.2) for specific antibody. Antibody positive wells with the lowest initial seeding numbers were recloned twice. After the final cloning the clone was expanded to provide supernatant for characterisation by immunoblotting (Section 3.3.1). Antibodies produced from the NS1 line were numbered from 1 and those produced from the NSO line were numbered from 101. Positive clones were expanded for further characterisation.

3.3.3.8 Production of ascites.

A mouse was injected intraperitoneally (ip) with 0.5ml of pristane (2,6,10,14-tetramethylpentadecane, Aldridge Chemical Co). Twenty ml of cell suspension was harvested by centrifugation (150g for 10min) and resuspended in 0.5ml of sterile PBS. This was injected into the mouse ip (4-60 days after pre-treatment with pristane). 7-10 days after injection, the ascitic fluid was collected using a 5ml syringe while the mouse was anaesthetised. A 10% v/v solution of EDTA-sodium azide was added to prevent clot formation; the ascitic fluid was centrifuged for 10min at 200 x g and the supernatant collected and stored at -20°C.

3.3.4 Immunofluorescence techniques.

Immunofluorescence was performed by a modification of the procedure of Hofstra *et al* (1979). Iron-limited and iron-replete cell cultures were grown and the cells were harvested by centrifugation. The cells were washed once in 0.85% saline and then resuspended to an A_{470nm} of 1.0. The bacterial suspensions were smeared onto a glass slide, allowed to air dry then fixed for 5min in ice-cold acetone. The slides were washed in PBS then monoclonal antibody tissue culture supernatants or ascitic fluid (diluted 1:100 in PBS) was added to the slide and incubated for 1hr at 37°C. The slides were washed in PBS and then a 1:25 dilution of rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) was added and incubated for 30min at 37°C. The slides were washed once again then mounted in PBS. The bacteria were examined at 492nm by epi-illumination with an ultra-violet microscope.

3.3.5 Immunogold labelling of *P. aeruginosa*.

P. aeruginosa PAO1 was cultivated as described in CDM-Fe (section 2.5) for 18hrs at 37°C with shaking. Cells were harvested by centrifugation at 10,000 x g for 5min. The cells were washed twice in 0.85% sterile saline, then resuspended to an A_{470nm} of 1.0. One ml of bacterial suspension was fixed with 0.1% glutaraldehyde or formaldehyde for 10min. The fixatives were washed from the cells using 3 PBS washes (centrifugation at 13,000 x g in an Eppendorf centrifuge); cells were then resuspended to 200µl and 10µl aliquots were distributed into Eppendorf tubes.

Ninety µl polyclonal serum or ascites fluid (diluted 1:10 or 1:100 in PBS) or end supernatant (diluted 1:2) was added to the cells and incubated, with rotation, at 37°C for 60min. A negative control ascites (mouse anti-human luteinising hormone, IgG_1 subclass) was used as a negative control. After incubation, cells were washed as before and resuspended to 40µl in PBS. Twenty µl of this cell suspension, either neat or diluted 1:2, was pipetted onto parafilm and electron microscope grids were suspended, inverted, on top of the samples. The grids were left for 10min to allow the bacteria to adhere; they were then removed, excess cell suspension was blotted from them, and then they were washed by placing on drops of PBS for 1min. They were then blotted again, placed on a 1:10 dilution of immunogold conjugate (see section 2.2) and left in a closed petri dish overnight at 4°C, with some damp filter paper underneath the parafilm. The following day the grids were washed in water, then counterstained by incubation in 0.5% phosphotungstic acid for 1min. They were washed once again and examined under the electron microscope (Phillips EM 300).

3.3.6 Protection studies.

3.3.6.1 Determination of LD_{50} values.

Cells of *P. aeruginosa* serotypes 1-5 (PAO1 representing serotype 5) and AK1282 were grown as described in section 2.5 in iron-depleted CDM, shaking at 37°C. To obtain cells in log-phase growth, a 1% inoculum of an overnight culture in CDM-Fe was added to pre-warmed CDM-Fe. The cells were cultured for 5hr, then harvested by centrifugation at 10,000 x g for 5min. Cells were washed in 0.85% NaCl, then resuspended to an A_{470nm} of the highest required for the challenge. Dilutions of this cell suspension were made in 0.85% NaCl, the A_{470nm} read, and then the cell suspensions which had the required A_{470nm} were loaded into 2ml syringes. Groups of 8 or 10 mice were challenged with 0.5ml of live bacteria administered ip. Deaths were monitored for 7 days after challenge, and the LD_{50} values were calculated following the method of Reed and Muench (1938).

3.3.6.2 Passive protection studies.

Passive protection antiserum, ascitic fluid or control serum was administered ip or iv, 1.5hrs prior to bacterial challenge. Viable counts were performed on all cell suspensions given to the mice, and deaths were monitored for 7 days after the challenge.

4-7 Results and discussions.

4 Studies on the purification of IRMPs.

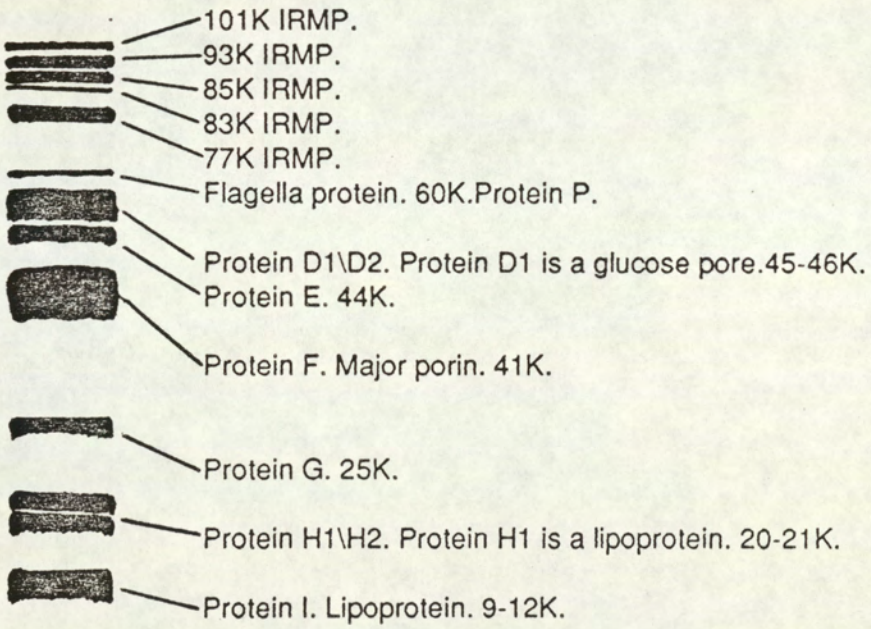
4.1 Introduction.

To study the expression and immunological roles of the IRMPs produced by *P. aeruginosa*, methods were devised to purify these proteins in sufficient quantities for immunological examination. One of two techniques were used, either selective solubilisation followed by gel-filtration or ion-exchange chromatography, or excision and elution of these proteins from SDS-polyacrylamide gels. This section describes the preparation of antigens that were utilised to produce polyclonal and monoclonal antibodies specific for the IRMPs; these antibodies were subsequently used in studies of the expression (surface-exposure and cross-reactivity) of IRMPs and the potential of these proteins to elicit the production of protective antibodies.

4.2 Preparation and analysis of *P. aeruginosa* OMs.

IRMPs were induced in the OM of bacteria cultivated in the chemically defined medium described by Noy (1982) designed to restrict the supply of iron. The OMP profile of *P. aeruginosa* was visualised by staining an electrophoretically separated OM preparation with 0.1% Coomassie brilliant blue (a diagrammatic form is shown in fig. 4.2.1); the molecular weights (determined by comparison of R_f values with those of known molecular weight markers) and functions (where known) are shown. The OMP profile was found to conform to that described by Mizuno and Kageyama (1979). SDS-PAGE gel scans of OMs prepared from iron-restricted cultures indicated that IRMPs represented 22.5% of the Coomassie brilliant blue-stained material; they were therefore considered to be major

Fig. 4.2.1. Digrammatic representation of the OMP profile of *P. aeruginosa*, (when this organism is grown under iron-restricted conditions) as visualised on a Coomassie brilliant blue-stained 12% SDS-Polyacrylamide gel.



OMPs under these growth conditions. Despite this fact, it was necessary to start from a large initial cell mass. Generally, 4 x 2L cell batch cultures of CDM-Fe were grown to give the starting material during developmental protein purification. A large batch culture (40L) was grown, but iron-contamination of the fermenter produced 134g cell paste of bacteria that did not express IRMPs.

The presence of 10mM EDTA in the N-lauryl sarcosine buffer used in the preparation of bacterial OMs was found to reduce the amount of LPS in the preparation 5-10 fold, as measured by KDO assay, and EDTA was therefore included in all incubations even though it resulted in a 30% lowering of protein content in the OM preparation (data not shown).

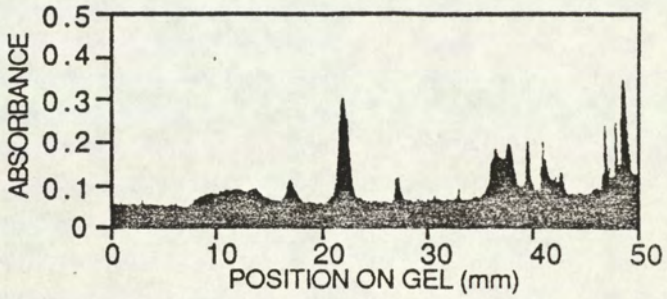
IRMPs were induced in the OMs of bacteria in the simple salts medium and therefore there was no requirement for the preparation of chelexed complex medium. Cultures not expressing IRMPs could be obtained by growing cells in CDM+Fe. However, it was found that cells had to be harvested before the stationary phase of growth, or the bacteria exhibited a low expression of IRMPs.

4.3 Solubilisation of OM preparations.

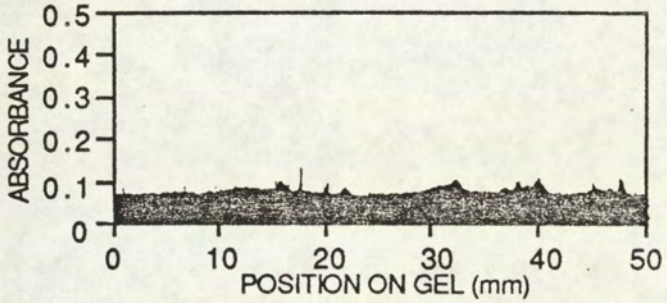
In order to purify the proteins, a range of detergents was tested for their ability to solubilise IRMPs of *P. aeruginosa.* It is advantageous to convert the solubilisation step into the first stage of protein purification; this can be achieved by the use of a detergent that selectively solubilises the proteins of interest. Other factors that must be taken into consideration in the choice of a detergent are that it must be effective at low concentrations and temperatures, and that it must not alter the antigenicity of important antigenic determinants. Additionally, the detergent should be ineffective at solubilising LPS and it should be compatible with ion-exchange purification techniques.

Fig. 4.3.1. Laser scans of SDS-PAGE gels showing solubilisation of OMs of *P. aeruginosa* PAO1 with different detergents.

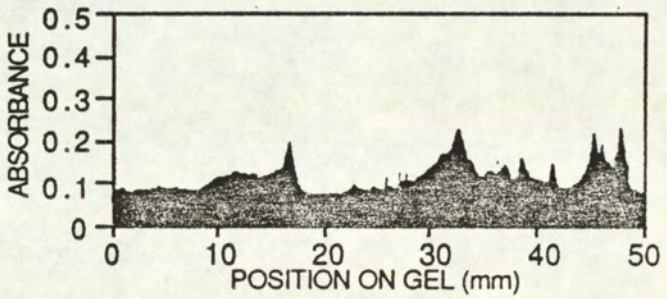
a. OM profile of *P. aeruginosa* PAO1 grown in CDM-Fe.
IRMPs = 22.5%
Total intensity = 2456.5



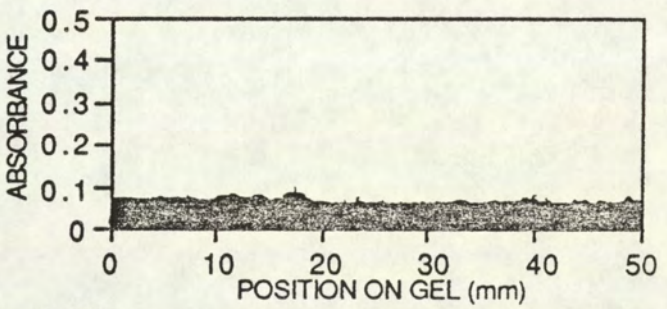
b. 0.5% Deoxycholate solubilised OM.
IRMPs = 17.9%
Total intensity = 467.2



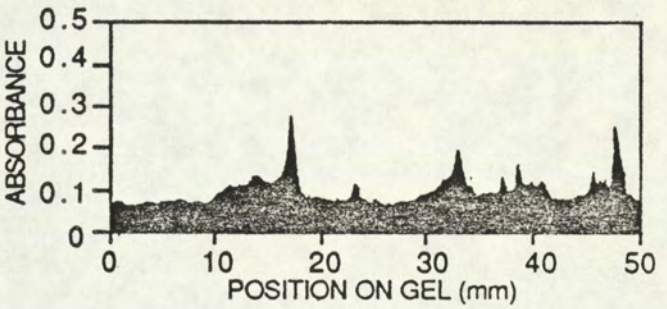
c. 0.5% SDS solubilised OM.
IRMPs = 23.9%
Total intensity = 2227.0



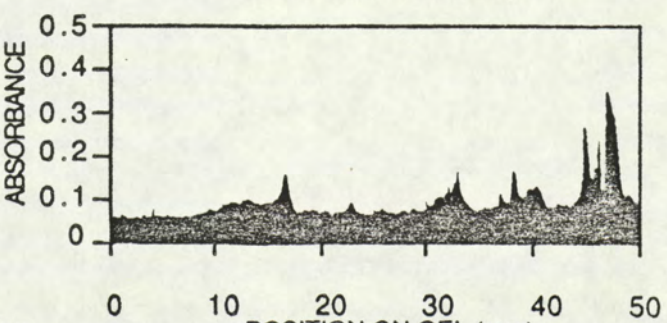
d. 0.5% Cetyl dodecyl trimethyl ammonium bromide solubilised OM.
IRMPs = 1.2%
Total intensity = 77.8



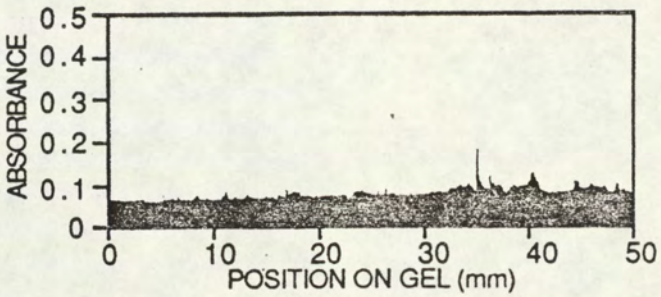
e. 0.5% 3- (3-Cholamidopropyl) dimethylammonio -1-propanesulfonate (CHAPS) solubilised OM.
IRMPs = 21.8%
Total intensity = 1628.4



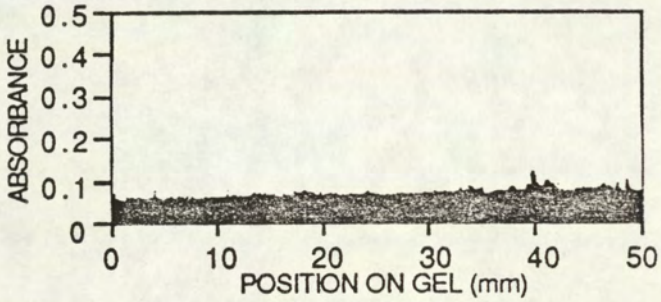
f. 0.15% Empigen BB solubilised OM.
IRMPs = 51.4%
Total intensity = 2155.5



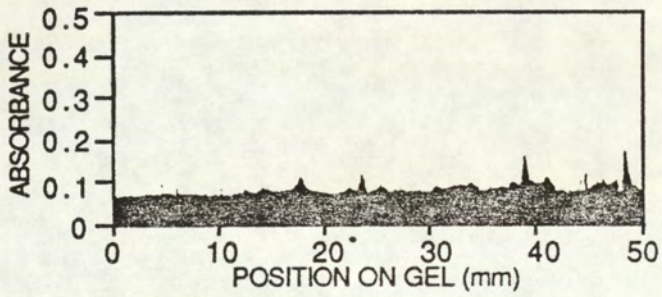
g. 0.5% Polyoxyethylene 23 lauryl ether (Brij 35) solubilised OM.
IRMPs = 32.8%
Total intensity = 224.4



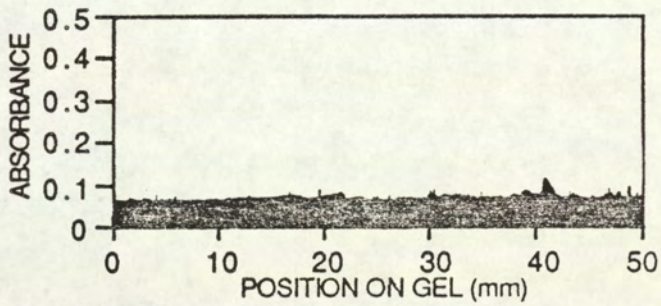
h. 0.5% Polyoxyethylene ether W-1 solubilised OM.
IRMPs = 28.8%
Total intensity = 178.2



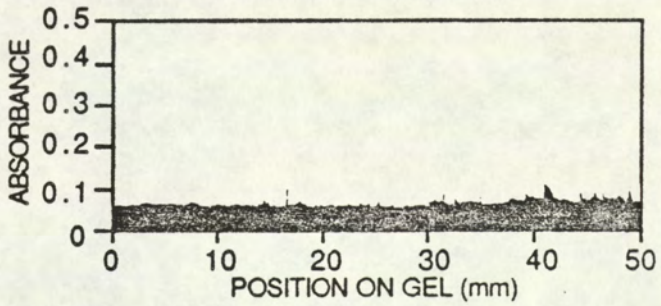
i. 0.5% Triton x 100 solubilised OM.
IRMPs = 27.7%
Total intensity = 558.6



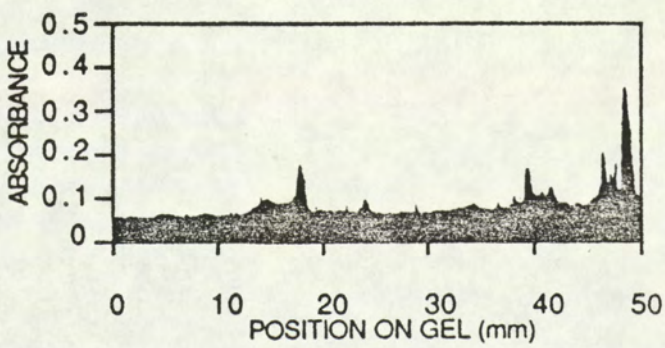
j. 0.5% Tween 20 solubilised OM.
IRMPs = 15.6%
Total intensity = 87.4



k. 0.5% Tween 80 solubilised OM.
IRMPs = 19.5%
Total intensity = 97.2



l. 0.5% Nonidet P 40 solubilised OM.
IRMPs = 51.7%
Total intensity = 1509.8



OMs of *P. aeruginosa* strain PAO1 were prepared and extracted with the detergents detailed in fig. 4.3.1. Solubilised extracts were analysed on SDS-PAGE for both their protein and LPS contents and quantitative assays were performed using protein and KDO assays. The solubilisation profiles are shown as scans of Coomassie brilliant blue stained gels (fig. 4.3.1), in which the IRMPs are represented as the 3-4 peaks on the right hand side of the traces.

Selective solubilisation of IRMPs was achieved with some of the detergents tested. The percentage of the total Coomassie brilliant blue-stained material which the IRMPs represented in the solubilised extracts varied from 1.2% (for cetyl trimethyl ammonium bromide) to 51.7% (for Nonidet NP40) as compared to 22.5% for the OM preparation. These figures must be considered together with the total intensity of the gel scans, as this is a measure of the efficacy of the detergent.

In general, integral proteins can be extracted from membrane preparations by anionic detergents (like SDS) and, in fact, SDS was very effective at solubilising the OM components. There are, however, drawbacks associated with the use of anionic detergents in purification strategies since they may not be compatible with ion exchange techniques. In addition, the resulting SDS-protein complexes exhibit loss of biological activity due to the cooperative denaturation produced by SDS (Helenius and Simons, 1975). The retention of antigenic properties was very important for the immunological studies, so SDS was not chosen for these reasons and also because it did not selectively solubilise the IRMPs.

In contrast to ionic detergents, non-ionic and zwitterionic surfactants often exhibit low solubilising efficiency of membrane proteins and do not interfere with the biological activity of the solubilised proteins (Helenius and Simons, 1975). These detergents, despite their low efficacy, have been suggested as being useful for purification

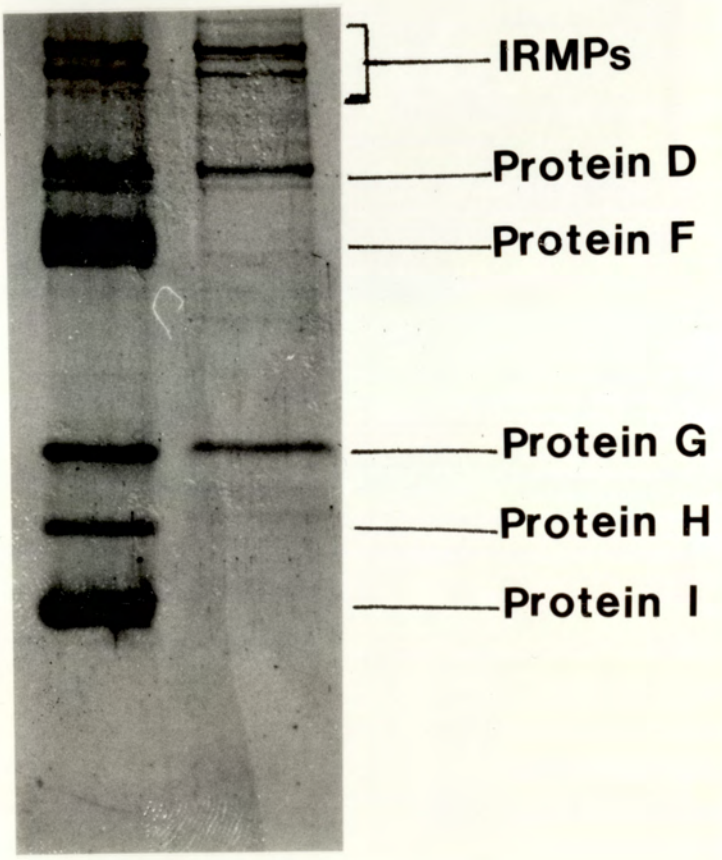
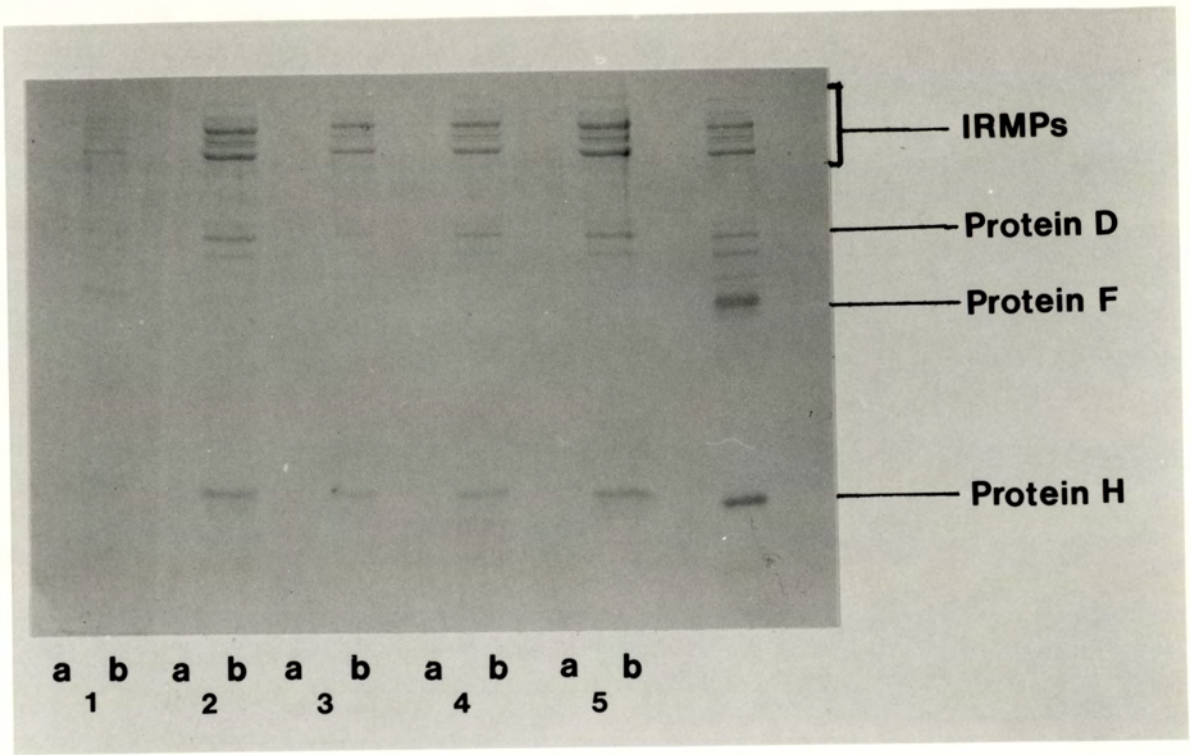


Fig. 4.3.2. 0.1% Coomassie blue stained 12% SDS-PAGE gel of an OM preparation of *P. aeruginosa* strain PAO1 (lane 1) and a 0.5% Nonidet (+10 mM EDTA) extract of this preparation (lane 2). This figure shows selective solubilisation of the IRMPs.

a



b

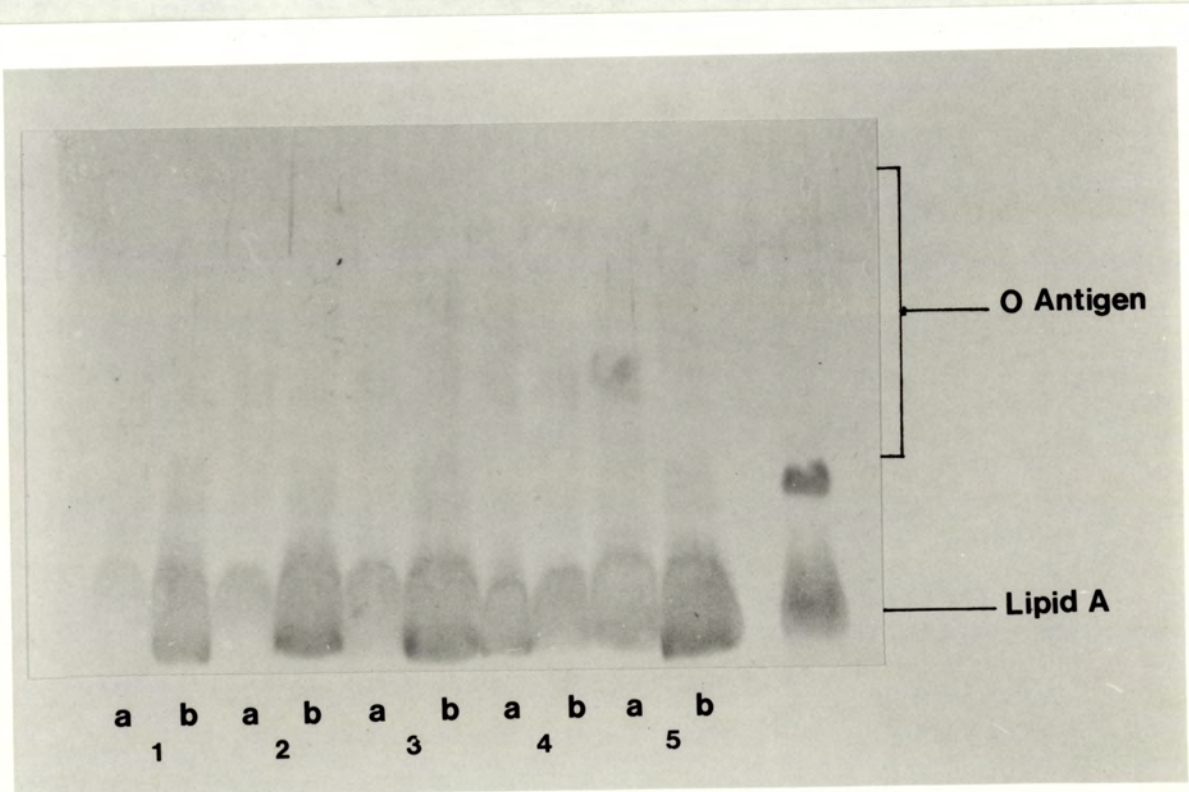


Fig. 4.3.3. Effect of concentration of detergent and inclusion of EDTA in the solubilisation buffer on the solubilisation of IRMPs. 0.1% Coomassie brilliant blue (a) and silver nitrate stained (b) 12 % SDS-PAGE gels showing selective solubilisation of IRMPs of P. *aeruginosa* strain 6750. 0.0625% (lane 1, a and b), 0.125% (lane 2, a and b), 0.25% (lane 3, a and b), 0.5% (lane 4, a and b) and 1% (lane 5, a and b) Nonidet NP40-solubilised extracts are shown. 10mM EDTA was included in the solubilisation buffer for samples in tracks labelled b. In addition, the OM preparation is shown on the right side of the gels.

applications (Gonenne and Ernst, 1978).

Both Empigen BB (zwitterionic) and Nonidet NP40 (non-ionic) were highly effective at selectively solubilising IRMPs and therefore these detergents were chosen. Fig. 4.3.2 shows a Coomassie brilliant blue stained SDS-PAGE gel of a 0.5% Nonidet solubilised extract of *P. aeruginosa* strain PAO1.

The effect of the inclusion of 10mM EDTA in the solubilisation buffer on the ability of various concentrations of Nonidet NP40 to solubilise OM components was investigated. Protein and LPS gels (fig. 4.3.3) indicated that the amount of LPS solubilised was greater in the presence of EDTA, but there was a greater relative solubilisation of the OM proteins. EDTA was therefore included in all solubilisation buffers.

4.4 Gel-filtration chromatography.

IRMPs were futher purified by gel-filtration chromatography using a detergent system. The solubilised extracts of OM preparations were applied to a Superose 12 gel-filtration column, and initially Nonidet NP40, Empigen BB and SDS buffer systems were examined.

Fractions eluting from the columns were analysed for their protein and LPS contents. It was found that the gel-filtration technique using these buffer systems effected a good separation of IRMPs from other OM proteins, however, when the corresponding LPS contents were examined, it was found that there was a large amount of LPS contaminating the fractions containing the IRMPs.

Due to the failure of these chromatography systems to separate IRMPs from other OM components, a deoxycholate gel-filtration system was employed (Heckels, 1981) reported to disrupt protein-LPS complexes. Sodium cholate and deoxycholate break down LPS into

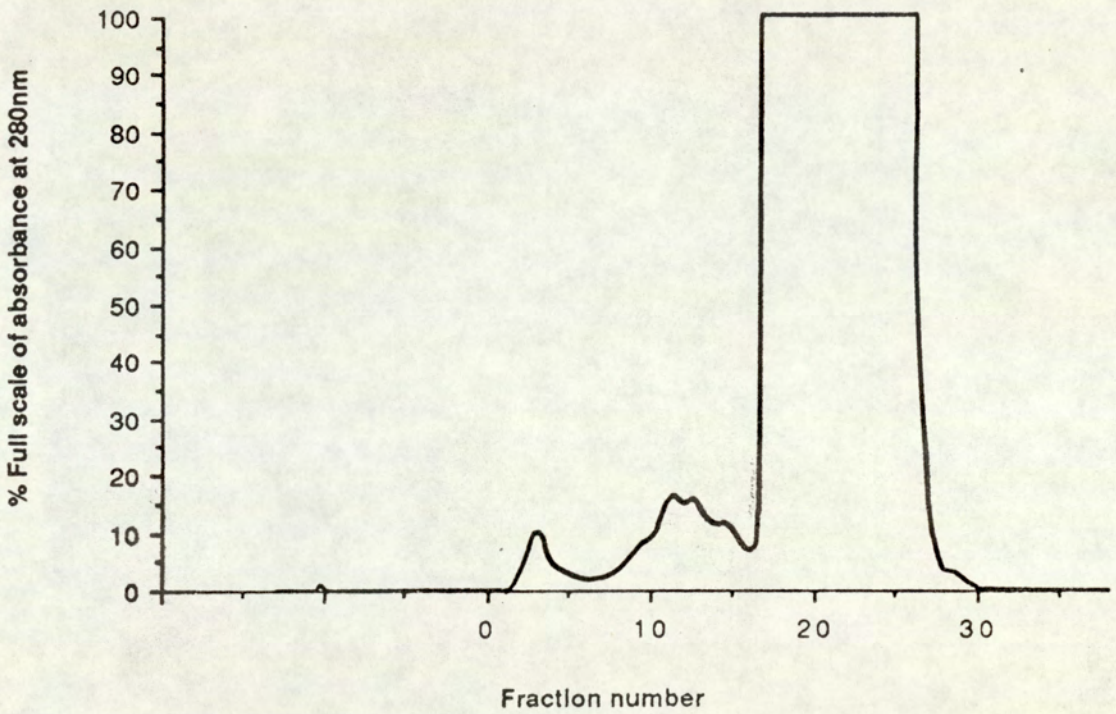


Fig. 4.4.1. Elution profile of a 1% deoxycholate gel-filtration column loaded with 500µl of a 0.5% Nonidet NP40-solubilised extract of *P. aeruginosa* strain 6750. The elution buffer contained 1% deoxycholate, 0.1M glycine, 10mM EDTA, 0.001M PMSF, 0.02% sodium azide and 0.1M sodium chloride, pH 9.5. All fractions were analysed SDS-PAGE for their protein and LPS content: the Coomassie brilliant blue stained gel is shown in fig. 4.4.2. The flow rate was 0.5ml/min and the full scale detection at A_{280nm} was 0.2.

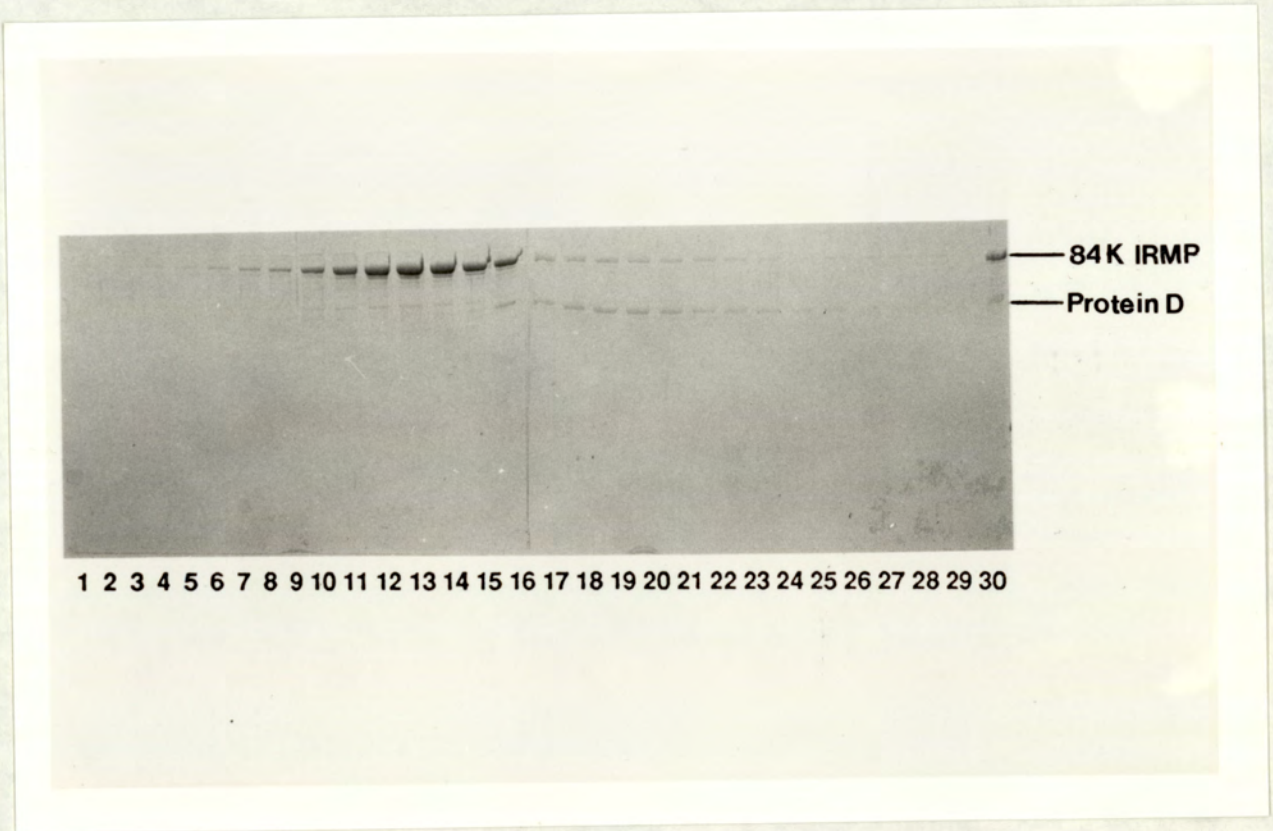


Fig. 4.4.2. Coomassie brilliant blue stained 12% SDS-PAGE gel analysis of fractions 1-29 (lanes 1-29) eluted from a 1% deoxycholate gel-filtration chromatography column. The 0.5% Nonidet Fe- OM extract of *P. aeruginosa* strain 6750 which was loaded onto the column is shown in lane 30. A flow rate of 0.5ml/min was used.

monomeric sub-units of about 7K (Heckels, 1981; Ribi *et al*, 1966); these monomers have a longer retension time on a gel-filtration column. Using the Superose 12 column and a flow rate of 0.5 ml/min, a 0.5% Nonidet NP40 OM extract of *P. aeruginosa* strain 6750 was chromatographically separated. Two hundred µl fractions were collected from 12 minutes after sample injection. A distinctive elution profile was produced (fig. 4.4.1); the large peak was lipid A, eluting from the column late in the chromatography program (fractions 17-28). Protein SDS-PAGE gel analysis revealed that the IRMPs were collected mainly into fractions 9-15 (fig. 4.4.2). Silver-stained LPS gels demonstrated an effective separation of IRMPs from lipid A, but it was unclear whether O-antigen was contaminating the proteins.

Immunological detection is more sensitive than staining on SDS-PAGE and therefore analysis of fractions was performed using this technique. The deoxycholate-based separation procedure, with the alteration that an extract from LPS smooth strain PAO1 was used, (instead of 6750) was repeated. The elution profile is shown in fig. 4.4.3. Fractions, collected from 12 minutes after the injection of the sample and examined in immunoblotting assays (using rabbit serum hyperimmune to OMs Fe- of *P. aeruginosa* PAO1 as a probe), revealed that the proteins were not as highly purified as at first thought (figs. 4.4.4 and 4.4.5). The characteristic bands of the O-antigen were fractionated but still eluted from the column in the same fractions as the IRMPs and therefore this technique produced a preparation of IRMPs that was contaminated with O-antigen. The immunoblots confirmed that there was a good separation of proteins from lipid A.

Immunoblot analysis additionally revealed that the proteins were not eluted from the column according to their apparent molecular weights (as determined by SDS-PAGE). This was thought to be because the proteins were separated on the column in a non-denatured state, but analysed by SDS-PAGE in a denatured form. Protein D and a protein of

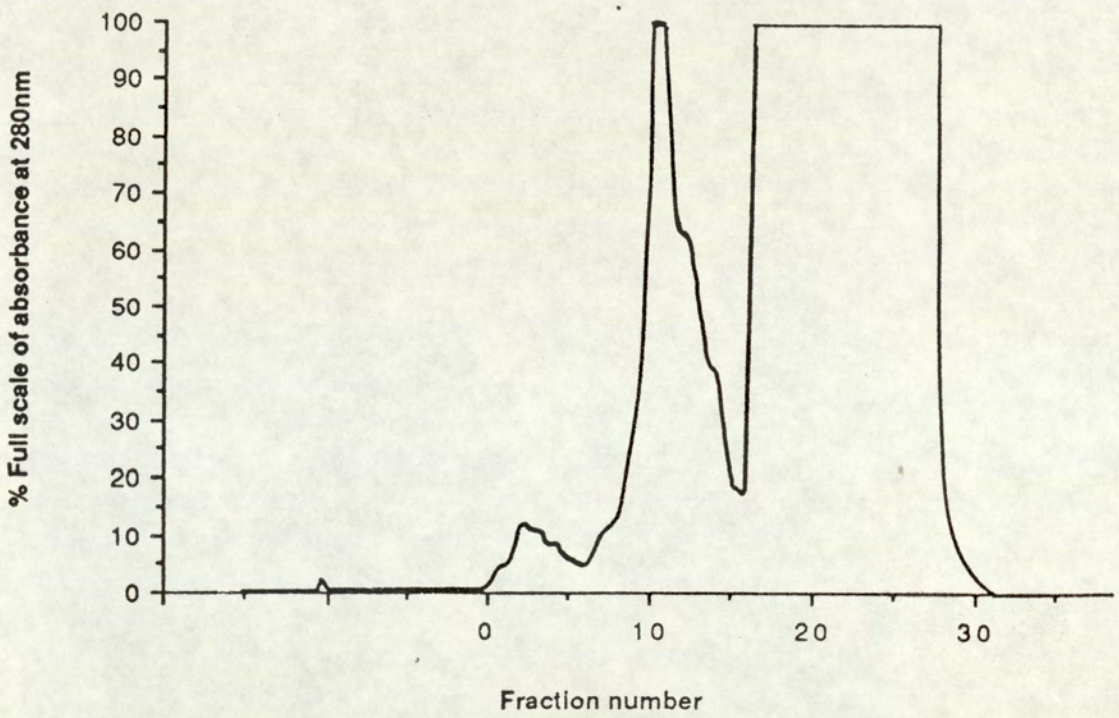


Fig. 4.4.3. Chromatography trace of a 1% deoxycholate gel-filtration column. The sample applied to the Superose 12 column was a 0.3% Empigen BB-solubilised extract of *P. aeruginosa* PAO1 Fe- OMs. The elution buffer contained 1% deoxycholate, 0.1M glycine, 10mM EDTA, 0.001M PMSF, 0.02% sodium azide and 0.1M sodium chloride, pH 9.5. All fractions were analysed by SDS-PAGE and immunoblotting for their protein and LPS contents: immunoblots are shown in figs. 4.4.4 and 4.4.5. The flow rate was 0.5ml/min and the full scale detection at A_{280nm} was 0.2.

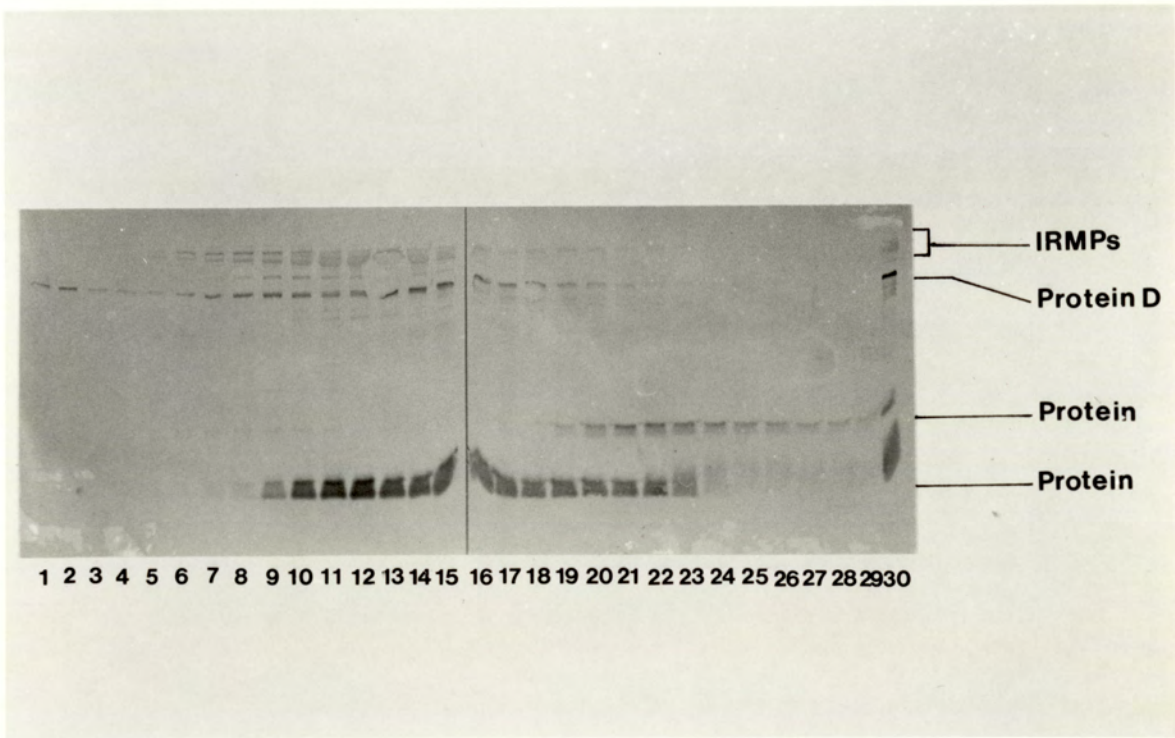


Fig. 4.4.4. Protein immunoblot analysis of fractions 1-29 (tracks 1-29) eluting from a 1% deoxycholate Superose 12 gel-filtration column that was loaded with a 0.3% Empigen BB solubilised OM extract from *P. aeruginosa* strain PAO1. The blot, which was probed with serum raised to OMs Fe- of *P. aeruginosa* strain PAO1, corresponds to the chromatograph depicted in fig. 4.4.3. Track 30 shows the antigens in the extract recognised by the serum. IRMPs eluted in fractions 6-22. Protein A-AP conjugate.

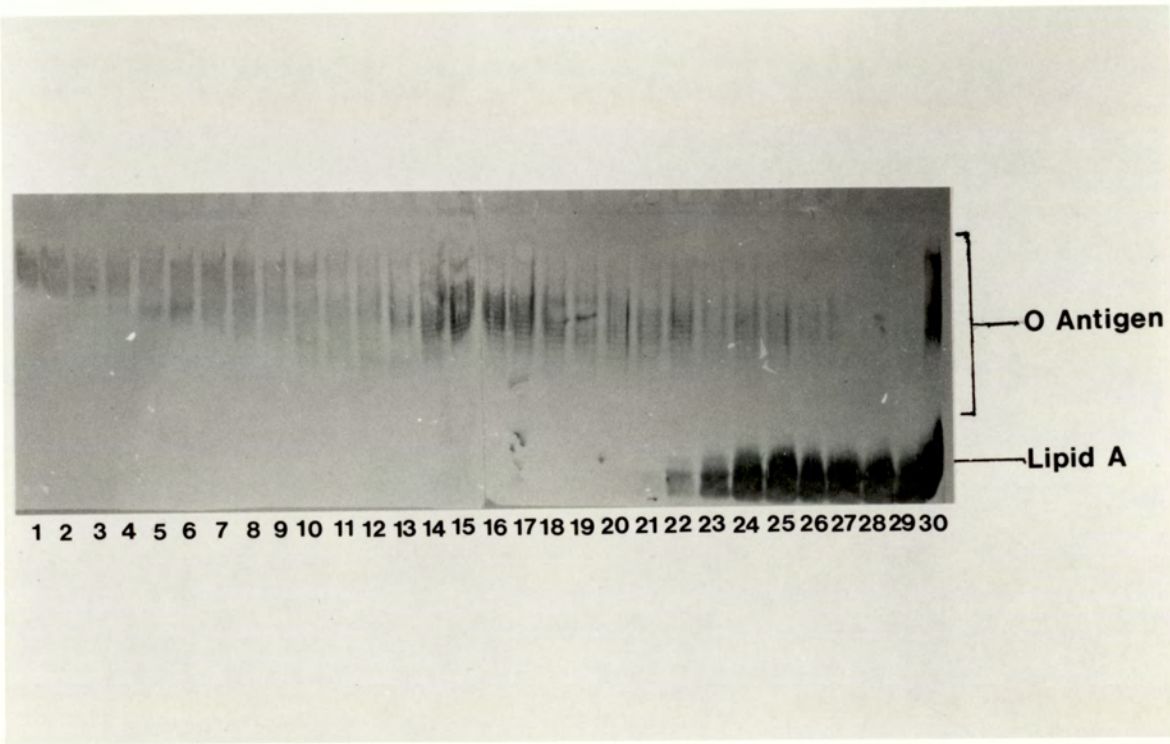


Fig. 4.4.5. LPS immunoblot analysis of fractions 1-29 (tracks 1-29) eluting from a 1% deoxycholate Superose 12 gel-filtration column that was loaded with a 0.3% Empigen BB solubilised OM extract from *P. aeruginosa* strain PAO1. The blot, which was probed with serum raised to OMs Fe- of the same strain, corresponds to the chromatograph depicted in fig. 4.4.3. Samples were treated with proteinase K to digest proteins and electrophorectically separated on a 12% SDS-PAGE gel. The figure shows fractionation of the O-antigenic moiety of the LPS molecule and, additionally, the lack of lipid A in fractions 1-21. Track 30 shows the antigens in the solubilised extract recognised by the serum. Protein A-AP conjugate.

approximate molecular weight of 10K (possibly protein I) were particularly noticeable. Protein D eluted before the IRMPs from the Superose 12 column. The 10K protein eluted very early from the column (and would therefore be considered to be of a large molecular size), however on denaturation, it had a relatively low molecular weight. It is suggested that perhaps this protein migrated down the column as a complex with other proteins or OM components. On SDS-PAGE, the protein migrated at the same rate as the lipid A moiety of LPS which normally masks its detection, but it was distinguished as the lipid A component had been separated from the other OM components, (except proteins H1 and H2). This component was recognised on a protein immunoblot, but was sensitive to proteinase K and was therefore not detected on an LPS immunoblot of proteinase K digested samples.

4.5 Selection of a rough mutant of *P. aeruginosa.*

Initially, a smooth strain of *P. aeruginosa* was chosen until it was established that the deoxycholate gel-filtration system was effective at separation of the proteins of interest from lipid A, but not from O-antigen. This finding forced the selection of a rough mutant of *P. aeruginosa* to circumvent the problem. Strains 7/1, 18s and CF003 (Pitt *et al*, 1986) obtained from Colindale were examined by SDS-PAGE gel analysis. An LPS gel of these strains, together with the parent strains of 7/1 and 18s are shown in fig. 4.5.1. From this investigation, strain 7/1 (which was selected by resistance to phage 7 from the phage typing set described by Asheshov, 1974) gave a banding pattern on SDS-PAGE similar to that of the parent LPS, but lacking the high molecular weight components. Strain 18s appeared to be the more promising, as it appeared on silver stained SDS-PAGE gels to have dramatically less O-antigen, leaving only the core polysaccharide and lipid A components of the LPS remaining; this confirmed the findings of Pitt *et al*, (1986). 18s was isolated from the same cystic fibrosis sputum as a serum-resistant culture (18r) and was thought to be derived from 18r on the basis of pyocin type and reversion of 18s to 18r

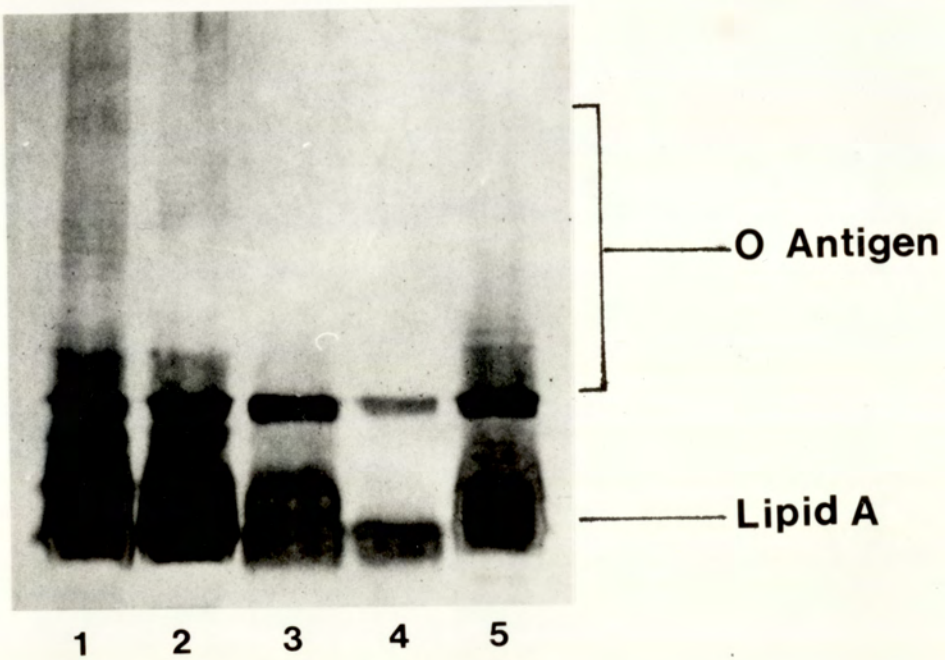


Fig. 4.5.1. Selection of a rough mutant of *P. aeruginosa.* 12% SDS-PAGE gel of strains 7 (lane 1), 7/1 (lane 2), 18r (lane 3), 18s (lane 4) and CF003 (lane 5), stained with silver nitrate for detection of LPS.

(expression of O-antigen 5d). Both 18s and 18r were lysed by the LPS-specific phage E79 (Jarrell and Kropinski, 1976; 1977). Strain 18s was not selected for further use, however, as it had poor growth characteristics in the CDM-Fe.

Other rough mutants which have been used by researchers interested in the contribution of LPS to the pathogenicity of Gram-negative bacteria, have been isolated (Jarrel and Kropinski, 1976; 1977; Kropinski and Chadwick, 1975; Temple *et al*, 1986). These LPS-defective mutants have been selected by their resistance to LPS-specific phages. The LPS-specific phage øPLS27, used to isolate *P. aeruginosa* AK1282 has been characterised extensively (Jarrell and Kropinski, 1981a; 1981b). Strain AK1282 was resistant to øPLS27 and also to E79 and PBI. Meadow and Wells (1978) showed that the receptor sites for bacteriophage E79 were in the core part of LPS of *P. aeruginosa.* Strains AK1282 and AK1012 were obtained from A.M. Kropinski and analysed by SDS-PAGE to investigate their protein and LPS profiles. Strain AK1282 was selected as this organism grew well in the CDM-Fe medium, fully expressed IRMPs and lacked O-antigen. Additionally, an advantage of using this strain to continue the development of a purification strategy was that it was derived from *P. aeruginosa* strain PAO1, and therefore serum that had been raised to PAO1 could be used to probe immunoblots of strain AK1282.

OMs of the rough mutant AK1282 were prepared and solubilised in 0.5% Nonidet NP40 before being subjected to the 1% deoxycholate gel-filtration system developed using the smooth strains. A characteristic elution profile is shown in fig. 4.5.2. It was noted that the peak corresponding to lipid A was decreased considerably: the chromatogram was measured at 0.1 full scale detection, rather than 0.2 as in the previous traces. The peak produced by the IRMPs was well defined. This run was repeated 12 times in order to prepare sufficient antigen to use for passive protection studies 3 and 4 (see section 7) and also to raise monoclonal antibodies (fusion C, section 6). The chromatography was very reproducible as the 12 chromatographs could be superimposed.

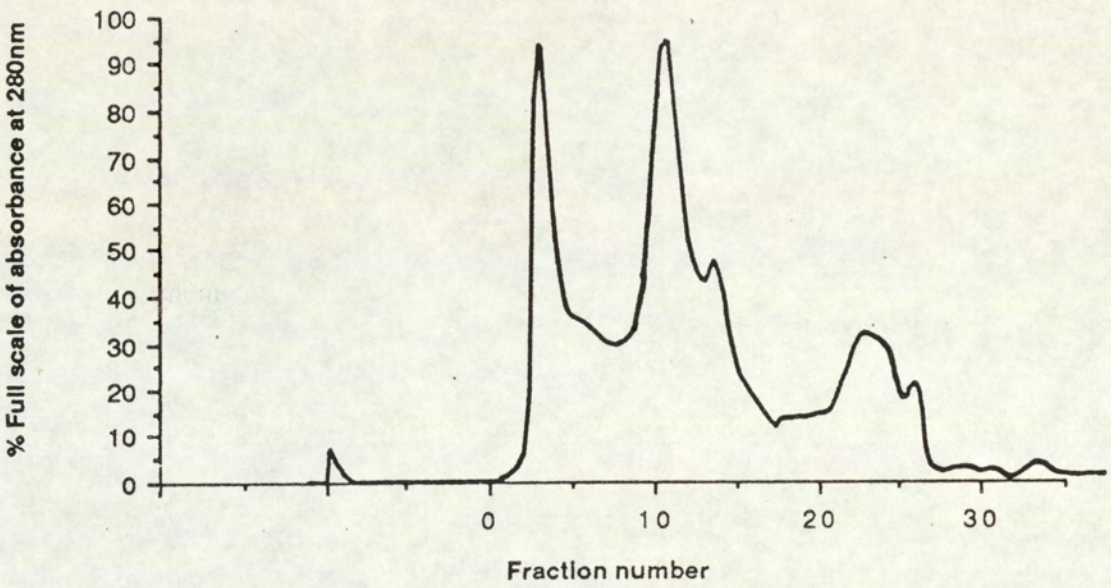


Fig. 4.5.2. Chromatography trace of a 1% deoxycholate gel-filtration column. The sample applied to the Superose 12 column was a 0.3% Empigen BB solubilised extract of *P. aeruginosa* AK1282 Fe- OMs . The elution buffer contained 1% deoxycholate, 0.1M glycine, 0.001M PMSF, 10mM EDTA, 0.02% sodium azide and 0.1M sodium chloride, pH 9.5. All fractions were analysed by SDS-PAGE and immunoblotting for their protein and LPS contents.The flow rate was 0.5ml/min and the full scale detection at A_{280nm} was 0.1.

SDS-PAGE and immunoblot analysis revealed that an effective separation of proteins from lipid A was achieved, and no O-antigen could be detected. The extract prepared from this set of chromatography was very enriched for IRMPs, and consequently, good separation of IRMPs from other OM proteins was obtained.

4.6 Iso-electric focusing.

Another approach applied to the purification of IRMPs was ion-exchange. To design efficient ion exchange or chromatofocusing separation procedures, electrophoretic titration curve analysis and iso-electric focusing were performed to determine the pH at which charge differences of the proteins in the solubilised OM extract were maximal. A Phast system (Pharmacia) was used as this system required only minute amounts of protein, in contrast to those used in larger systems.

Initially, iso-electric focusing (IEF) was performed on standard Pharmacia gels, however it was found that the proteins in a solubilised preparation of OMs precipitated at the point of application. Consequently, a detergent-soaked iso-electric focusing system was developed which was applicable to the separation of membrane proteins. Coomassie brilliant blue-stained and silver-stained IEF gels did not produce a clear result; the solubilised OM extract was too complex to allow identification of the IRMPs. Immunoblots of IEF gels, using serum raised to IRMPs purified by 1% deoxycholate gel-filtration, did not yield a clearer result. A Coomassie brilliant blue-stained titration curve produced using the detergent system, however, indicated that the major IRMPs had pI values of approximately 6.5. This result was confirmed by investigation of the binding of proteins to Q-Sepharose Fast Flow (Pharmacia) at various pH values (data not shown). The IRMPs were found to bind to the matrix at pH values of 7, 8 and 9. From these results, the pH for the ion-exchange systems was selected as 7.5 as the proteins would bind to the anion-exchange matrix at this pH, but the interaction would not be too strong.

4.7 Ion-exchange chromatography.

Ion-exchange using a Mono Q column and a 0.3% Empigen BB buffer system was performed at pH 7.5. Initially, a 0.3% Empigen BB solubilised extract of *P. aeruginosa* strain E1 was used. The elution profile is shown in fig. 4.7.1. The IRMPs were displaced from the matrix at a salt concentration of 0.25M, and a good separation of IRMPs and other proteins was achieved. However, the IRMPs were contaminated with O-antigen, and therefore, the rough mutant of *P. aeruginosa* was used in order to circumvent O-antigen contamination. The elution profile shown in fig. 4.7.2 represents the chromatographic separation of a 0.3% Empigen BB extract of *P. aeruginosa* strain AK1282, in a 0.3% Empigen BB buffer system. An immunoblot of fractions 12-18, probed with rabbit serum hyperimmune to OMs Fe- of strain AK1282, is shown in fig. 4.7.3. The IRMPs and protein D were again eluted at a salt concentration of 0.25M. The two other peaks on the graph represent the material which did not bind to the column and that which was only eluted at 0.9-1M salt. A corresponding LPS immunoblot showed that the proteins were contaminated to a minimal extent with lipid A.

4.8 Other methods used for removal of LPS.

Detoxi-gel, a ligand reported to have a specificity for endotoxin, was found to retain both LPS and proteins from a solubilised extract loaded on to a column. It was thought that proteins would not bind to the matrix, especially in the presence of 0.3M sodium chloride, however, due to their intrinsic association with LPS which would bind to the ligand, this method was not successful at separating OMPs and LPS. The presence of deoxycholic acid did not appear to be effective at disrupting protein-LPS interactions in this system.

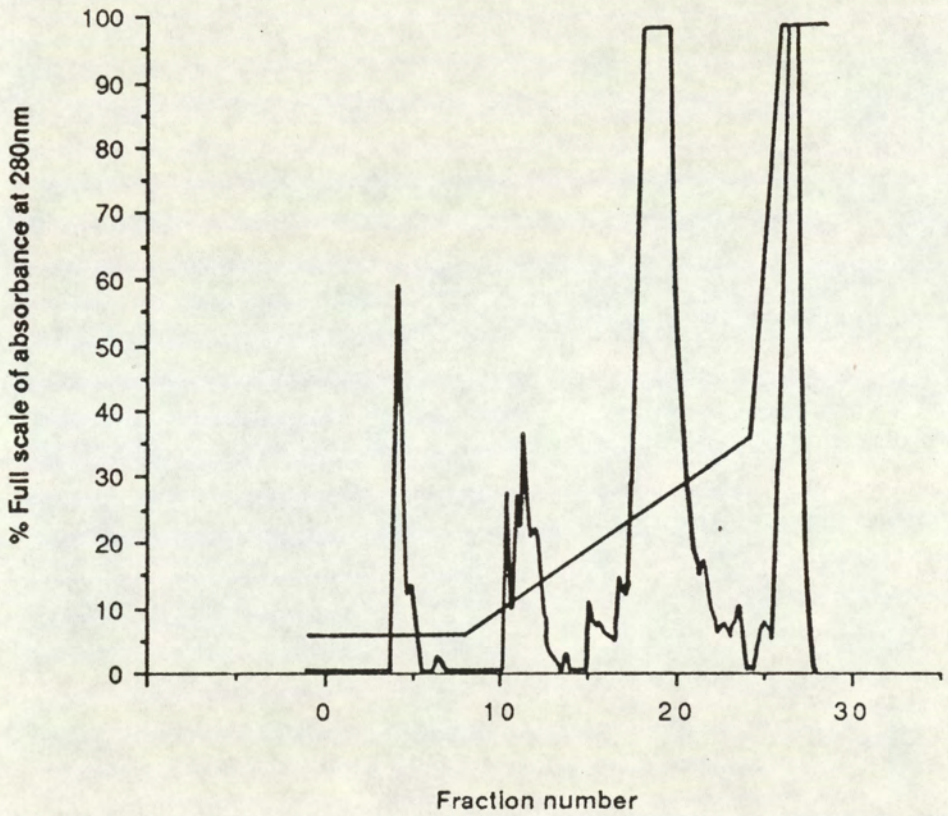


Fig. 4.7.1. Chromatogram of material eluted from a Mono Q ion-exchange column. A 0.3% Empigen BB solubilised OM Fe- extract (0.8148 mg/ml) of *P. aeruginosa* strain E1 was applied to the column and any material that did not bind to the matrix was displaced. The elution buffer contained 0.3% Empigen BB, 20mM triethanolamine, and 0.001M PMSF (1M sodium chloride in the high salt buffer), pH 7.5. Full scale detection at A_{280nm} was 0.2. and the flow rate was 1.0 ml/min. All the fractions were analysed for their protein and LPS contents.

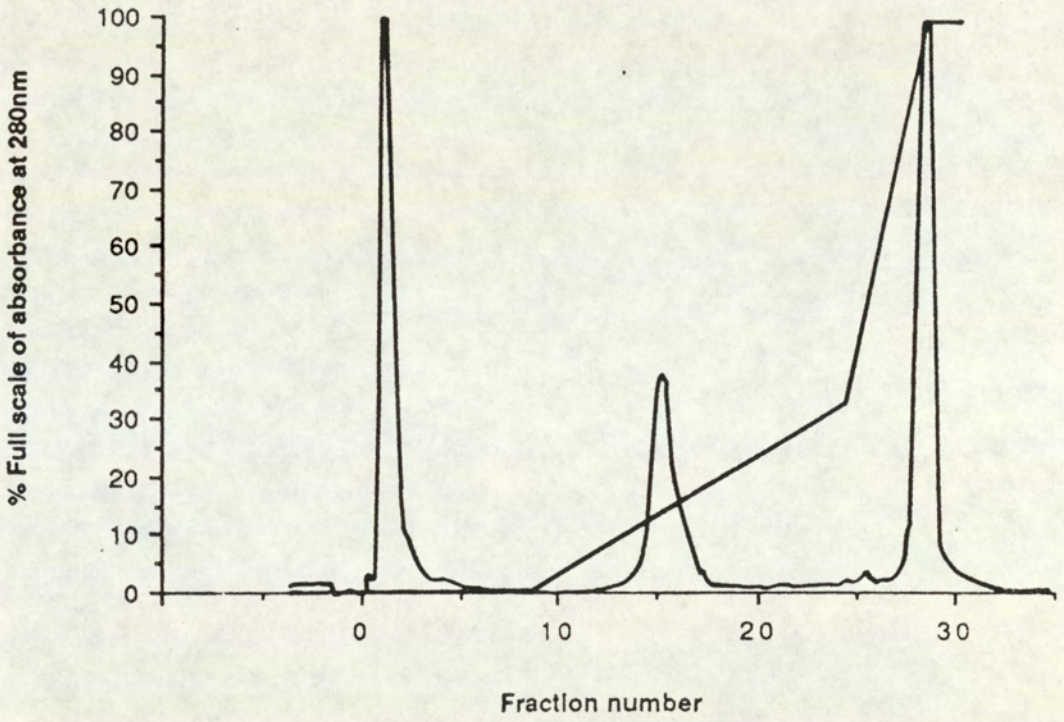


Fig. 4.7.2. Chromatogram of material eluted from a Mono Q ion-exchange column. A 0.3% Empigen BB solubilised extract of AK1282 OMs Fe- was applied to the column. The elution buffer contained 0.3% Empigen BB, 20mM triethanolamine, and 0.001M PMSF (1M sodium chloride in the high salt buffer), pH 7.5. Full scale detection at A_{280nm} was 0.2, and the flow rate was 1.0 ml/min. Fractions collected after material that did not bind to the matrix had been washed from the column, were analysed for their protein and LPS contents. Immunoblots of fractions 12 to 18 are shown in fig. 4.7.3.

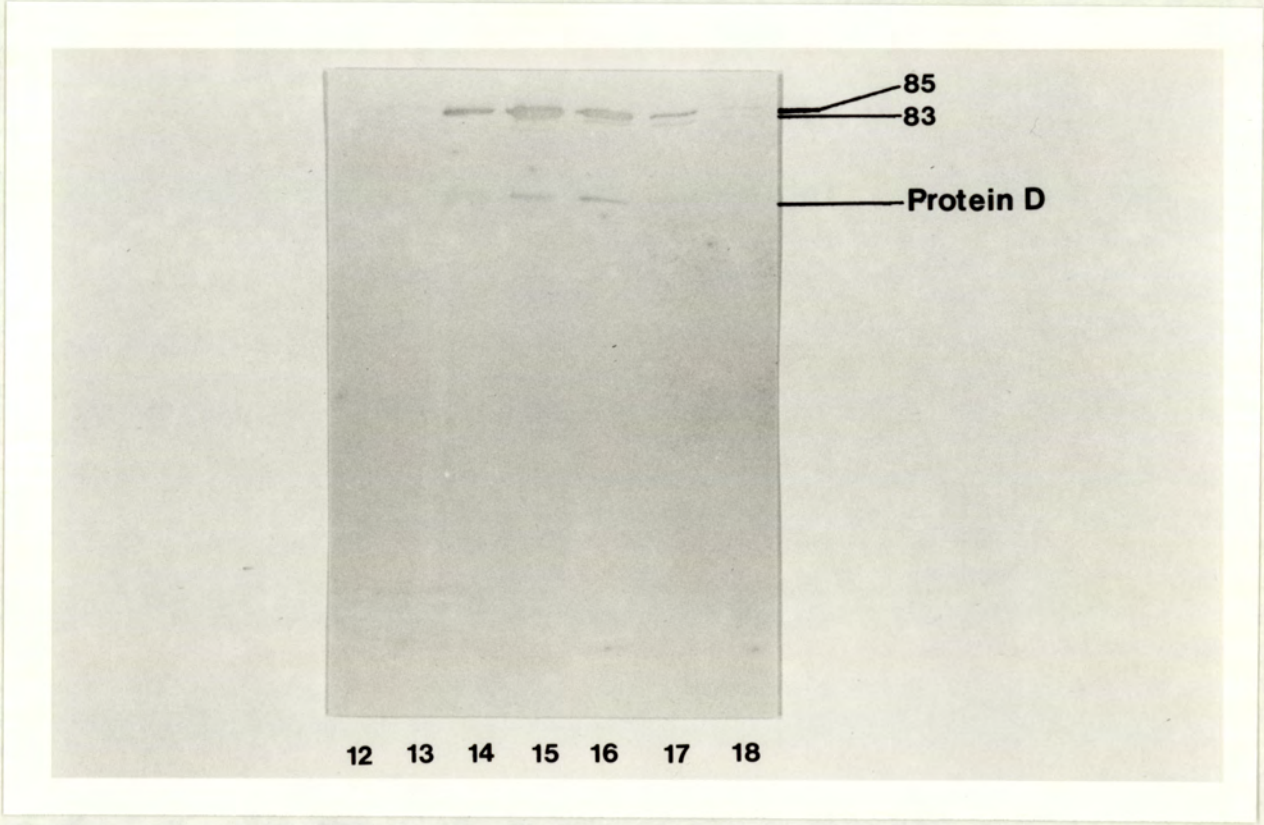


Fig. 4.7.3. Protein immunoblot of fractions 12-18 eluted from a Mono Q column loaded with a 0.3% Empigen BB OM Fe- extract of *P. aeruginosa* strain AK1282. The blot was probed with rabbit serum raised to whole OM preparations from the same strain of *P. aeruginosa*, and developed using a protein A-AP conjugate.

An additional approach from an immunological basis was used to investigate the IRMPs, ie. an attempt was made to mask the antigenicity of LPS. Polymyxin B has been reported by Applemelk *et al* (1988) and Rifkind (1967) to exhibit a neutralising effect on LPS endotoxicity: incubation of LPS with polymyxin B resulted in a 20-fold increase in LD_{50} (Rifkind, 1967). Colistin methanesulfonate (the sodium salt of methanesulfonic acid derivative of polymyxin E) was investigated for its ability to mask the antigenicity of LPS. OMs Fe- were prepared from *P. aeruginosa* strain PAO1 and aliquots of 20µl were incubated with 80µl of various concentrations of colistin (ranging from 0-80000 units/ml) for 60min at 37°C. After incubation, the OM preparations were treated with proteinase K, electrophoretically separated on 12% SDS-PAGE gels and stained with silver nitrate or blotted onto nitrocellulose. Immunoblots were probed with rabbit serum hyperimmune to OMs Fe- of PAO1 (fig. 4.8.1). Both the gels and blots showed a distortion of the LPS; the disruption of this molecule increasing with increased colistin concentration. The intensity of the blots did not alter with increasing drug concentration; the effect appeared to be more one of a change in the migration of the LPS through the polyacrylamide gel. This would suggest that the molecular conformation of the LPS molecule had been altered, rather than that the LPS had been immunologically masked.

The effect of colistin was further investigated using two approaches. Firstly, Fe- OM preparations of *P. aeruginosa* PAO1 were separated by SDS-PAGE and blotted prior to treatment with colistin. Strip blots of OMs were incubated in a range (0-80000 units/ml) of concentrations of colistin for 60 minutes, then reacted with the hyperimmune rabbit serum as above. No gradient of blot intensity was demonstrated, however this may have been due to the serum (which had a high titre), masking any effects of the colistin. The second approach taken involved an ELISA technique: *P. aeruginosa* strains PaSA and PaWH were grown in CDM-Fe and harvested by centrifugation. Washed cells were either treated with colistin or left untreated. These

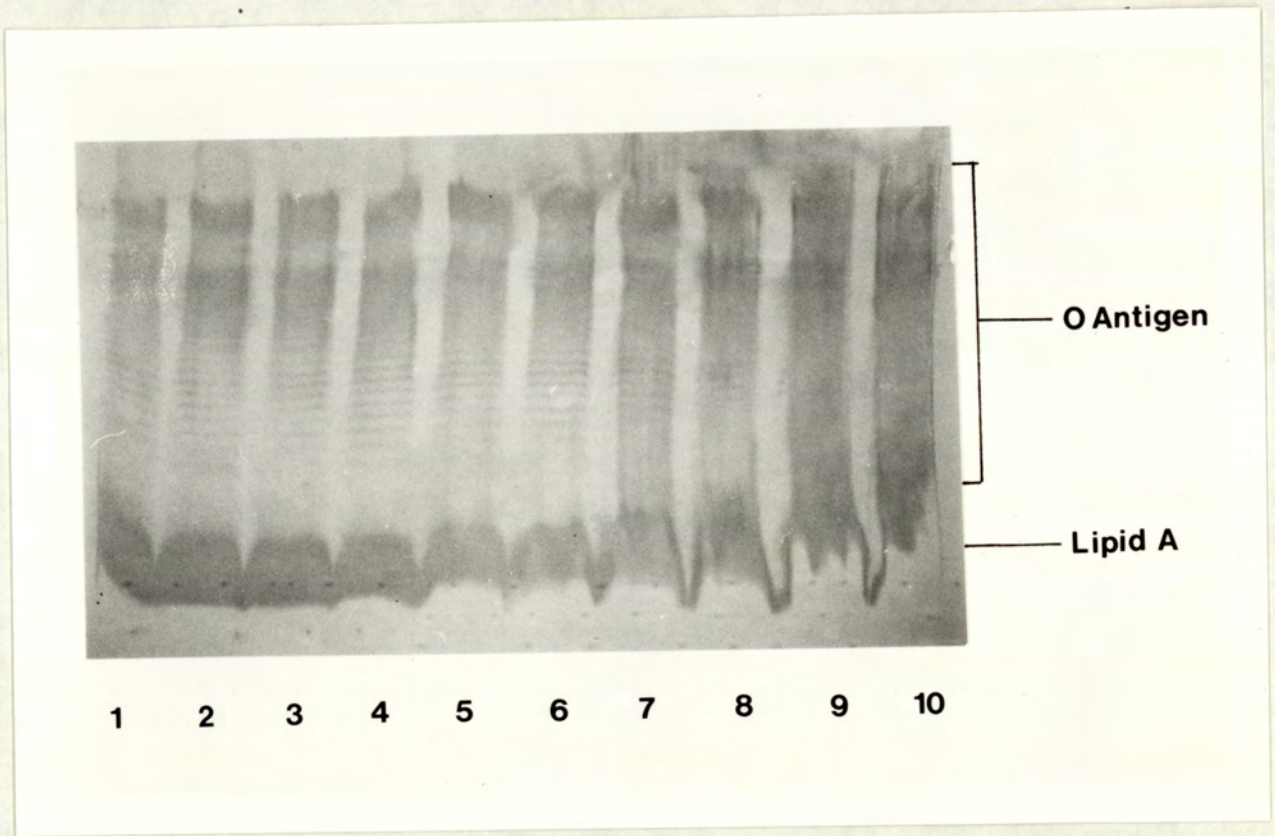


Fig. 4.8.1. Effect of colistin on the migration of LPS in SDS-PAGE gels. OMs Fe- of *P. aeruginosa* strain PAO1 were incubated in a range of concentrations of colistin, prior to separation on SDS-PAGE. The fig. shows an LPS blot probed with rabbit anti-OMs Fe- of strain PAO1, using a protein A-AP conjugate for visualisation of the antigenic sites recognised by the serum. Concentrations of colistin used were 0 (lane 1); 100 (lane 2); 250 (lane 3); 1000 (lane 4); 2500 (lane 5); 5000 (lane 6); 10000 (lane 7); 25000 (lane 8); 50000 (lane 9) and 80000 (lane 10) units/ml.

cells were used to absorb antibodies from serum obtained from the cystic fibrosis patients from which the organisms were isolated; the serum was subsequently used in an ELISA assay, using whole cells of the two *P. aeruginosa* strains as the coating antigen. It was hypothesised that cells treated with colistin would be able to absorb out fewer antibodies from the serum samples than the untreated cells, and therefore serum absorbed with treated cells would have a higher titre. A large set of ELISA data (5 repeat runs using unabsorbed serum, serum absorbed with normal cells and serum absorbed with cells treated with four concentrations of colistin for the two strains of *P. aeruginosa*) failed to show this pattern.

It was suggested (Applemelk *et al*, 1988) that polymyxin B contains highly charged as well as hydrophobic parts, which, by interacting with their counterparts in the lipid A-KDO region of LPS either shield or deform LPS, thus reducing endotoxicity. Moore *et al* (1986) demonstrated that polymyxin B bound to multiple sites on LPS, including sites which bind aminoglycoside antibiotics and other polycationic compounds. Peterson *et al* (1985) found that positive charges on the polycationic antibiotics were attracted to the negatively charged groups on LPS of *P. aeruginosa*, which resulted in a consequent change in the conformation of LPS aggregate structure by disrupting LPS-LPS interactions, and additionally, Morrison and Jacobs (1976) demonstrated binding of polymyxin B to the lipid A portion of LPS.

It is suggested that in the intact cell, the core is probably masked by O-antigen and therefore no alteration in the immunogenicity of LPS was demonstrated as the ELISA assays performed utilised whole cells. Incubation of blots in colistin prior to probing with antibody, however, failed to reveal any antigenic masking of LPS.

4.9 Excision of IRMPs from SDS-PAGE gels and production of polyclonal antibodies directed towards OM components.

Fig. 4.9.1 shows a scan of a Coomassie blue-stained 12% SDS-PAGE gel loaded with (a) OMs Fe- strain AK1282 and (b) an excised, eluted preparation of IRMPs from the OM preparation. The scans show that there was an effective separation of IRMPs (the 4 peaks on the right hand side of the scan) from other OM proteins using this purification technique.

Screening of pre-immune serum from animals was undertaked routinely to ensure that the serum was 'clean' before any immunisation schedules were started. An immunoblot assay was performed using SDS-PAGE separated OM components of *P. aeruginosa* as the antigens. In many cases there was a weak reponse to the OM antigens in the pre-immune serum. Griffiths *et al* (1985) investigated the natural occurence of antibodies in the serum of humans, rabbits, mice, and guinea pigs and found the presence of antibodies reacting with IRMPS of two *E. coli* strains, 0111 and 018, as well as other OM components.

Antiserum was raised in rabbits and mice to various OM preparations and it was found that a high titre of antibodies could be raised using the immunisation procedure outlined in section 2.2. IRMPs purified from a smooth strain (PAO1) by the method of excision and elution from SDS-PAGE (or excision from nitrocellulose blots) also induced the production of a high titre of antibodies; these proteins were therefore found to be not denatured by the purification procedure, which confirmed the results of Hjerten, (1983). When this response was further investigated in immunoblotting assays, however, it was found that a large proportion of the response was directed towards O-antigen. Since the antigenicity of LPS is well recognised, it was not surprising that anti-O-antigen antibodies were present in the serum (fig. 4.9.2). It was noted that there

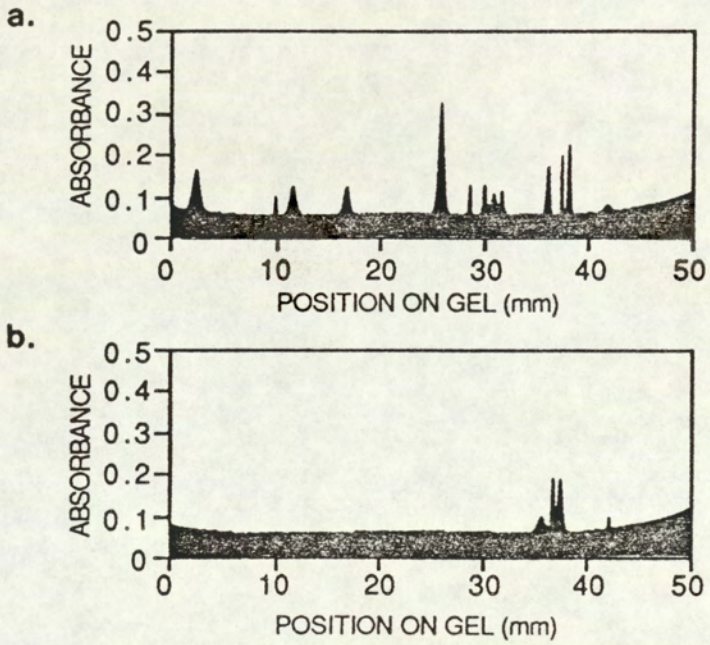


Fig. 4.9.1. Scans of 12% SDS-PAGE gels showing OM components stained with Coomassie brilliant blue detected in (a) an Fe- OM preparation of *P. aeruginosa* strain AK1282 and (b) a preparation of IRMPs excised and eluted from the OM preparation. The figure shows that there was an effective isolation of the IRMPs.

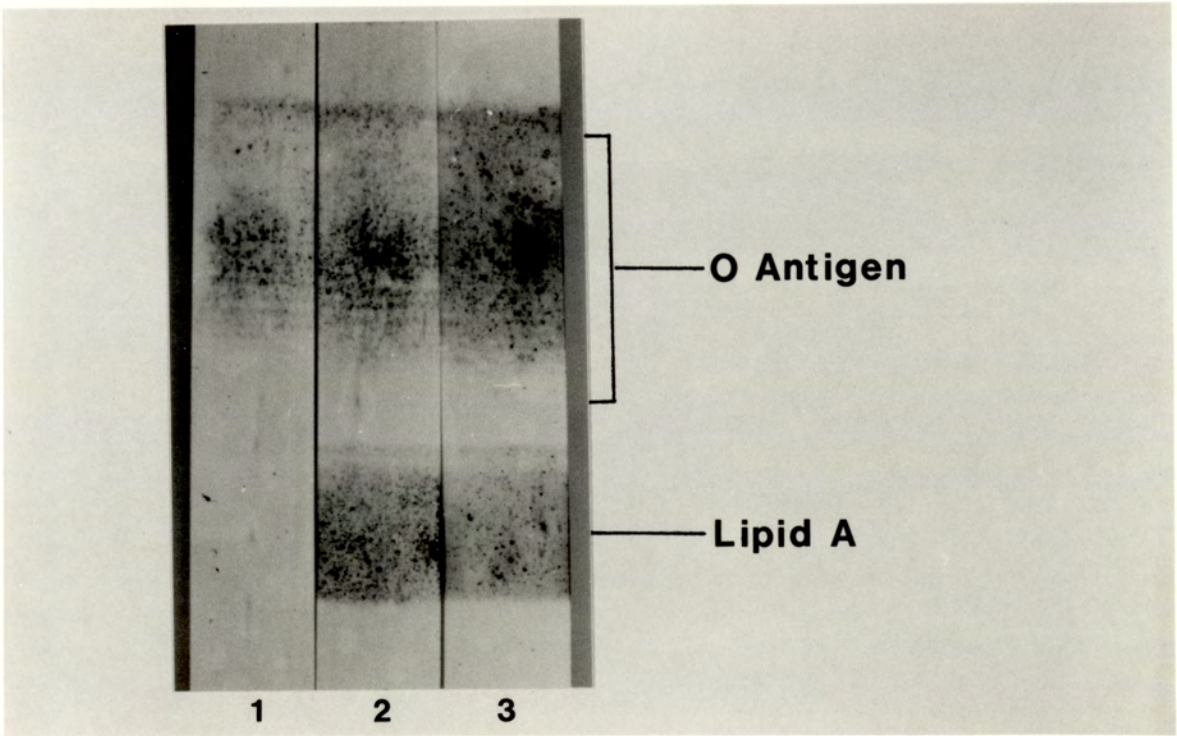


Fig. 4.9.2. LPS immunoblot analysis of electrophoretically separated OM antigens of *P. aeruginosa* strain PAO1. Samples are (lane 1) IRMPs excised and eluted from 12% SDS-PAGE gels, (lane 2) OM preparation from cells grown in CDM+Fe, and (lane 3) OM preparation from cells grown in CDM-Fe. This figure demonstrates the presence of O-antigen and the absence of lipid A in the excised preparation.

were no anti-lipid A antibodies evident.

IRMPs were also purified by the method of selective solubilisation combined with 1% deoxycholate gel-filtration chromatography. Although the purity of these preparations was checked before administration to the animals, and the animals were screened to check that they did not have antibodies to *P. aeruginosa* prior to immunisation, a clean response to just IRMPs was never achieved, although responses were more specific those that produced by immunisation with excised proteins. The specificities of this serum were also investigated (see section 5).

A dose to response relationship was produced using a range of amounts of OMP preparations to immunise mice. The titres produced by the mice were monitored by an ELISA assay. The effect of encorporation of OMP preparations in liposomes was studied, however no measure of adjuvant activity was detected.

4.10 Discussion.

The selection of the strain and growth conditions had to be given careful consideration to ensure the maximum production of IRMPs before commencing the extraction and purification procedure. Growth conditions were used which induced the production of IRMPs to an extent to which they were considered to be major OM constituents. IRMPs were found to have molecular weights of between 77 and 101K. Several papers have reported the induction of a 14K IRMP (the receptor for ferripyochelin) under conditions of iron-limitation (Sokol and Woods, 1983; 1984; Sokol, 1984). This 14K protein was not detected in the outer membranes analysed during this study. The initial study by Sokol and Woods, (1983), that demonstrated the presence of the 14K protein, relied on the incubation of OM preparations with ^{59}Fe-pyochelin prior to SDS-PAGE analysis. It is unlikely that the binding of ^{59}Fe to pyochelin or of ^{59}Fe-pyochelin to receptor protein

would withstand SDS denaturation unless the links between molecules were covalent. There is no evidence to suggest that this is the case, and no SDS-polyacrylamide gels of ^{59}Fe-pyochelin running alone have been shown. It is suggested that the protein represents a fragment of a larger protein that binds ^{59}Fe-pyochelin.

Cultivation in a medium which induces the maximum production of the proteins of interest is an approach that has been taken by several workers interested in the purification of OM proteins. For example, for the purification of protein H1 from *P. aeruginosa*, the strain selected by Bell and Hancock (1989) was cultured in a divalent cation depleted medium that induced the production of this protein.

Difficulties encountered during the purification of IRMPs from *P. aeruginosa* strains PAO1 and 6750 forced the selection of a rough mutant to circumvent the problems associated with the smooth phenotype. The strains obtained from Colindale (Pitt *et al*, 1986) were confirmed to be lacking the O-antigen moiety of LPS by examination on SDS-PAGE. The strain with no apparant O-antigen visible on the gels (18s), however, did not grow well in CDM-Fe and so this strain was not selected. The other promising strain, CF003 was also not selected as it was a cystic fibrosis isolate and of mucoid phenotype.

P. aeruginosa strain AK1282 was described by Jarrell and Kropinski, (1981b) as having a very defective LPS. Jarrell and Kropinski (1981b) demonstrated that this mutant of PAO contained only lipid A, 2 keto-3-deoxyoctonate, heptose and alanine as major components. The LPS lacked galactosamine which was present in the inner core of the LPS of other rough mutants analysed. The use of this strain enabled effective separation of IRMPs and O-antigen by gel-filtration chromatographic techniques by circumventing the problem ie. using a strain that did not produce O-antigen. Other workers have selected rough strains to use in purification procedures. Verstreate *et al* (1982) found that the process of extraction and purification of OMPs of *Brucella abortus* was easier and more

complete with the rough strain used than the smooth strains. They sugested that the difficulties encountered with smooth strains were due to interactions of LPS with the OMPs.

The selection of a strain that over-produced the IRMPs would have been useful. Mutants obtained from K. Hantke, (Hantke, 1981; 1987) were not available until a late stage of the research project. Other workers (Chart and Griffiths, 1985; Bell and Hancock, 1989) have selected strains that overproduced the proteins in which they were interested. Chart and Griffiths (1985) selected *E. coli* 0111 as this strain produced a particularly large amount of the 81K ferric enterobactin receptor protein under iron-restricted conditions. Additionally, Bell and Hancock (1989) described the purification of protein H1 from *P. aeruginosa* from a mutant strain of *P. aeruginosa* PAO1 selected for it's overproduction of this protein (strain H181).

The next stage of purification of IRMPs, after selection of a suitable strain and preparation of OMs from bacteria grown in a medium which induced the expression of the proteins of interest, was the selective solubilisation of OMs; this yielded a preparation that was enriched for IRMPs. Meitzner *et al* (1987) purified the major IRMP of *N. gonorrhoeae* using selective solubilisation with cetyl trimethyl ammonium bromide. It was found that this detergent gave the least effective solubilisation of the IRMPs of *P. aeruginosa* out of all the surfactants tested. Triton X-114 has been reported to be effective at solubilising membrane proteins (Pryde, 1986; Pryde and Phillips, 1986), however this detergent also did not give an effective solubilisation of IRMPs.

Nonidet NP40 and Empigen BB were selected as these surfactants gave an effective, selective solubilisation of IRMPs and were compatible with ion-exchange chromatography techniques. Problems arose in the use of Nonidet NP40, however, as the structure of this detergent was modified slightly by the manufacturers half way through the research

project. This modification caused the Nonidet to become absorbent at A_{280nm}, which meant that it could not be used as it interfered with the detection of protein components.

Both Gabay *et al* (1985) (purification of the major OMP of *Legionella pneumophila*) and Blake and Gotschlich (1984) (purification of opacity-associated proteins of *N. gonorrhoeae*) used selective extraction with Zwittergent-3,14 in the presence of calcium ions in their studies. It was hypothesized by these workers that in the presence of a high concentration of calcium, LPS would aggregate and decrease it's association with membrane proteins allowing the zwitterionic detergent to interact with exposed hydrophobic sites of the proteins and solubilise them (Blake and Gotschlich, 1984). Using a different approach in the purification of the IRMPs, it was demonstrated that chelation of divalent cations by EDTA (which would remove the stabilising cross-bridges in the KDO-lipid A region of LPS) increased the amount of LPS solubilised, but also effected a greater relative solubilisation of the OMPs. Other workers have utilised the chelating ability of EDTA in their purification procedures. For example, for the purification of protein H1 from *P. aeruginosa*, Bell and Hancock (1989) found that they could selectively extract the protein by solubilising twice in Triton X100-Tris-HCL (pH8) and twice in Triton X100-Tris-HCL (pH8), containing EDTA, and collecting the second two supernatants.

The major problem in the purification of Gram-negative bacterial OM proteins is separation of these proteins from the LPS with which they are intrinsically associated. The next stage of protein purification was designed around this problem. Gel-filtration chromatography using 1% deoxycholic acid effected a good separation of IRMPs from the lipid A moiety of LPS in the rough strain *P. aeruginosa* AK1282. It was observed that LPS was readily dissociated in the deoxycholic acid system, allowing this component to be separated without denaturing the protein antigens (as discussed by Shands and Chun,

1980, and Heckels, 1981).

Other workers have investigated the problem of LPS contamination in other ways. Verstreate *et al* (1982) isolated OM proteins of *Brucella abortus* using solubilisation with Zwittergent followed by sequential anion-exchange and gel-filtration chromatography. These workers found that membrane proteins contained substantial quantities of tightly adherent LPS that could be reduced but not eliminated by extraction of cells with trichloroacetic acid before disruption. Foulaki *et al* (1989), on the other hand, found that isolation of a 55K surface protein from *Salmonella typhimurium* was possible, by extraction with urea followed by ion-exchange and gel-filtration chromatography. Preparations were shown to be LPS free by analysis of the ß-hydroxymyristic acid content and by the fact that immunisation of rabbits with isolated protein led to the production of specific antibodies. Additionally, Carniel *et al* (1989a) took advantage of the high molecular weights (190,000 and 240,000) of the IRMPs of *Yersinia entercolitica* serovar 0:8 to purify these proteins by gel-filtration chromatography. The preparations were of high enough purity to produce specific antibodies in an animal.

A crude purification method involving excision of the proteins of interest from SDS-PAGE techniques has been shown to have potential as a method of purification of bacterial OM proteins. The results presented here confirmed the potential of this method to be an effective method of separation, however, it was noted that due to the comigration of O-antigen and OM protein components, the use of a rough strain of *P. aeruginosa* was a critical factor in this purification strategy. Bell and Hancock (1989) compared anion-exchange chromatography and preparative SDS-PAGE for their application to the purification of protein H1 from *P. aeruginosa*. These workers found that both methods gave a good separation of protein H1 from other membrane proteins, however the protein purified by chromatography contained substantial amounts of LPS (shown by silver-staining SDS-PAGE). They noted that the LPS contaminating the protein contained a

higher proportion of molecules with O side chains than did the bulk LPS of the OMs. Bell and Hancock (1989) found that protein H1 of *P. aeruginosa* purified by excision from SDS-PAGE contained no detectable LPS. Additional evidence indicating the application of this method for the purification of bacterial OM proteins was presented by Chart and Griffiths (1985), who attempted to isolate the 81K ferric enterobactin receptor protein from *E. coli* 0111, using gel-excision from preparative SDS-PAGE gels as a method of purifing the protein; they found this method to be a successful way of raising specific antibodies. Coulton (1982) successfully isolated the ferrichrome-iron receptor OM protein from *E. coli* K-12 by preparative SDS-PAGE gel electrophoresis and electroelution.

Matthews-Greer and Gilleland (1987) prepared antisera in mice to protein F of *P. aeruginosa*, by immunisation with a preparation of this protein purified by excision from SDS-PAGE. The sera reacted with the protein F band on an immunoblot and also with protein H. These workers suggested that OM proteins F and H may share common epitopes responsible for producing cross-reactivity, or that the protein F preparation had minute amounts of protein H contamination. The technique of gel-excision was effective in the preparation of the 84K IRMP of *Pasteurella multocida* strain P-1059. Ikeda and Hirsh (1988) demonstrated in immunoblotting assays that an antibody response specific for the 84K protein was achieved by immunising turkeys with proteins eluted from SDS-PAGE gels.

Excision from SDS-PAGE gels is a simpler technique than selective solubilisation followed by chromatography and appears, in some cases to effectively purify the protein. Some workers have, however, suggested that excised proteins may not be highly purified, due to the co-migration of proteins and LPS in SDS-PAGE (Hitchcock, 1984; Poxton *et al*, 1985; Rocque *et al*, 1987). Rocque *et al* (1987) detected LPS bound to isolated porin protein extracted from *E. coli* using a carbohydrate-specific silver stain on SDS-PAGE

gels and an anti-lipid A monoclonal antibody on immunoblots. These workers demonstrated that isolated porin could be separated from loosely associated LPS by SDS-PAGE, but a fraction of tightly bound LPS could not be dissociated, even with additional heating at 100°C and two electrophoretic separation procedures.

Additionally, Hitchcock (1984) probed whole cell lysates and LPS extracted from *N. gonorrhoeae* separated on a SDS-PAGE. Results obtained using a stain that preferentially stained LPS, indicated a stable association of LPS with proteins (23-36K), in particular with the major OM protein I. Hitchcock (1984) also analysed the gels by immunoblotting with rabbit antisera prepared from protein I bands excised from SDS-PAGE gels. The serum revealed the presence of antigens below protein I (in the region of the LPS). Antibodies to these constituents were removed by absorption of antisera with purified LPS. Immunoblotting with a monoclonal antibody specific for LPS demonstrated the reactivity of the antibody with LPS and with protein I. It was concluded that protein I and perhaps other proteins in the whole cell lysate were stably associated with LPS, an interaction that was resistant to dissociation in SDS at high temperature (100°C). Analysis of two-dimensional polyacrylamide gels, however, showed that the LPS migrated in a unique fashion and did dissociate from the proteins; this result was unexplained.

Production of antibodies specific for IRMPs proved difficult owing to the dominant response to LPS. It is likely that these OM proteins functioned in some measure as carriers for the tightly associated LPS determinants. Other workers have encountered difficulties in raising specific antibodies to OM proteins. Kelly *et al* (1989) attempted to raise antisera to IRMPs of *P. aeruginosa* by immunisation of rabbits with IRMPs obtained by selective solubilisation and excision from SDS-PAGE. The resultant antibody preparation reacted very weakly with only one of the four IRMPs in strains AK1012 and PAO1. This could be an indication of the poor immunogenicity of highly purified OM protein components. Chart and Griffiths (1985) showed that an antiserum was raised to

an 81K IRMP (isolated by gel-excision) from *E. coli* 0111, produced a weak reaction with the 81K IRMP and antigen of 24K molecular weight. This antiserum also reacted on immunoblots with LPS in *E. coli* 0111, 018 and W3110. This was also demonstrated in ELISA assays, using purified LPS as the coating antigen. It was thought that there was co-migration of the protein and LPS (Chart and Griffiths, 1985). Additionally, it was suggested that the 81K and the 24K proteins might share common epitopes, as the 24K protein was recognised in strains that did not have the 81K protein.

The inclusion of OM preparations in solid liposomes failed to exert an adjuvant effect. It is suggested that the use of liposomes and other delivery systems may be more applicable to the mucosal delivery routes, rather than ip administration. The IRMP preparations always evoked a strong immune response in animals when administered with Freund's adjuvant, and there was no requirement for the use of a tailored delivery system with ip administration.

In summary, good induction of IRMP expression was achieved in the iron-deprived cultures of *P. aeruginosa.* Selective solubilisation was highly effective at enriching the OM preparations for IRMPs. It was found that use of a rough strain was the only way to eliminate O-antigen from the purified preparations and the gel-filtration system developed was effective at separation of lipid A from the IRMPs. Ion-exchange chromatography effected a good separation of OM proteins from each other, however, it was ineffective at disrupting protein-LPS interactions.

The preparation of IRMPs was not of high enough purity to produce specific polyclonal antibodies, but more drastic efforts to separate residual O antigens from purified IRMPs were not made as a meaningful study of antigenic relationships could only be carried out with proteins in their native configuration. It would be useful if individual OM components could be purified, in order to study the contribution of each to the pathogenicity of *P.*

aeruginosa, and also so that any critical surface antigens can be identified which may be useful for inclusion in immunising preparations.

5 Cross-reactivity, expression *in vivo* and surface expression of *P. aeruginosa* antigens.

5.1 Introduction.

Antibodies to IRMPs may be important as immunotherapeutic agents. In order for their potential to be realised, however, the epitopes that they interact with must be expressed *in vivo*, surface-accessible *in vivo* and not specific to only a limited number of strains of the pathogen. These aspects of IRMP expression are investigated in this section.

5.2 Cross-reactivity of polyclonal anti-IRMP antibodies with the seventeen serotypes of *P. aeruginosa.*

Immunoblots prepared of OMs Fe- of representatives of the seventeen serotype strains of *P. aeruginosa* and strain PAO1 were probed with rabbit antibodies raised to OMs Fe- of *P. aeruginosa* PAO1. Fig. 5.2.1 is a Coomassie blue-stained SDS-PAGE gel which confirmed the presence of the IRMPs in all of the samples. It can be seen that a variable number of IRMPs were expressed. At this stage it is not clear whether this is attributable to different protein species or a varying amount of bound LPS, both of which could be strain-dependent and sensitive to slight changes in either growth conditions or preparation procedure. It was demonstrated that the 77 and 83K IRMPs were common to all strains examined.

No anti-*P. aeruginosa* antibodies were found in the pre-immune serum (data not shown), and anti-protein and LPS specific antibodies were found to be present in the post-immune serum. The protein immunoblot shown in fig. 5.2.2 demonstrated that there was a good cross-reaction of the antibodies raised to strain PAO1 with antigens, especially proteins F and H1/H2, in all the strains examined.

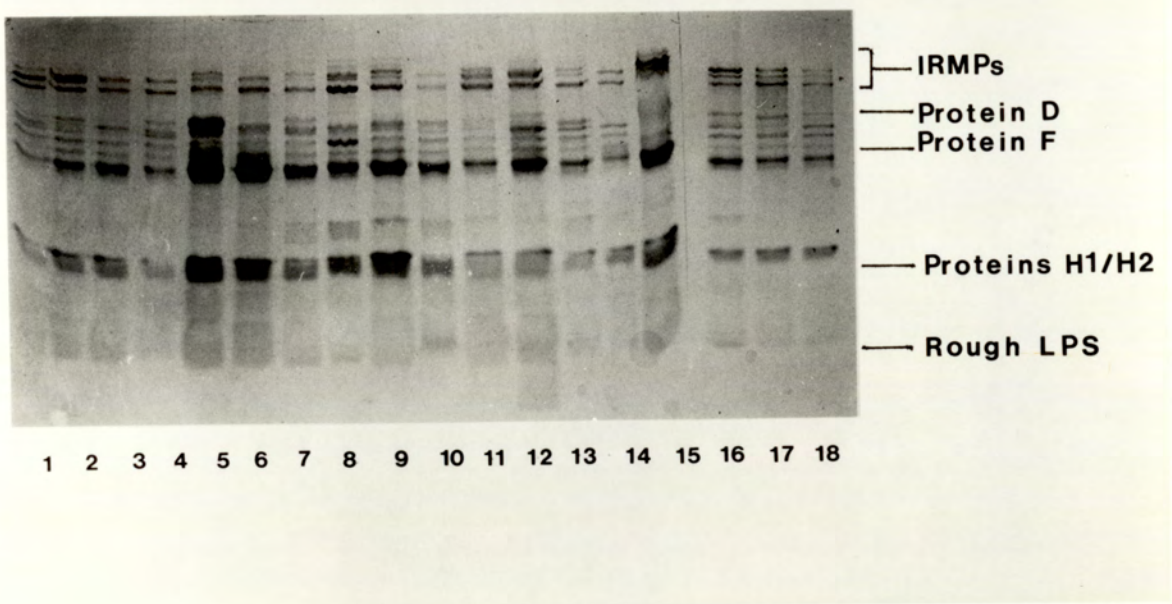


Fig. 5.2.1. 12% SDS-PAGE analysis of OM preparations of representatives of the seventeen serotypes of *P. aeruginosa* (lanes 1-17) and strain PAO1 (lane 18). Staining with 0.1% Coomassie blue confirms the presence of all major OMPs, including the IRMPs in all the preparations (which were extracted from bacteria grown in CDM-Fe). Replicate protein and LPS gels were prepared, blotted onto nitrocellulose and developed as described in figs. 5.2.2 and 5.2.3.

Fig. 5.2.2. Protein immunoblot analysis of OM components extracted from representatives of the seventeen serotypes of *P. aeruginosa* (lanes 1-17) and strain PAO1 (lane 18). OM preparations were electrophorectically separated on a 12% SDS-PAGE gel before blotting onto a nitrocellulose membrane. The immunoblot was developed with serum raised to OMs Fe- of *P. aeruginosa* strain PAO1, with a protein A-AP conjugate.

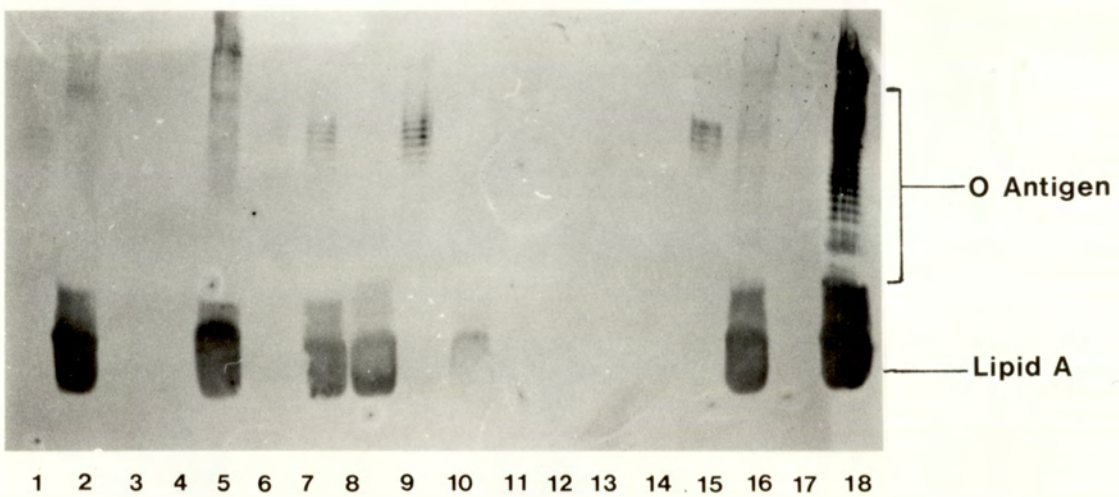


Fig. 5.2.3. LPS immunoblot analysis of OM components extracted from representatives of the seventeen serotypes of *P. aeruginosa* (lanes 1-17) and strain PAO1 (lane 18). Samples were treated with proteinase K to digest proteins prior to electrophorectic separation on a 12% SDS-PAGE gel and blotting onto nitrocellulose. The immunoblot was probed with serum raised to OMs Fe- of *P. aeruginosa* strain PAO1 and developed with a protein A-AP conjugate.

The 83K IRMP was detected in the OMs of serotypes 1, 2, 5, 7, 9, 11, 13, 15, 16 and 17. In addition there was a strong detection of all the IRMPs expressed by the serotype 5 organism and strain PAO1.

An immunoblot examination of the anti-LPS activity of the serum revealed that it recognised LPS antigenic determinants in serotypes 2, 5, 7, 8, 9, 10, 15 and 16 in addition to strain PAO1. Not all of these determinants presented by lipid A, which suggested that the band marked rough LPS in fig. 5.2.2 may be a combination of lipid A and protein I.

LPS adsorption removed anti-lipid A antibodies more effectively than the anti-O-antigen antibodies. This was shown by probing an immunoblot with the anti-OMs Fe- serum which had been absorbed once with LPS to immunoprecipitate anti-LPS antibodies (data not shown); the O-antigen response remained high with all cross-reacting serotypes (2, 5, 7, 9, 15, 16 and strain PAO1), while the intensities of the bands corresponding to lipid A were very diminished. Absorption repeated three times was effective at precipitating anti-LPS antibodies, and the incubation process did not appear to cause the denaturation of antibodies directed against proteins.

The same form of immunoblot analysis of polyclonal antibodies raised to the IRMPs of strain PAO1 excised from SDS-PAGE gels was performed. This serum was subsequently used for passive protection studies 1 and 2 (see chapter 7). Again many of the OM antigens were detected in all of the isolates examined. IRMPs were detected in serotypes 1, 5, 7, 9, 11, 13, 15, 16, 17 and strain PAO1 (data not shown). Only the reaction with serotype 2 was different from the serum raised to whole OMs of the same organism. Cross-reactivity of the polyclonal antibodies was also investigated using ELISA techniques, and again, the highest response was observed with the representative strain of serotype 5 and strain PAO1. To immunoprecipitate anti-LPS antibodies from serum, it was repeatedly absorbed

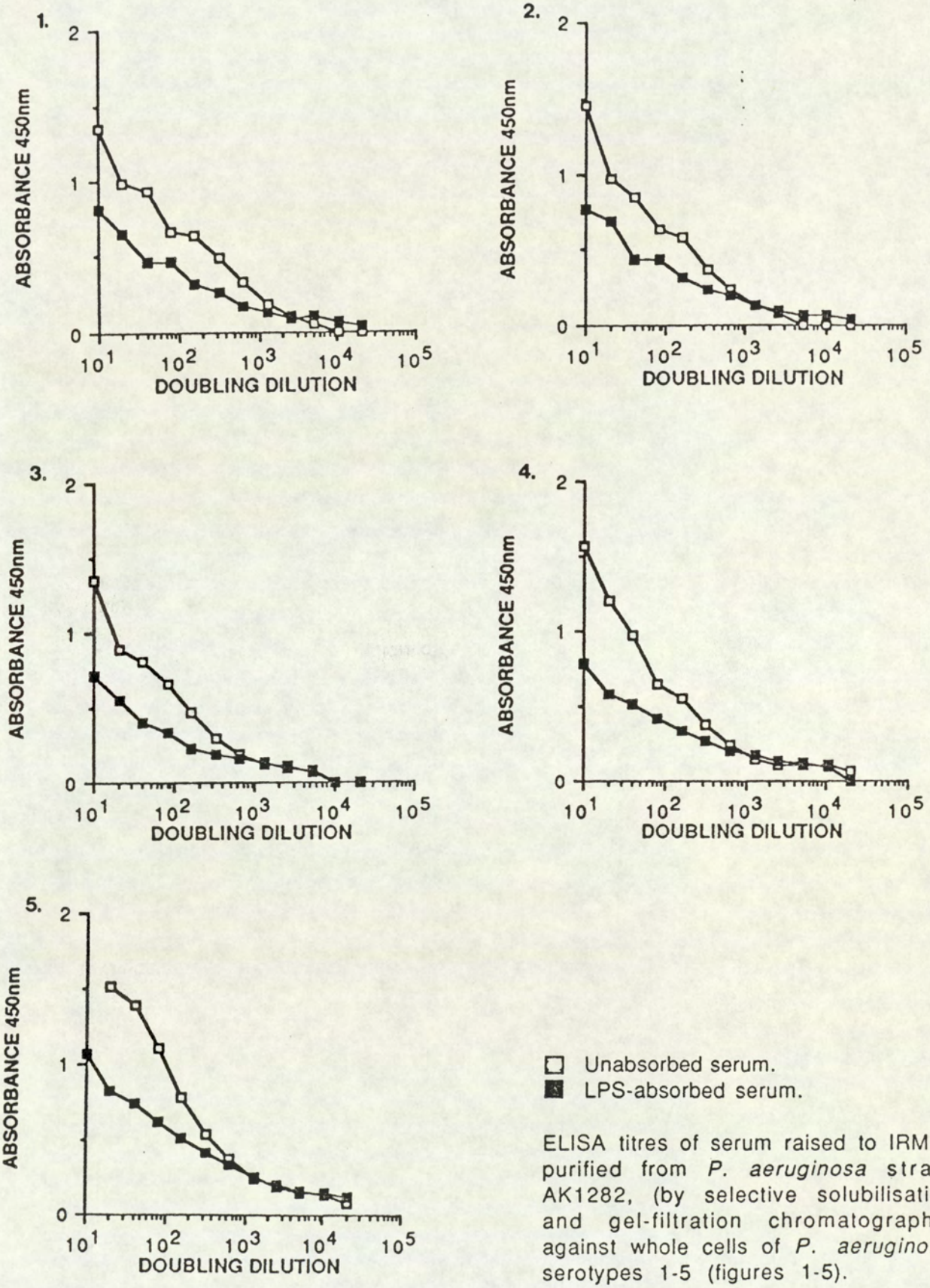
with purified LPS (using LPS recovered from the phenol or aqueous phases formed during LPS purification): the IgG titre of serum against whole cells before and after absorption was examined. It was seen that in general, absorption of serum with LPS led to a decrease in IgG titre against whole cells, but there was no difference between the capacity of aqueous and phenol-phase LPS to immunoprecipitate anti-LPS antibodies (data not shown). After precipitation of anti-LPS antibodies, a high IgG titre was still seen in the serum raised to IRMPs purified by excision from SDS-PAGE gels.

The serum used in passive protection studies 3 and 4 (see chapter 7) was investigated in ELISA assays for it's ability to bind to whole cells of *P. aeruginosa* of the seventeen serotype strains. The results from this assay are depicted in fig. 5.2.4. Only serotypes 1-5 are shown, however, a similar pattern was observed for serotypes 6-17. The polyclonal antibodies, raised to IRMPs purified from strain AK1282 by selective solubilisation and gel-filtration chromatography, were able to interact with whole cells of all serotypes. A decrease in the titre was observed when the serum was absorbed with LPS prior to assay by ELISA, but the titre remained high, confirming the presence of antibodies directed towards non-LPS OM components. Immunoblot analysis revealed that the predominant specificity of the serum was anti-IRMPs.

5.3 Interaction of monoclonal antibodies with OMs of the seventeen serotypes of *P. aeruginosa*.

The abilities of monoclonal antibodies A2, B112.2.1 and B8.1 to interact with epitopes expressed by the different serotypes strains of *P. aeruginosa* was investigated using an immunoblot assay. The anti-O-antigen antibody A2 reacted on an LPS immunoblot with serotypes 2, 4, 6, 8, 10 and 11 and strain PAO1 (data not shown); this cross-reactivity was different from that observed with the polyclonal antibodies raised to the same strain, the monoclonal antibodies being more specific.

Fig. 5.2.4. Cross-reactivity of anti-IRMP antibodies with representative strains of various serotypes of *P. aeruginosa*.



ELISA titres of serum raised to IRMPs purified from *P. aeruginosa* strain AK1282, (by selective solubilisation and gel-filtration chromatography) against whole cells of *P. aeruginosa* serotypes 1-5 (figures 1-5).

Protein immunoblot analysis determined that monoclonal antibodies B112.2.1 and B8.1 reacted with the 83 and 85K IRMPs in serotypes 2, 5 and 7, and with protein D in serotypes 2, 3, 4, 5, 6, 7, 16 and 17 (data not shown).

5.4 Cross-reactivity of monoclonal antibodies with proteins from other bacterial species.

An immunoblot was performed to investigate the cross-reactivities of monoclonal antibodies B8.1, B112.2.1 and B118.1 with OM components of two other Gram-negative bacterial species. *Klebsiella pneumoniae* and *E. coli* were chosen and, in addition, *P. aeruginosa* was run on the immunoblots as a positive control. The immunoblot shown in fig. 5.4.1 demonstrates the reaction of antibody B112.2.1 with the antigenic determinants expressed by these bacteria under conditions of iron depletion. The other two antibodies gave a similar reaction; all three antibodies reacted with the IRMPs and protein D of *P. aeruginosa*, as expected, but also recognised the 35.5K and 39K porin proteins in the OM of *K. pneumoniae*, and the Omp C and Omp F (36K and 39K respectively) porins of *E. coli.* The polyclonal serum reacted similarly, but also recognised other proteins in the OM of *P. aeruginosa.*

5.5 Surface expression of IRMPs.

Monoclonal antibodies produced from the fusions detailed in chapter 6 were used in an investigation of the surface-exposure of *P. aeruginosa* antigens, using immunofluorescence and immunogold labelling techniques. Immunofluorescence results are shown in table 5.5.1; out of a selection of 20 antibodies tested, 7 were found to be positive.

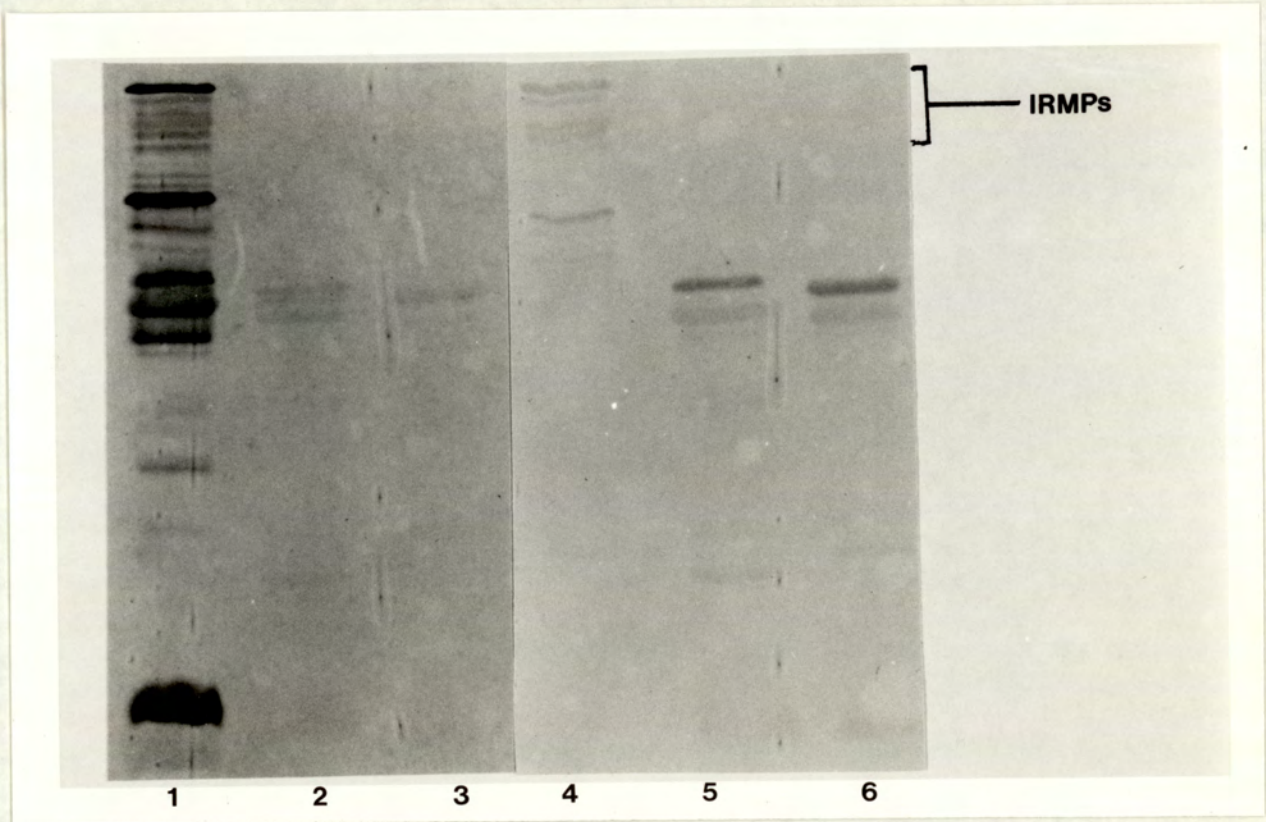


Fig. 5.4.1. Protein immunoblots of OM preparations from iron-restricted cultures of *P. aeruginosa* strain AK1282 (lanes 1 and 4), *Klebsiella pneumoniae* (lanes 2 and 5) and *E. coli* (lanes 3 and 6). Blots 1-3 were probed with serum raised to IRMPs purified from *P. aeruginosa* strain AK1282. Blots 4-6 were probed with monoclonal antibody B112.2.1. An anti-mouse F(ab'$_2$) fragments-HRP conjugate was used.

Table 5.5.1. Immunofluorescence of whole bacterial cells using monoclonal antibodies to detect surface antigenic sites.

	Fe- cells	Fe+ cells
PAO1		
Serum raised to excised IRMPs of PAO1 (1:100 dilution)	+ve	+ve
Negative control ascites (anti-human leutenising hormone)	-ve	-ve
A2 (1:100 dilution of ascites)	+ve	+ve
B112.2.1 (1:100 dilution of ascites)	+ve	(-ve)
B118.1 (1:100 dilution of ascites)	+ve	(-ve)
AK1282		
B112.2.1 (neat end supernatant)	+ve	not tested
B118.1 (neat end supernatant)	+ve	not tested
C103.3 (neat end supernatant)	++ve	not tested
C9.3 (neat end supernatant)	++ve	not tested
C23.3 (neat end supernatant)	++ve	not tested
C28.3 (neat end supernatant)	++ve	not tested

Immunogold staining of whole bacterial cells was performed to support the results obtained from the immunofluorescence study. Fig. 5.5.1 shows antigenic sites recognised by the positive control serum (anti-IRMPs excised from SDS-PAGE gels of strain PAO1), on the surface of *P. aeruginosa* whole cells which had been fixed with glutaraldehyde. It can be seen that specific staining occurred, however, it was demonstrated that a greater degree of specific staining occurred with the rough *P. aeruginosa* strain: the same dilution of the antiserum reacting with whole cells of *P. aeruginosa* strain AK1282 is shown in fig. 5.5.2. The staining of strain AK1282, however, appeared to be unevenly distributed, with only 9.27% of cells being very positively stained and the rest being only weakly positive. Fig. 5.6.1 shows a low magnification of the electron microscope grid to demonstrate this. There is no explanation for this, however it may have been due to different growth phases of the cells but this is unlikely as the culture was harvested at stationary phase. Fig. 5.5.3 shows the negative control antibody (mouse anti-human leutenising hormone, IgG_1 isotype) which proved that the gold label was not staining non-specifically. Formaldehyde

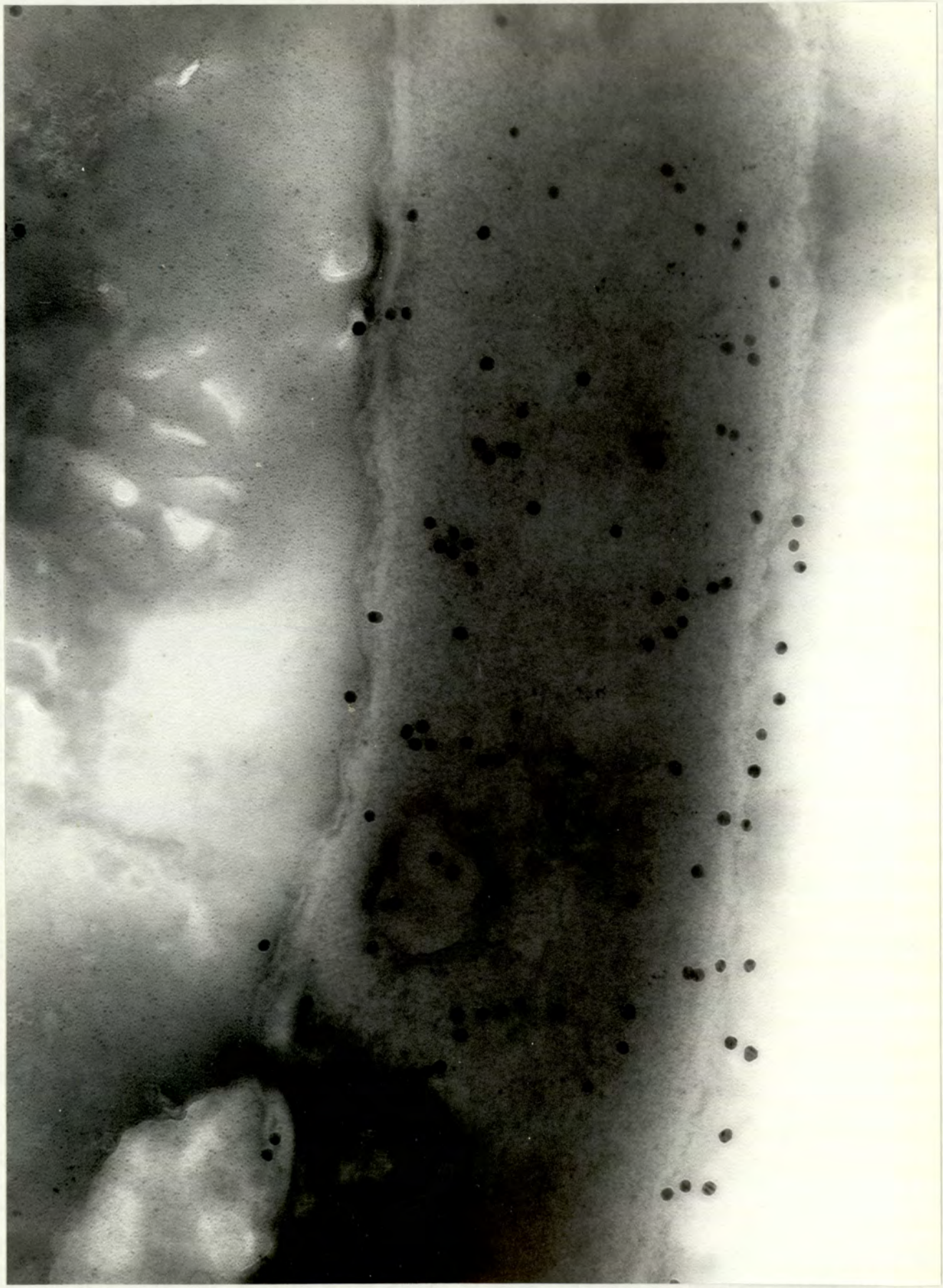


Fig. 5.5.1. Electron micrograph showing immunogold detection of surface antigenic sites of *P. aeruginosa* PAO1. Iron-depleted whole cells of *P. aeruginosa* PAO1 were fixed with 0.1% glutaraldehyde and reacted with a 1:100 dilution of positive control serum (mouse anti-IRMPs *P. aeruginosa* PAO1, purified by gel-excision). A 15nm particle size anti-mouse IgG immunogold conjugate was used. Magnification of 42K, photo at x3.

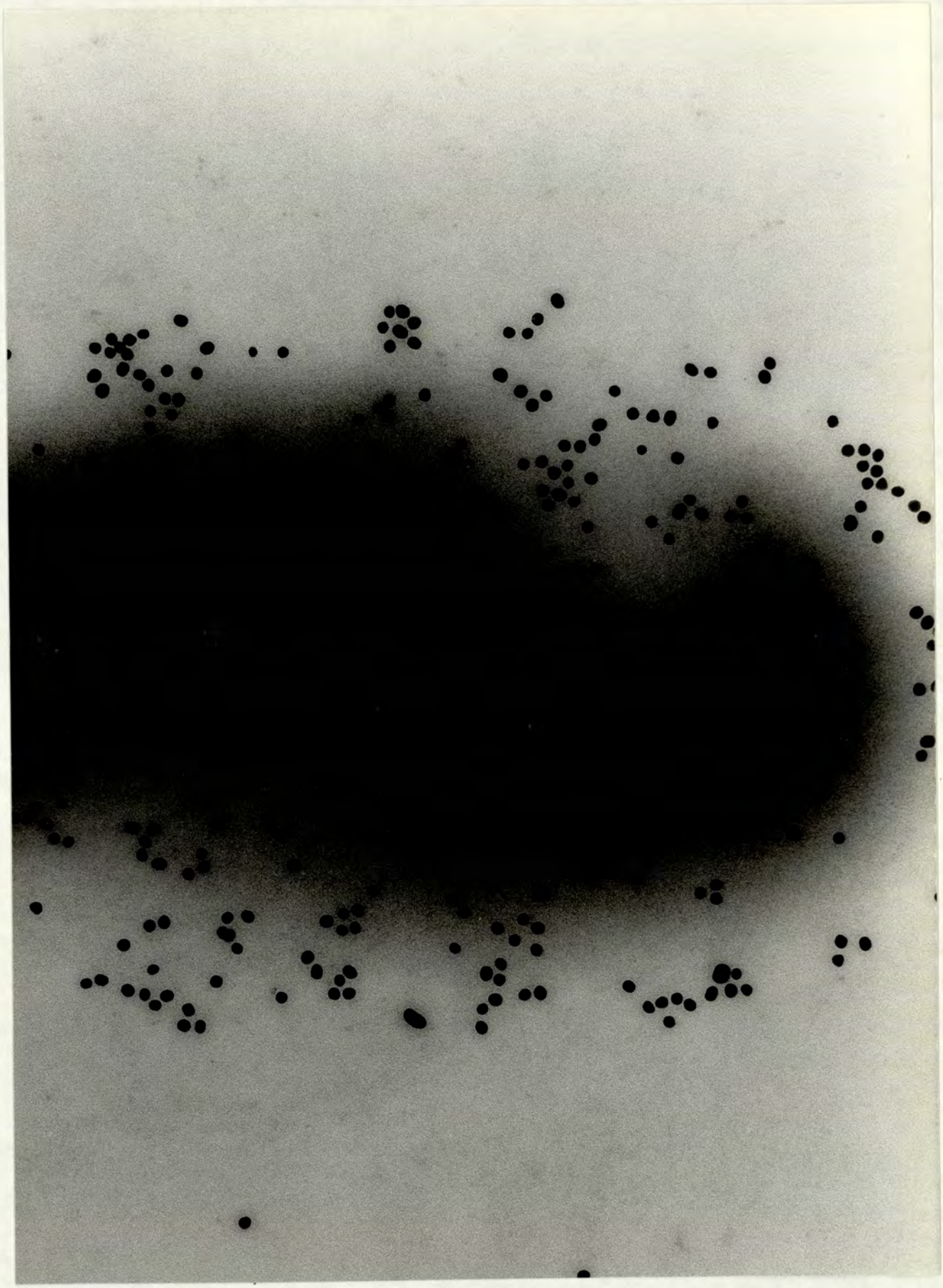


Fig. 5.5.2. Electron micrograph showing immunogold detection of surface antigenic sites of *P. aeruginosa* AK1282. Iron-depleted whole cells of *P. aeruginosa* AK1282 were fixed in 0.1% formaldehyde and reacted with a 1:100 dilution of positive control serum (mouse anti-IRMPs *P. aeruginosa* PAO1, purified by gel-excision). A 15nm particle size anti-mouse IgG immunogold conjugate was used. In this immunogold assay, only 9.27% of the bacteria were strongly positive for gold staining. Magnification of 90K, photo at x3.

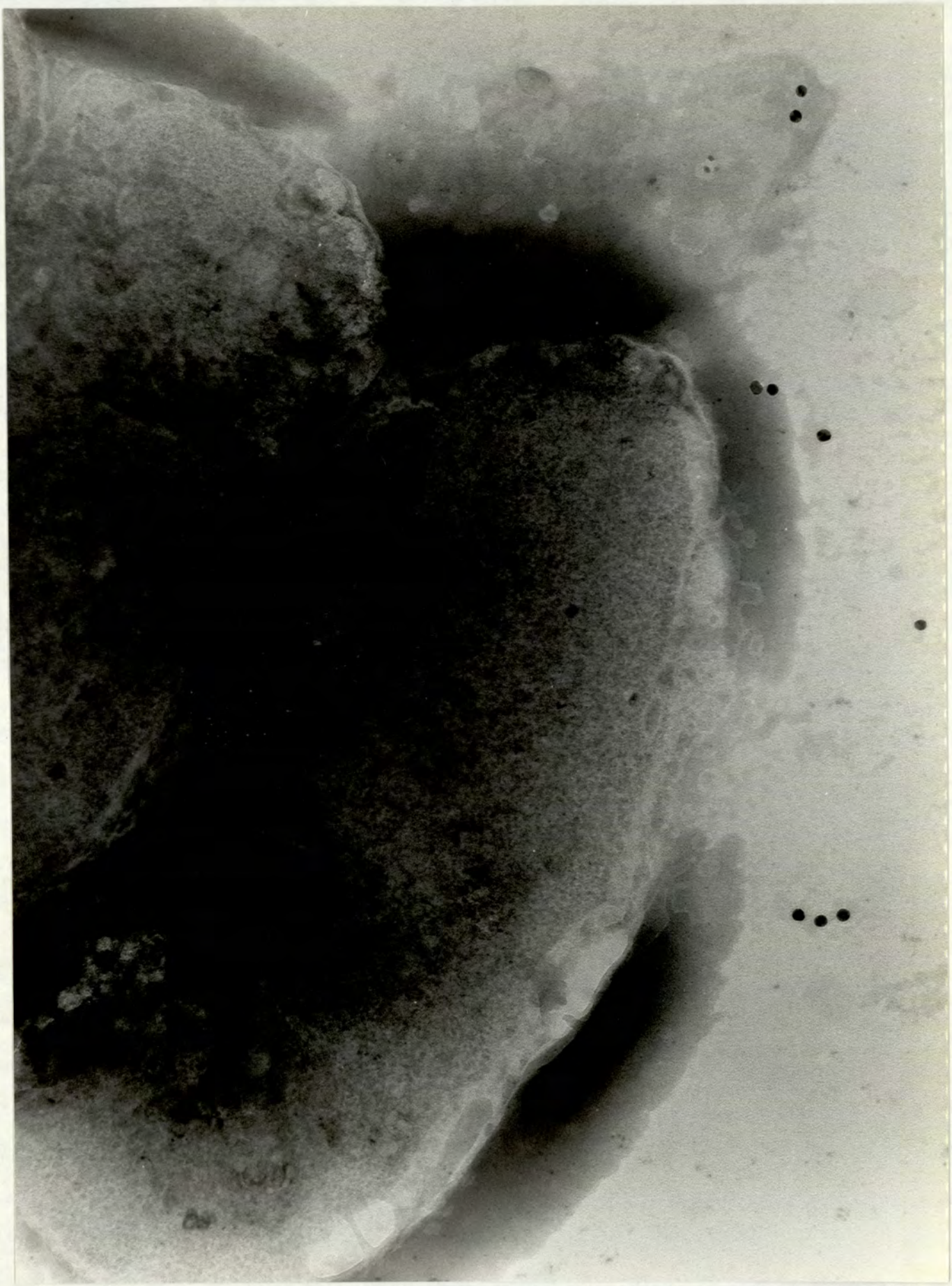


Fig. 5.5.3. Negative control for immunogold detection of surface antigenic sites of *P. aeruginosa*. Iron-depleted whole cells of P. *aeruginosa* strain PAO1 were reacted with a 1:10 dilution of a negative control ascites (mouse anti-human leutenising hormone, IgG_1 isotype). This negative control confirmed a lack of non-specific staining by the anti-mouse IgG immunogold conjugate. Magnification of 42K, photo at x3.

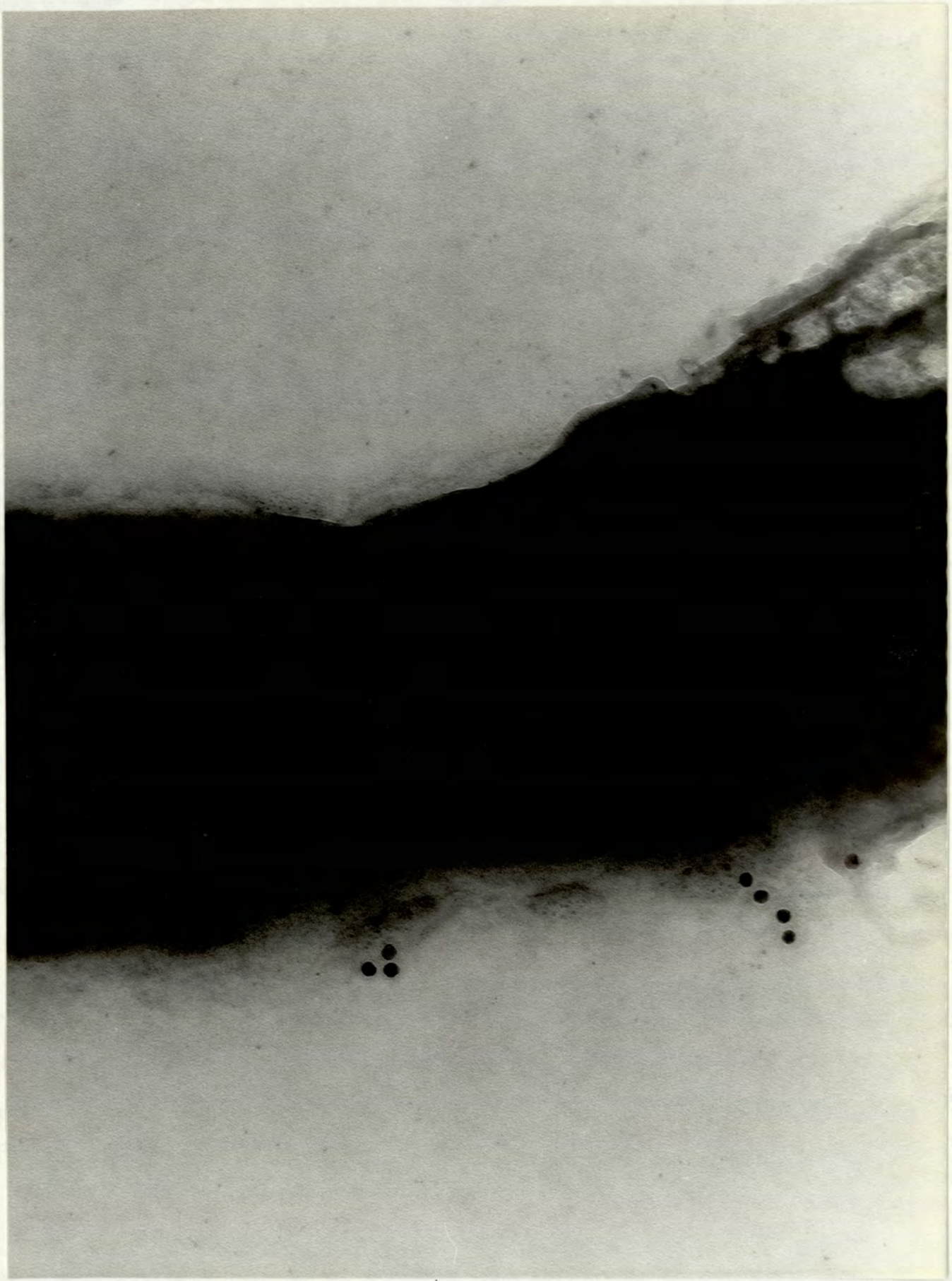


Fig. 5.5.4. Electron micrograph showing immunogold detection of surface antigenic sites of *P. aeruginosa* strain PAO1. Iron-depleted whole cells were were fixed in 0.1% glutaraldehyde and reacted with a 1:100 dilution of monoclonal antibody A2. Antibody A2 was shown in immunoblotting assays to be directed at O-antigen. 15nm particle size anti-mouse IgG immunogold was used as the conjugate. Magnification of 55K, photo at x3.

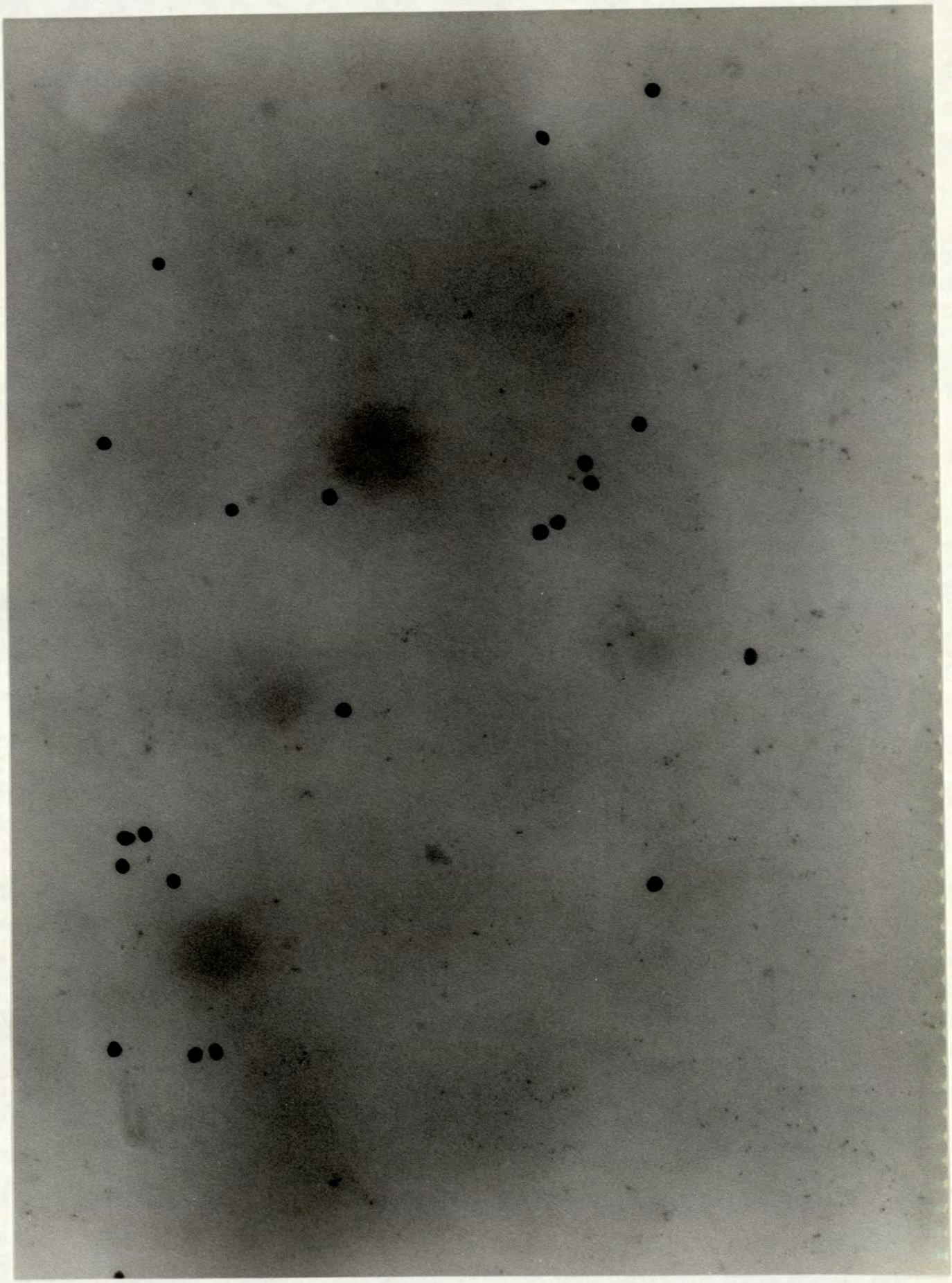


Fig. 5.5.5. Immunogold detection of surface antigenic sites of *P. aeruginosa* AK1282. Specific staining of *P. aeruginosa* was observed when iron-depleted whole cells were reacted with a 1:10 dilution of monoclonal antibody B112.2.1. This antibody was shown in immunoblotting assays to be directed to the IRMPs and protein D. Anti-mouse IgG immunogold conjugate, 15nm particle size was used as the conjugate. The bacteria were fixed in 0.1% formaldehyde. Magnification of 55K, photo at x3.

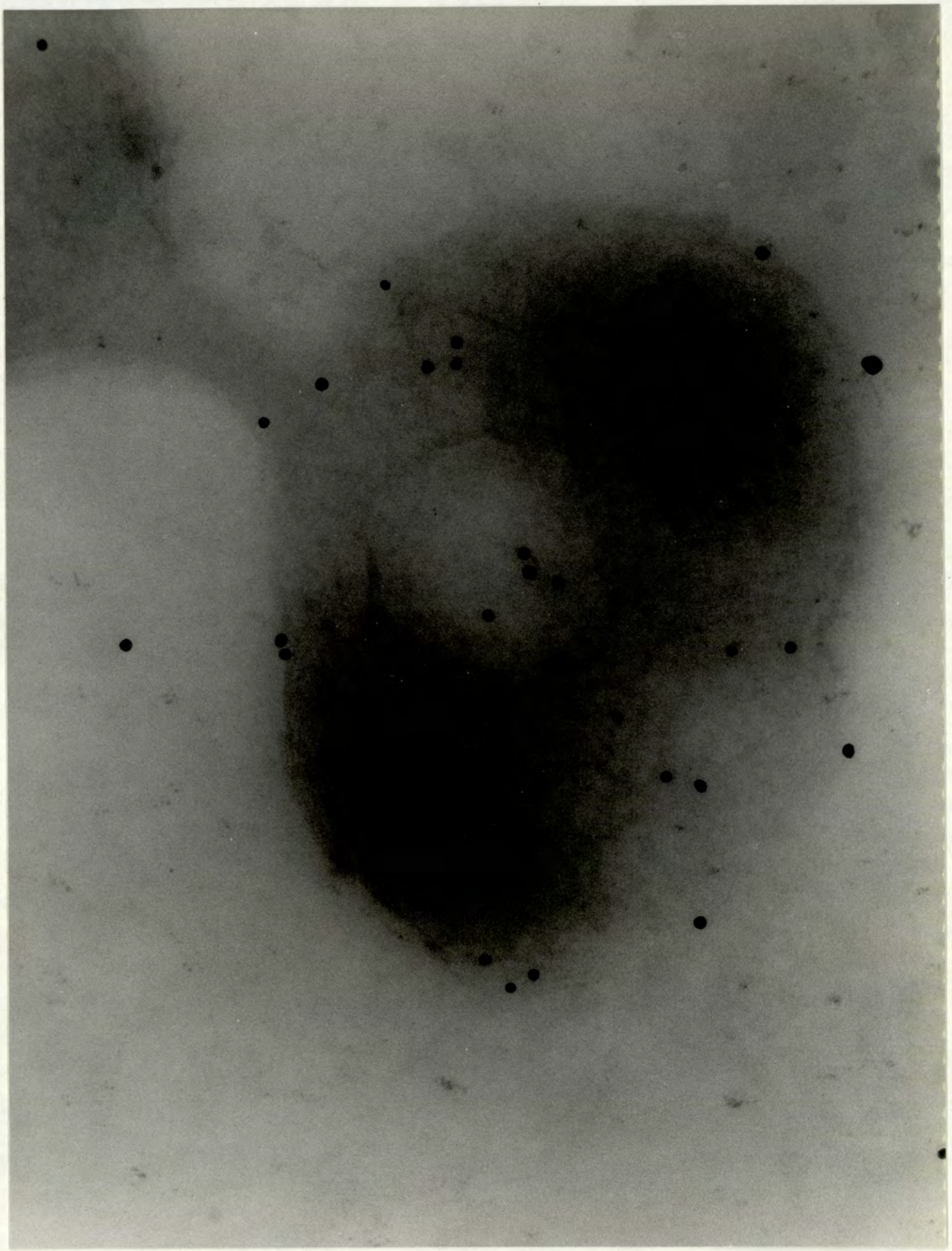


Fig. 5.5.6. Immunogold detection of surface antigenic sites of *P. aeruginosa* AK1282. Specific staining of *P. aeruginosa* was observed when iron-depleted whole cells were reacted with a 1:10 dilution of monoclonal antibody B8.1. This antibody was shown in immunoblotting assays to be directed to the IRMPs and protein D. Anti-mouse IgG immunogold conjugate, 15nm particle size was used to detect bound antibody. The bacteria were fixed in 0.1% formaldehyde. Magnification of 42K, photo at x3.

was found to be a more effective fixative than glutaraldehyde, producing a clearly defined outline of the bacterial cells. The positive and negative controls worked well and therefore the abilities of a selection of monoclonal antibodies generated (chapter 6) was tested for binding to intact bacterial cells.

Fig. 5.5.4 shows monoclonal antibody A2 and it can be seen that specific staining occurred. This was an expected result as antibody A2 is an anti-O-antigen antibody and LPS is one of the major surface-exposed components of the cell envelope (Lam *et al*, 1987a).

The reactions of monoclonal antibodies B112.2.1 and B8.1 are shown in figs. 5.5.5 and 5.5.6 respectively. These antibodies and antibody C103.3 were positive, suggesting that the IRMPs and/or protein D are surface exposed antigens. Only a small selection of antibodies was assayed in the immunogold study, but the assay was repeated five times to confirm results. Antibody B8.1 was positive, even though it is of the IgM isotype, and an anti-IgG conjugate was used.

5.6 Agglutination of *P. aeruginosa.*

Antibodies were analysed for their abilities to agglutinate whole bacterial cells. Fig. 5.6.2 shows agglutination of strain AK1282 by monoclonal antibody C19.3. Monoclonal antibodies B8.1, C16.3 and C103.3 were also effective at agglutination of whole bacterial cells. The mouse polyclonal antibodies raised to excised and eluted IRMPs of *P. aeruginosa* strain PAO1 failed to agglutinate whole cells of strains PAO1 or AK1282 (fig. 5.6.1). The antibodies that were noted as being able to agglutinate whole cells were of various isotype (C19.3 IgG_{2a}; B8.1 IgM; C 16.3 IgG_{2b} and C103.3 IgG_{2a}).

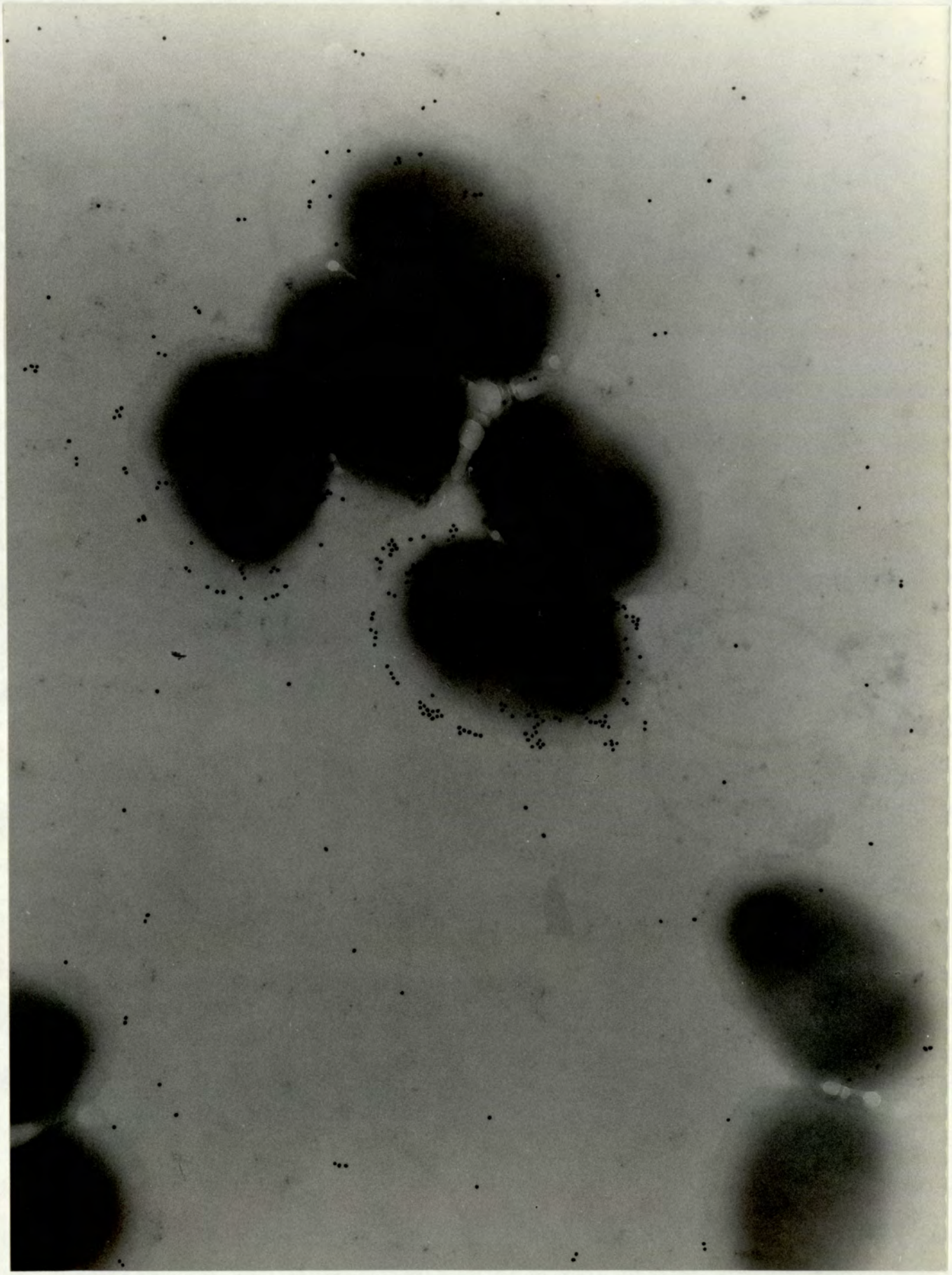


Fig. 5.6.1. Agglutination of *P. aeruginosa* strain AK1282. Iron-depleted whole cells of *P. aeruginosa* AK1282 were reacted with a 1:100 dilution of positive control serum (mouse anti-IRMPs *P. aeruginosa* PAO1, purified by gel-excision). Low magnification (13K) of the electron micrograph shows non-agglutination of bacterial cells. The bacteria were fixed in 0.1% formaldehyde. Anti-mouse IgG immunogold conjugate, 15nm particle size shows specific staining. Photo at x3.

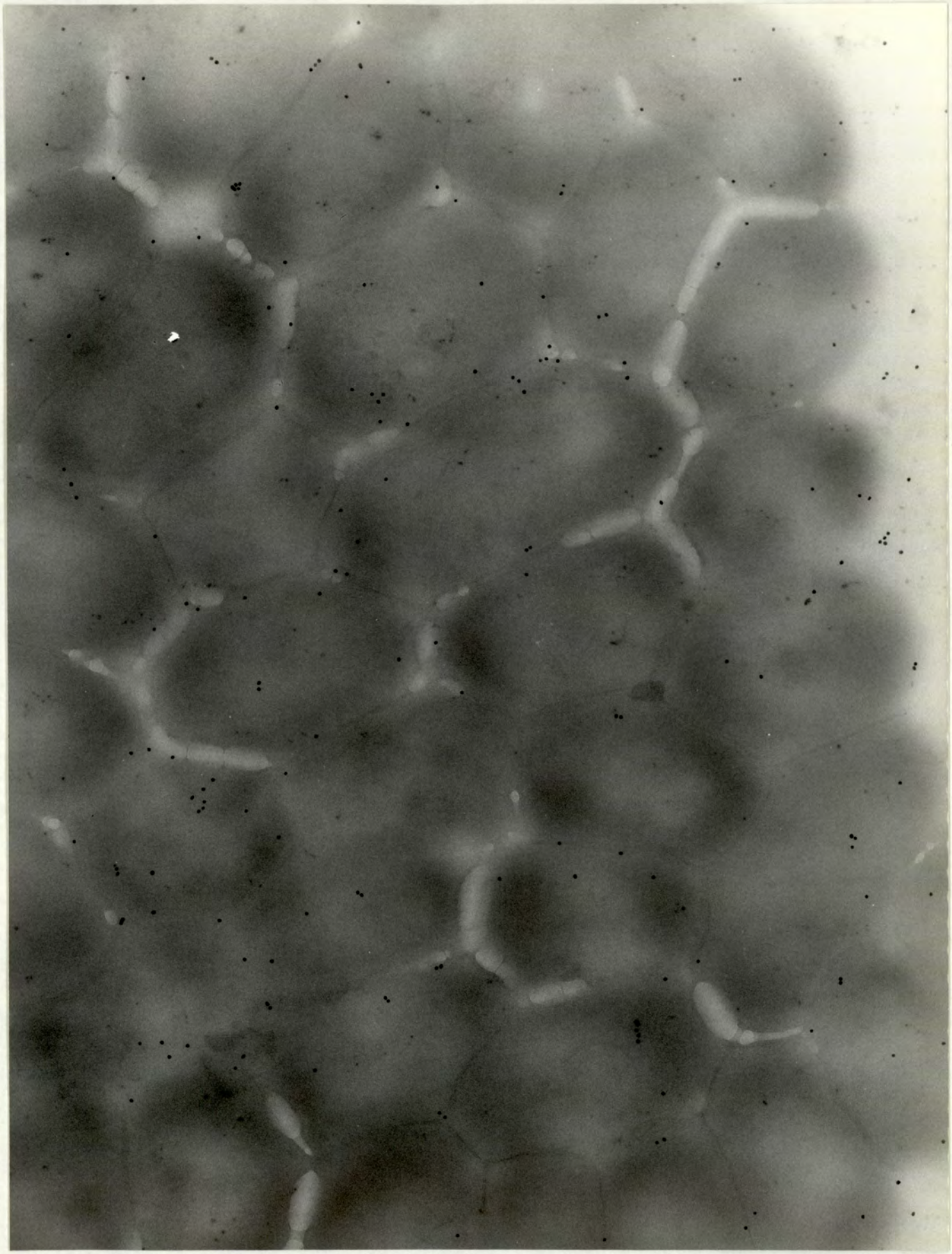


Fig. 5.6.2. Agglutination of *P. aeruginosa* strain AK1282. Iron-depleted whole cells of *P. aeruginosa* fixed in 0.1% formaldehyde were reacted with a 1:10 dilution of monoclonal antibody C19.3. Low magnification (13K) of the electron micrograph shows agglutination of bacterial cells. Anti-mouse IgG immunogold conjugate, 15nm particle size shows a low degree of specific staining. Monoclonal antibody C103.3 showed similar strong agglutination, with very few single cells on the grids. Photo at x3.

5.7 Expression of IRMPs *in vivo.*

A clinical isolate and sample of serum obtained from a diver who was suffering from *Otitis externa* were investigated in immunoblot assays. The infecting organism was grown on CDM-Fe medium to mimic the iron-depleted growth conditions found *in vivo.* Immunoblotting, using the serum and OMs extracted from the isolate, revealed the presence of antibodies to the IRMPs expressed by the organism, as well as antibodies to other OM proteins and to LPS antigenic determinants.

The effect of growth in a hyperbaric oxy-helium environment was investigated. An increased resistance to antibiotics (with the exception of Neomycin, see table 5.7.1) was demonstrated, and it was noted that the clinical isolate was affected by the growth conditions to a greater extent than the two other strains used in the study (NCIB 8295 and NCTC 10662). Results are given as a mean of 5 experiments.

Table 5.7.1. Effect of growth in a hyperbaric oxy-helium enviroment on resistance to antibiotics.

Antibiotic.	Diameter of inhibition of growth (mm).	
	Normobaric growth conditions	Hyperbaric growth conditions
Chlorotetracycline	15.0	12.8
Chloramphenicol	14.7	9.8
Furazolidone	15.3	13.4
Sulphafurazole	14.6	10.8
Neomycin	13.6	14.0
Penicillin G	9.0	9.0
Streptomycin	10.4	10.2
Oxytetracycline	18.8	14.0

It was found that pressure did not affect antibiotic diffusion significantly. Slight changes in the expression of OM proteins were induced during growth in the hyperbaric oxy-helium environment; the expression of minor OM proteins of apparant molecular

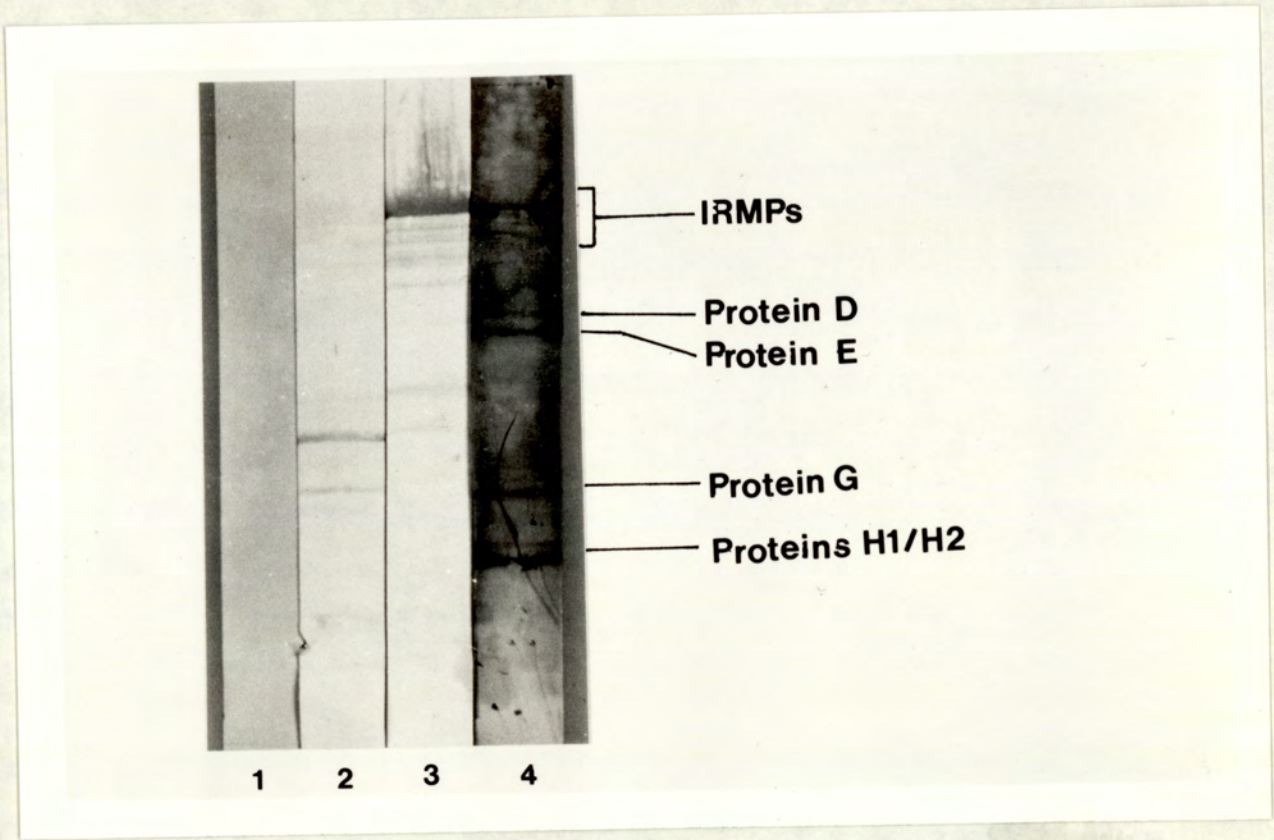


Fig. 5.7.1. Protein immunoblot showing the antigens in an Fe- OM extract of *P. aeruginosa* strain P1 probed with pre-immune serum (lane 2) and post-immune serum (lane 3) from the patient with the pleural cavity infection. The protein A peroxidase conjugate blank is shown in lane 1, and an amido black stained blot is shown in lane 4.

weights of 30, 33, and 37K was frequently enhanced in OM preparations. This change was, however, not reproducable. The effect on physiology, growth rate and numerous other aspects of bacterial growth will have to be investigated before any interpretations are put forward.

Antibodies in the samples of pre- and post-infection serum and the lung fluid obtained from a patient with a pleural cavity infection were analysed to determine their specificities. Immunoblotting assays using electrophoretically-separated OM preparations of the clinical isolate P1 (grown *in vitro* in CDM-Fe) were performed. Fig. 5.7.1 shows that OM antigens were recognised by the post-infection serum, the IRMPs being recognised very strongly. The pre-immune serum demonstrated a very weak response to OM antigens and also the pleural fluid demonstrated a strong response to the IRMPs (data not shown).

An attempt was made to extract bacteria directly from the 1.5L sample of infected pleural fluid. This was unsuccessful and no distinct OM protein profile could be seen on SDS-PAGE or immunoblot analysis of various sarcosyl-insoluble fractions from sequential centrifugation pellets.

5.8 Discussion.

The polyclonal antibodies raised to the IRMPs of *P. aeruginosa* strains PAO1 and AK1282 exhibited cross-reaction with OM components, including the IRMPs, in the OMs of representatives of the seventeen serotypes of *P. aeruginosa.* It was hoped that anti-IRMP antibodies, in order to promote the potential of IRMPs as vaccine candidates, would be cross-reactive with all serotypes strains of *P. aeruginosa.* This was not the case with the antibodies raised to these two strains. Due to the variability of expression of IRMPs, it is possible that the IRMPs expressed by these strains may be atypical and exhibit less

cross-reactivity than those of other *P. aeruginosa* strains.

Recent evidence indicating the presence of classes of IRMPs was presented by Cornelis *et al* (1989). On examination of *P. aeruginosa* PAO1, ATCC 27853 and 89 clinical isolates, these workers found that an 80K IRMP is the receptor for the siderophore pyoverdine. There were, however, 3 pyoverdine specificity groups, as judged by the pyoverdine growth promotion activities in homologous and heterlogous pyoverdine-mediated ^{59}Fe uptakes. Demonstration of the presence of classes of receptors for different pyoverdine structural molecules was confirmed using a purified polyclonal antiserum against the 80K IRMP from *P. aeruginosa* strain PAO1, which was able to block the uptake of ^{59}Fe-pyoverdine in PAO1, and to detect the 80K IRMP on immunoblots of strains belonging to the same pyoverdine-specificity group. Another antiserum against the group 2 pyoverdine receptor showed the expected specificity, while an antiserum to the third group of pyoverdine receptors was less specific. This grouping of IRMPs reported by Cornelis *et al*, (1989) may help to explain the results obtained in this study when it was demonstrated in immunoblot assays that the IRMPs were not fully cross-reactive amongst the seventeen serotypes.

Other evidence acquired by Sokol and Woods (1986a) and Black *et al*, (1986), has indicated a good cross reactivity of IRMPs. Sokol and Woods (1986a) produced antibodies specific for FBP and found that they cross-reacted with FBP in OM preparations of all serotypes of *P. aeruginosa*. In addition, Black *et al*, (1986) demonstrated with immunoblotting studies that certain IRMPs of *Neisseria meningitidis* clinical isolates were antigenically related, by showing that rabbit antiserum against the 70K IRMP from one of the disease isolates contained IgG and IgM antibodies that reacted with the 70K IRMP from 4 meningococcal disease isolates.

Additional evidence suggesting the presence of common epitopes is presented in chapter 1. The conclusions drawn from the evidence presented here, and that of other workers discussed both here and in chapter 1, is that iron-acquiring systems are most likely conserved within a given bacterial species and therefore IRMPs demonstrate potential to be utilised as a cross-reactive antigen in immunotherapeutic trials.

The interaction of antibodies specific for proteins H2, F and I with various serotype strains has been investigated by Hancock *et al* (1982), Mutharia and Hancock (1983) and Pfaller *et al* (1989). The data presented here agrees with the previous reports suggesting that these proteins have antigenic sites common to all *P. aeruginosa* serotypes. Evidence acquired by Hancock *et al* (1982) indicated that monoclonal antibodies to protein H2 were able to interact with the OMs of strains representing the seventeen serotypes of *P. aeruginosa*. In addition, the antibodies generated by Mutharia and Hancock (1983) (MA1-3, anti-protein I and MA1-6, anti-protein H2) were found to interact with these proteins in the OMs isolated from strains representing the seventeen serotypes of *P. aeruginosa*, and from another 15 clinical isolates from patients with cystic fibrosis. The antigenic sites recognised by these antibodies were therefore common to all *P. aeruginosa* serotypes. Anti-protein F monoclonal antibodies are already commercially exploited as immunodiagnostic agents; a direct fluorescent monoclonal antibody to *P. aeruginosa* porin protein F is the basis of a diagnostic test marketed by Genetic Systems Corporation, Washington. This antibody has been found to be highly effective at detection of all serotypes strains of *P. aeruginosa*, demonstrating a 100% detection in blood cultures, with no detection of other bacteria (Pfaller *et al*, 1989).

The anti-O-antigen antibody A2 bound to determinants in several serotypes of *P. aeruginosa*. This was unexpected as *P. aeruginosa* is serologically heterogeneous with seventeen LPS type-specific O-antigens described by the IATS, the differences among the O-antigen side chains forming the basis of the serotyping system (Liu *et al*, 1983). Other

workers, however, have demonstrated cross-reactivity between heterologous O antigen side chains. For example, Hancock *et al* (1982) generated a monoclonal antibody (MA1-8) specific for the O-antigen of *P. aeruginosa* strain PAO1 which reacted strongly with the OM of a serotype 5 strain (PAO1 is classified as Type 5 in the IATS, Cox,1979), but also interacted weakly with the O-antigen of type 17.

It was noted that there was a difference in the cross-reactivity of polyclonal and monoclonal antibodies in that the polyclonal antibodies appeared to be less specific. This is in agreement with Sokol and Woods (1986a) who found that the interaction of monoclonal antibodies with FBPs from *Pseudomonas* spp. other than *P. aeruginosa* was more specific than that observed with a polyclonal rabbit anti-FBP IgG immunoglobulin.

The monoclonal antibodies produced reacted with antigens in the OMs of the two other Gram-negative bacterial species examined, although IRMPs expressed by these species were not detected. Cross-reactivity of antigens between different bacterial species has been demonstrated by Carniel *et al* (1989a), who showed that two of the high molecular weight IRMPs of *Yersinia entercolitica* serovar 0:8 had common epitopes with the high molecular weight IRMPs of the high-virulence-phenotype species *Y. pestis, Y. pseudotuberculosis* and a different strain of *Y. entercolitica* serovar 0:8. Additional evidence indicating cross-reactivity was reported by Mietzner *et al* (1986), who investigated the presence of proteins antigenically related to the 37K IRMP of *N. gonorrhoeae.* These workers purified the 37K protein from *N. gonorrhoeae* F62 and prepared both monospecific rabbit antisera and murine monoclonal antibodies. Fifty seven strains from nine species of *Neisseria* and *Branhamella catarrhalis* were probed using immunoblotting and ELISA assays to identify cross-reactivity. Forty of the strains examined reacted with monospecific antiserum raised to the purified protein and two of seven monoclonal antibodies were broadly cross-reactive, recognising proteins that reacted with the monospecific antiserum in all the species examined. The other five

antibodies were more discriminating, only recognising these proteins from certain species.

Additional evidence indicating the conservation of antigenic sites was presented by Sokol and Woods (1986b), who used monoclonal antibodies to FBP as probes to investigate the antigenic conservation of FBP among *Pseudomonas* strains and other bacterial species. Two of the monoclonal antibodies they produced reacted with FBP in strains of *P. putida, P. fluorescens* and *P. stutzeri*, but no cross-reaction with OM proteins from other Gram-negative bacteria (*P. cepacia*, *P. multivorans* and *P. maltophila*, among others) was detected. Other evidence concerning the cross-reactivity of monoclonal antibodies specific for OM antigens has been presented by Mutharia and Hancock (1983), who investigated the cross-reactivity of protein F-specific monoclonal antibodies with OM proteins from other bacterial species. The monoclonal antibodies did not react with unrelated species, with the exception that there was a slight recognition of protein F in the OM of *P. putida*. Additionally, Hancock *et al* (1982) produced a monoclonal antibody reacting with OM protein H2 of *P. aeruginosa* and found that the antigenic site against which this monoclonal antibody was reactive was present in the OMs of two *P. fluorescens* strains, two *P. putida* strains, a *P. anguilliseptica* strain and an *Azotobacter vinelandii* strain, but not in the OMs of five other bacterial species examined.

LPS antibodies were found to be immunoprecipitated from sera by incubation with purified LPS. Chart and Griffiths (1985) found that incubation of serum with purified LPS from *E. coli* strain 0111 effectively absorbed out anti-LPS antibodies in serum raised to a preparation of the 81K IRMP of *E. coli* 0111. Indeed, some of the protection studies reported have relied on effective removal of anti-LPS antibodies by this method to demonstrate the protective activity of anti-protein components of a polyclonal antisera (Bolin and Jensen, 1987; Loeb, 1987).

It was noted that both polyclonal and monoclonal antibodies bound to the rough strain (AK1282) of *P. aeruginosa* more easily than they bound to the smooth strain PAO1. This is probably due to either an unmasking of antigenic determinants (by loss of the O-antigenic side chains of LPS) or a rearrangement of the OM components of the rough strains. Studies by Mutharia and Hancock (1983) were in agreement with this finding. These workers investigated the interaction of a monoclonal antibody (directed towards protein H2 of *P. aeruginosa*) with whole cells of this bacterium. The antibody showed no fluorescence on intact, smooth *P. aeruginosa* bacterial cells, but was able to interact with a rough, LPS-deficient mutant. Additionally, van der Ley *et al* (1986), who investigated the binding of antibody molecules to the outer membrane pore protein PhoE in intact cells of members of the *Enterobacteriaceae*, found a direct correlation between the presence of O-antigenic side chains and the inability of anti-PhoE protein-specific antibodies to bind to the cells.

The interaction of antibodies with rough strains is significant as these strains are frequently isolated from patients with cystic fibrosis (Hancock *et al*, 1983; Ojeniyi *et al*, 1989) and therefore these bacteria might present OM proteins as surface antigens to a greater extent than smooth organisms. The effect of the mucoid phenotype on the interaction of antibodies with *P. aeruginosa* (a factor to take into account when considering cystic fibrosis isolates) must also be investigated (Baltimore and Mitchell, 1980), and there are other important complications in cystic fibrosis, for example the formation of immune complexes.

Another indication that IRMPs and protein D are surface-exposed antigens was demonstrated using the whole-cell ELISA technique, which offers the advantage of measuring antibodies directed to surface-exposed antigenic determinants (Borowski *et al*, 1984). Monoclonal antibodies to IRMPs and protein D and serum raised to purified IRMPs reacted with whole cells of *P. aeruginosa* (data not shown).

The ability to react with intact cells, or even with cell envelopes rather than purified components, is more indicative of protective antibodies as the epitopes involved in the reaction with purified components may not be exposed on the surface of the intact cell. The fact that monoclonal antibodies specific for the IRMPs and protein D were able to bind to intact bacterial cells promotes the potential application of anti-IRMP antibodies to immunotherapy.

As no work has been published on the surface expression of protein D of *P. aeruginosa*, the results presented here cannot completely confirm that the IRMPs are surface exposed antigens: the possibility exists that the monoclonal antibodies were reacting with surface-exposed protein D. It is suggested, however, that as certain of the IRMPs have been implicated as being directly involved in iron-siderophore complex uptake (Cody and Gross, 1987; Cornelis *et al*, 1987; Icihara and Mizushima, 1978; Pierce and Earhart, 1986; Sokol and Woods, 1983; 1984), surface exposure of these proteins would be a necessity for their function.

Certain monoclonal antibodies, notably C103.3 and C19.3 were found to agglutinate whole bacterial cells. The property of agglutination of bacterial cells is advantageous when considering the potential of an antibody to be protective and therefore these antibodies were considered as being particularly promising as immunotherapeutic agents. Other workers have generated monoclonal antibodies which are capable of agglutinating bacteria. For example, Hancock *et al* (1982) produced an monoclonal antibody MA1-8, specific for the O-antigen of *P. aeruginosa* strain PAO1, which was able to agglutinate whole cells of strain PAO1, strain Z61 and a representative of serotype 5, but not any other serotyping strain used. MA1-3 (anti-protein I) and MA1-6 (anti-protein H2) failed to agglutinate any of the strains, including PAO1. This would suggest a correlation between anti-LPS specificity and agglutination activity, however the antibody produced here that was O-antigen specific was unable to agglutinate whole cells, whereas other OM

protein-specific antibodies were able to mediate agglutination.

Hancock *et al* (1982) found that a rabbit anti-whole OM serum agglutinated PAO1, Z61 and serotype 16 cells strongly, serotype 5 cells moderately and serotypes 4, 6, 7, and 17 cells weakly (Hancock *et al*, 1982), while the other 11 serotype strains were not agglutinated. The polyclonal serum described here did not agglutinate whole cells; it is suggested that the IgG component of the serum may possibly have had a higher avididity for binding sites on the cell surface and therefore block antibodies of the IgM isotype, which are usually indicated as being involved in agglutination reactions.

Research in the laboratory at Aston has contributed to the finding that IRMPs, integral components of the iron-uptake systems of Gram-negative bacteria, are powerful immunogens, expressed *in vivo* and recognised by the host's immune system. Bacteria recovered directly from infections of burn wounds (Ward *et al*, 1988), the urinary tract (Lam *et al*, 1984; Shand *et al*, 1985) and the cystic fibrotic lung (Anwar *et al*, 1984; Cochrane *et al*, 1987; 1988) have all been shown to express IRMPs. This, in fact, was the basis of the investigation into the expression and protective activities of antibodies directed towards IRMPs. To extend evidence on the recognition of these important antigenic determinants by the host's immune system, two other infection situations were investigated.

Saturation diving conditions provide a unique, extreme infection situation. Infection is promoted in the hyperbaric oxy-helium environment of a saturation complex due to the high humidity, high temperature and cramped conditions. "Diver's ear", *Otitis externa*, occurs frequently and the causative organism is often *P. aeruginosa.* Investigation into the antigenicity of *P. aeruginosa* in this infection situation was undertaken, using serum taken from an infected diver to probe immunoblots of OMs extracted from the corresponding clinical isolate, grown under hyperbaric or isobaric oxy-helium, iron-depleted

conditions. The IRMPs and other major OM proteins in addition to LPS antigens were recognised by antibodies in the patient's serum. No sample of pre-immune serum was available, so it could not be determined if the production of these antibodies was a direct response to the infection, however, it is suggested that this was the case, as the titre against *P. aeruginosa* among healthy individuals is generally low. Attempts were made to acquire further sera samples and clinical isolates from similar cases of *Otitis externa*, however, despite contacts with Naval and commercial medical centres, no samples were obtained due to the inaccessibility of the divers in saturation complexes.

It was shown that bacteria became more resistant to antibiotics when grown under conditions of hyperbaric oxy-helium. The results agreed with those of Kenward *et al* (1984). This increased antibiotic resistance has implications on the treatment of infections occuring in hyperbaric environments.

The immune response to OM antigens of *P. aeruginosa* in a pleural cavity infection was also investigated. Immunoblotting studies demonstrated that IRMPs were strongly recognised by post-infection serum and lung fluid collected from the patient with the infection. The serum was taken 7 days after the infection was detected and therefore the response to the IRMPs appeared to be one of the primary reactions to infection. This suggested that the pleural cavity infection was similar with respect to immunological response, to the acute infections (such as burn wound and urinary tract infections) reported previously, rather than the chronic *P. aeruginosa* lung infection which occurs in cystic fibrosis sufferers.

In summary, IRMPs were confirmed to be important antigens as they were recognised early in the course of a pleural cavity infection and an infection of *Otitis externa*. Specific antibodies to the IRMPs and protein D were able to interact with whole cells of *P. aeruginosa*, suggesting that these antigens are surface exposed immunogens. IRMPs were immunologically conserved among different serotype strains of *P. aeruginosa*; they are

therefore potential candidates for inclusion in immunisation preparations.

6 Production of a panel of monoclonal antibodies against OM antigens of *P. aeruginosa.*

6.1 Introduction.

A panel of antibodies specific for various OM components would be an invaluable tool in the investigation of the cell surface of *P. aeruginosa.* Selection of antibodies specific for the protein components of Gram-negative bacterial envelopes has, however, been severely restricted by the association of these proteins with immunodominant LPS molecules. Indeed, polyclonal antisera raised in rabbits to IRMPs purified by selective solubilisation and gel-filtration chromatography (section 4.9), were found to contain antibodies to LPS and to other OM proteins when there was only minimal contamination of immunising preparations detected on Coomassie blue-stained SDS-PAGE gels. By utilising the hybridoma technology developed by Kohler and Milstein (1975), however, relatively impure antigens can be used to produce highly specific antibodies. Preliminary purification of the antigen is highly desirable to minimise screening procedures and to avoid swamping the anti-IRMP response by strong immunogens such as LPS. Therefore, IRMPs purified either by selective solubilisation and gel-filtration chromatography (sections 3.1.2 and 3.2.5), or by the method of excision from SDS-PAGE gels (section 3.1.6.1) were used to generate antibodies specific for these antigens.

6.2 Production and characterisation of the monoclonal antibodies.

Three fusions were performed using the two myeloma cell lines or OKT3 hybridomas described in section 3.3.3.1, and the spleen cells taken from F_1 (CBA x BALB/c) hybrid mice immunised with the following antigen preparations:

Fusion:

A. IRMPs of *P. aeruginosa* PAO1 prepared by the method of gel excision (section 3.1.6.1). (Fusion with OKT3).

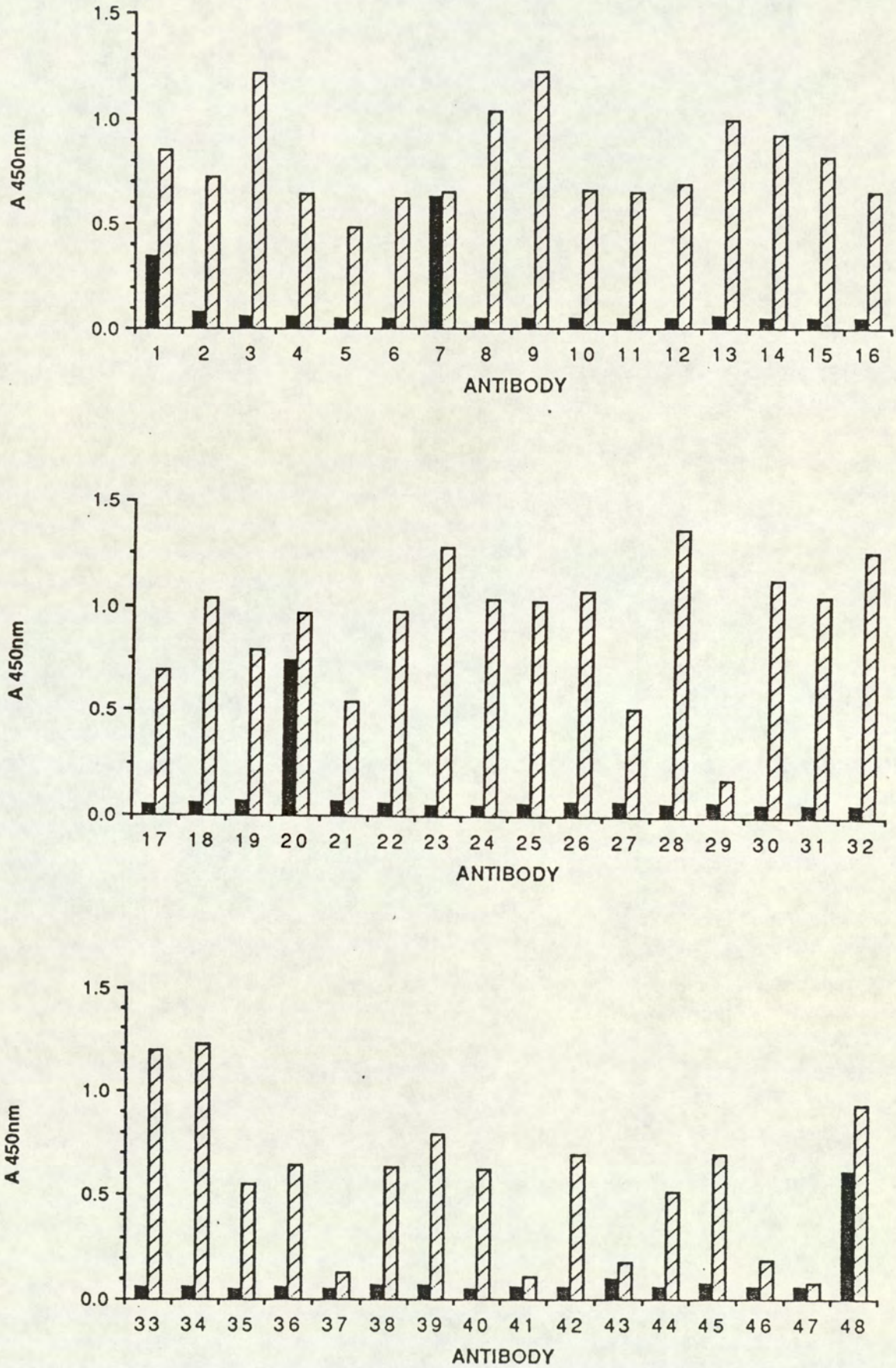
B. As for fusion A, except the fusion was with NS1 and NS0 myeloma cells.

C. IRMPs of *P. aeruginosa* AK1282 prepared by selective solubilisation and gel-filtration chromatography (sections 3.1.2 and 3.2.5). (Fusion with NS1 and NS0 myeloma cells).

The selection of clones from fusion C is detailed here; however clones from fusions A and B were screened and selected in a similar manner. The primary screen to detect specific antibody-positive clones was an ELISA assay which utilised OMs Fe- of *P. aeruginosa* AK1282 as the antigen. Forty six hybrid cell lines were positive in this primary screen and were further characterised by assay in an ELISA screen devised to differentiate between antibodies to LPS and to other OM components. ELISA plates were prepared with either purified AK1282 LPS (1mg/100mls) or OMs Fe- AK1282 (1:1000 dilution of OM preparation) being used as the coating antigen. One in ten dilutions of cell supernatants from the first set of 24-well plates (produced during the scale-up of selected hybrids) were used as a source of antibody, and a 1/1000 dilution of anti-mouse $F(ab')_2$ fragments conjugate linked to HRP was used to detect binding.

The results from this assay are depicted in fig. 6.2.1, in which antibodies 1-36 are colonies C1-C36 and antibodies 37-46 are colonies C101-C110. Antibody 47 is the PBS-Tween negative control, and antibody 48 is the positive control serum, taken from the mouse immediately prior to removal of the spleen for fusion C. Colonies C1, C7 and C20 reacted positively with purified LPS, and were therefore not maintained. All other wells, except the positive control, did not bind to the LPS. All of the colonies produced antibodies which bound to OMs Fe-, but hybrids C101, C105, C107 and C110 were discarded as the antibody bound only weakly to the OMs Fe-. C104, C106 and C109 were also discontinued, due to poor growth of these hybrids.

Fig. 6.2.1. Selection of clones from fusion C. Elisa data obtained during the screening of C monoclonal antibodies. This ELISA screen was developed to differentiate between antibodies directed to purified LPS and other OM components.



SEE TEXT FOR KEY TO ANTIBODIES USED IN THIS STUDY

■ PURIFIED AK1282 LPS USED TO COAT ELISA PLATE
▨ OMs Fe- AK1282 USED TO COAT ELISA PLATE

Further characterisation was performed using immunoblotting techniques. Specific antibodies were produced, despite the O-antigen contamination of the antigen preparations. Table 6.2.1 details the antibodies that were selected from the three fusions.

Table 6.2.1. Characterisation of the monoclonal antibodies produced from fusions A, B and C.

Monoclonal antibody	Isotype and reaction with Protein A	Specificity
A 1	IgG_1; Prot A -ve	IRMPs + D
A 2	$IgG_1/IgG_{2a/2b/3}$; Prot A +ve	O-antigen
B 1	Prot A -ve	
B 3.1	Prot A -ve	IRMPs + D
B 4	IgG_1; Prot A -ve	IRMPs + D + F
B 5	Prot A -ve	
B 6	$IgG_{2a/2b/3}$; Prot A +ve	
B 7	Prot A -ve	
B 8.1	IgM/ IgG_1; Prot A -ve	IRMPs + D
B 9	IgG_1; Prot A -ve	
B 10	Prot A -ve	
B 11	Prot A +ve	
B 12	Prot A -ve	
B 13	Prot A -ve	
B 14.1	Prot A -ve	IRMPs + D
B 15	IgM; Prot A -ve	Various
B 16	Prot A -ve	
B 17	Prot A -ve	
B 19	Prot A -ve	
B 20.1	$IgG_{2a/2b/3}$; Prot A +ve	IRMPs + D + E + H
B 21	Prot A -ve	
B 22.1.1	$IgG_{2a/2b/3}$; Prot A +ve	IRMPs + D + E + H
B 23.1	Prot A -ve	
B 24	Prot A +ve	
B 25	Prot A -ve	
B 26	IgM; Prot A -ve	
B 27.1	Prot A -ve	IRMPs + D + G
B 101.1	IgG_1; Prot A -ve	Various
B 102	Prot A -ve	
B 103.1	IgG_1; Prot A -ve	Not reactive
B 104.1.1	IgG_1; Prot A -ve	
B 105.1.1	Prot A -ve	
B 106.1	IgG_1; Prot A -ve	H
B 107.1	$IgG_{2a/\ 2b,/3}$/IgM; Prot A +ve	Lipid A
B 108	$IgG_{2a/2b/3}$; Prot A +ve	
B 109	Prot A +ve	
B 110.1	IgG_1; Prot A +ve	

B 111	IgM; Prot A +ve	
B 112.2.1	IgM	IRMPs + D
B 113	Prot A -ve	
B 114	Prot A -ve	
B 115	Prot A -ve	
B 116	$IgG_{2a/2b/3}$; Prot A +ve	
B 117	Prot A -ve	
B 118.1	IgG_1; Prot A -ve	IRMPs + D
C 1		LPS
C 2	IgG_{2a}; Prot A +ve	
C 4	IgG_{2a}	
C 5	Prot A +ve	
C 6	IgG_1; Prot A -ve	
C 7		LPS
C 8	Prot A -ve	
C 9	IgG_1; Prot A +ve	
C 10	IgG_1; Prot A -ve	
C 11	IgM; Prot A -ve	
C 12	IgG_{2a}	D+E+F
C 13	IgG_{2b}	
C 14	IgG_{2a}	
C 15	IgG_{2a}; Prot A +ve	IRMPs+D+E+F
C 16	IgG_{2b}; (Prot A +ve)	
C 17	IgG_1; (Prot A +ve)	
C 18	Prot A +ve	
C 19	IgG_{2a}; Prot A +ve	IRMPs+D+E+F
C 20		LPS
C 21	Prot A +ve	
C 22	IgG_{2a}; Prot A +ve	
C 23	IgG_{2a}; Prot A +ve	IRMPs+D+E+F
C 24	IgG_{2a}	D+E+F
C 28	IgG_{2a}; Prot A +ve	IRMPs+D+E+F
C 31	IgG_{2b}; Prot A +ve	
C 32	IgG_{2a}; Prot A +ve	
C 34	IgG_1; Prot A -ve	
C 102	IgG_{2b}	
C 103	IgG_{2a}; Prot A +ve	IRMPs+D+E+F
C 108	IgG_{2b}; (Prot A +ve)	IRMPs+D+E+F

6.3 Isotypes of monoclonal antibodies produced from fusions A, B and C.

A total of 75 antibodies that reacted with OMs Fe- of *P. aeruginosa* strains PAO1 or AK1282 were characterised. Antibodies from all subclasses, except IgA, were generated and of those isotyped, the predominant immunoglobulin subclass was IgG. A high proportion of the antibodies produced from fusion C were reactive with protein A.

6.4 Specificity of monoclonal antibodies produced from fusions A, B and C.

Figs. 6.4.1 to 6.4.3 show immunoblots demonstrating the reactions of various monoclonal antibodies with OM components of *P. aeruginosa*. The immunoblot screen was used to select an antibody that reacted with an IRMP, but had no reaction with other OM components.

Antibody A2 was shown to have an O-antigen specificity. Fig. 6.4.1, track 2 shows the characteristic pattern of the O-antigen bands detected by antibody A2 in the proteinase K treated OM samples. No response to lipid A was displayed by this antibody. Although antibody A2 was not specific for the IRMPs, the O-antigen antibody was useful as a positive control to confirm efficient transfer and good resolution of the O-antigen on the immunoblots performed, and to further characterise the expression of O-antigenic molecules on the surface of *P. aeruginosa* (see chapter 5).

Fig. 6.4.1 also shows protein and LPS immunoblots of *P. aeruginosa* Fe- OMs probed with the IRMP and protein D-specific antibody B112.2.1. This antibody bound to these protein components of the cell envelope and demonstrated no reaction with LPS antigenic determinants. Other antibodies found to be directed towards the three major IRMPs and protein D included B8.1 and 118.1. Fig. 6.4.2 shows the protein and LPS immunoblots of OMs Fe- reacted with antibodies B112.2.1 (lanes 1 and 2), B118.1 (lanes 3 and 4), B8.1 (lanes 5 and 6), B103.1 (lanes 7 and 8) and B101.1 (lanes 9 and 10). The amido

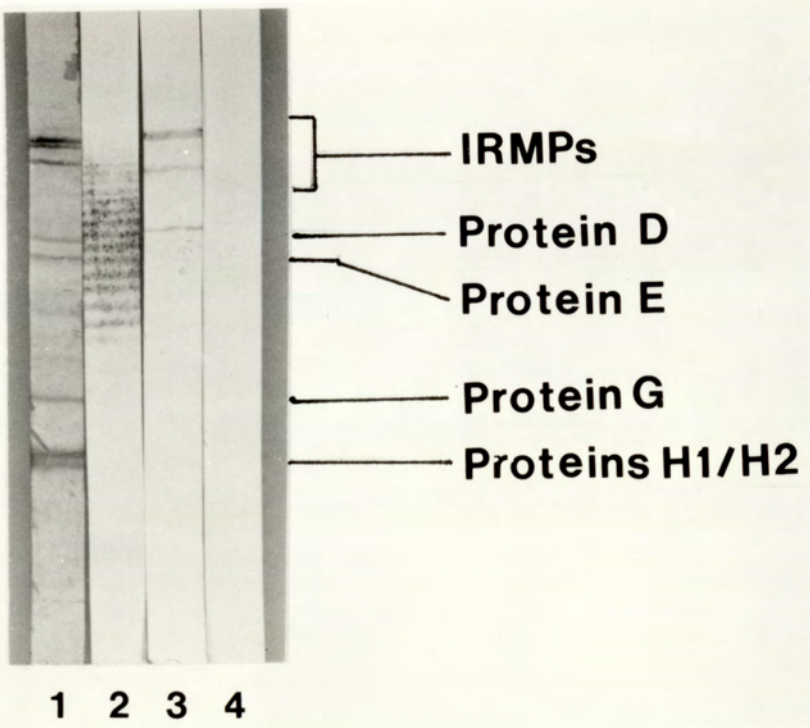


Fig. 6.4.1. LPS and Protein immunoblots of electrophorectically separated antigens from the OM of *P. aeruginosa* strain PAO1. Lane 1 is an amido black stained protein blot, which gives a clear identification of the protein antigens present in the sample. Lane 2 is an LPS blot probed with monoclonal antibody A2, an anti-O-antigen monoclonal, confirming effective transfer of LPS antigens onto the nitrocellulose sheet. Protein (lane 3) and LPS (lane 4) blots of the antigenic sites recognised by monoclonal antibody B112.2.1 are shown; this antibody recognised two IRMPs and protein D. There was no detection of O-antigen or lipid A. Biotinylated anti-mouse $F(ab'_2)$ fragments and streptavidin-HRP were used as the conjugates.

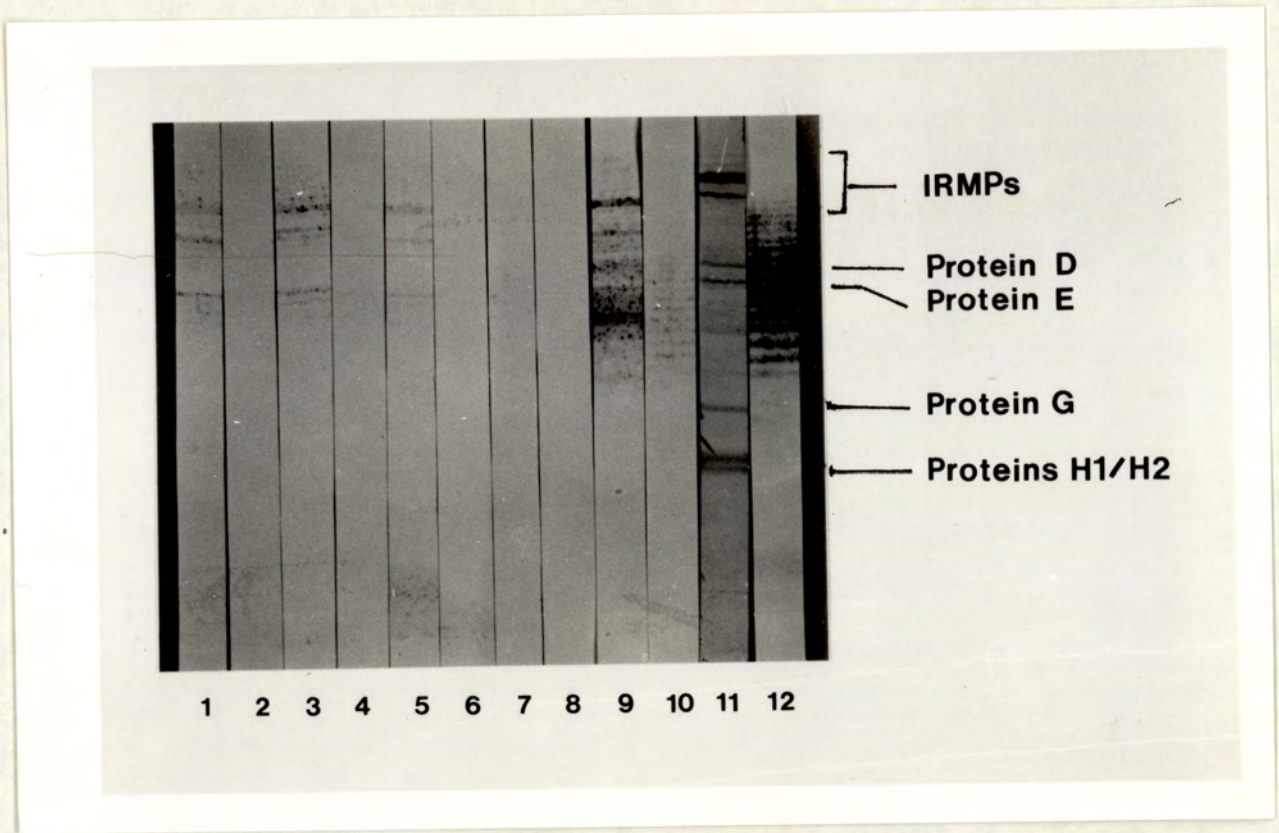


Fig. 6.4.2. LPS (lanes 2, 4, 6, 8, 10 and 12) and protein (lanes 1, 3, 5, 7, 9, 11) immunoblots of electrophoretically separated antigens from the OM of *P. aeruginosa* strain PAO1. Monoclonal antibodies B112.2.1 (lanes 1 and 2), B118.1 (lanes 3 and 4), B8.1 (lanes 5 and 6), B103.1 (lanes 7 and 8) and B101.1 (lanes 9 and 10) were used to probe the immunoblots. Lane 11 is a protein blot stained with amido black; this gives a clear identification of the protein antigens present in the sample. Lane 12 is an LPS blot reacted with monoclonal antibody A2, an anti-O-antigen monoclonal, confirming effective transfer of LPS antigens onto the nitrocellulose sheet. Biotinylated anti-mouse $F(ab'_2)$ fragments and streptavidin-HRP were used as the conjugates.

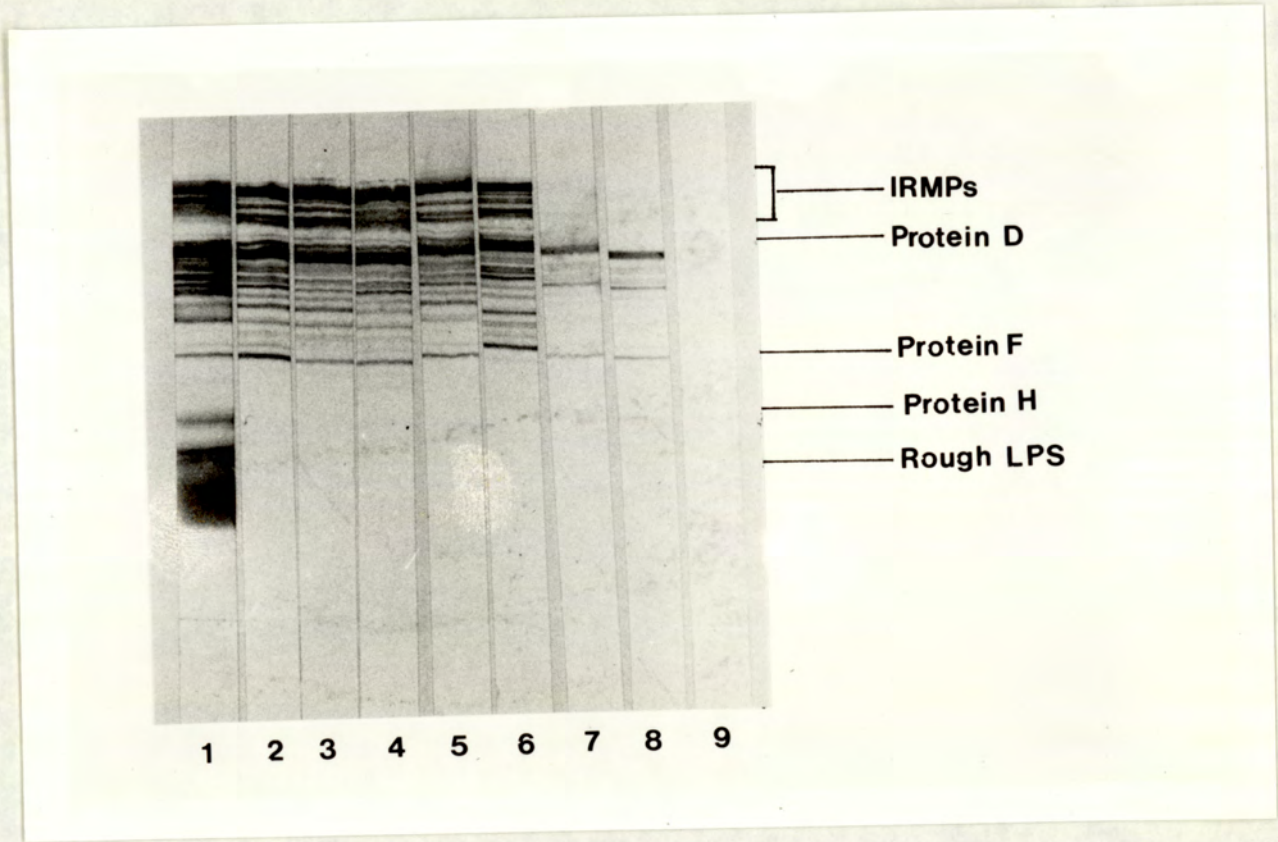


Fig. 6.4.3. Protein immunoblots of OMs Fe- of *P. aeruginosa* strain AK1282. The blots were probed with rabbit serum raised to OMs Fe- of *P. aeruginosa* strain PAO1 (lane 1, using a protein A-HRP conjugate) and monoclonal antibodies produced from fusion C. The monoclonal antibodies used were C28.3 (lane 2), C103.3 (lane 3), C108.3 (lane 4), C15.3 (lane 5), C23.3 (lane 6) C12.3 (lane 7) and C24.3 (lane 8). Anti-mouse $F(ab'_2)$ fragments-HRP conjugate was used and the conjugate blank is shown in lane 9.

black stained blot and positive LPS control antibody (A2) blot demonstrated the presence of all major OM proteins and a distinct O-antigen banding pattern on the nitrocellulose paper, (fig. 6.4.1, tracks 11 and 12).

Protein immunoblots of OMs Fe- of strain AK1282 were probed with the monoclonal antibodies generated from fusion C. A selection of immunoblots is shown in fig. 6.4.3, in which antibodies C28.3, C103.3, C108.3, C15.3 and C23.3 are shown to be specific for the IRMPs and proteins D, E and F (the resolution produced by the gel for the immunoblot was poor). Table 6.2.1 details the specificities of other monoclonal antibodies produced. Antibodies were subsequently characterised for their abilities to bind to intact cells (section 5.5), to react with representative strains of the seventeen serotypes of *P. aeruginosa* (section 5.3) and also other Gram-negative bacteria (section 5.4), to agglutinate whole cells (section 5.6) and to mediate passive protection in an experimental intraperitoneal challenge model (section 7.4).

6.5 Discussion.

A panel of monoclonal antibodies was produced which reacted with cell envelope components of *P. aeruginosa.* The first two fusions performed used an immunising preparation that had a high level of contamination with the O-antigenic moiety of LPS, as it had been extracted from a smooth strain of *P. aeruginosa* by the crude purification method of gel-excision. As expected, a large proportion of the antibodies produced from these fusions reacted with O-antigen, as determined by immunoblot analyses. In contrast, only three out of the 46 antibodies selected for characterisation produced from fusion C (which utilised a purer antigen) reacted with purified LPS in an ELISA assay.

Some of the antibodies generated in this study were found to be specific for the IRMPs and protein D. There have been some recent reports of the generation of monoclonal antibodies

specific for IRMPs, for example Sokol and Woods (1986b) describe the isolation of five hybridomas secreting antibodies against *P. aeruginosa* ferripyochelin binding protein (FBP, 14K) from a single fusion of NS1 cells and spleen cells from mice immunised with purified FBP. They detected positive clones using an ELISA sceen with purified FBP as the coating antigen, and demonstrated that the antibodies reacted with FBP in immunoblots of OM preparations from all serotypes of *P. aeruginosa*. Additionally, Cornelis *et al* (1987) isolated a monoclonal antibody (2H6), of the IgG_3 isotype, specific for the IRMPs of *P. aeruginosa* ATCC 27853. This was the only specific antibody that was generated out of several fusions of spleen cells from mice immunised with crude OM preparations. This emphasises the requirement to purify the antigen to minimise screening and promote the generation of specific antibodies. Antibody 2H6 displayed a strict specificity for the IRMPs and failed to react with either OM preparations on immunoblots or intact cells in ELISA assays from any strain except PA3 and PA10 (2 clinical isolates from CF and urinary tract infections respectively).

The antibodies produced from the fusions detailed here reacted with more than one band on an immunoblot. This compares with the observations of Cornelis *et al* (1987) who found that the antibody they generated (2H6) recognised multiple bands in the range of 72-115K on immunoblots. It is suggested that the monoclonal antibodies generated in this study, and antibody 2H6 (Cornelis *et al* 1987), may be recognising single epitopes shared by different proteins. An additional possibility is that the antigenic sites may be presented by different proteolytic cleavages or processing products of the same IRMP.

Another one of the antibodies generated, even though it was not specific for the IRMPs, was of particular interest. This was the anti-O-antigen antibody A2, which was subsequently used to provide additional evidence concerning the surface expression and cross-reactivity of O-antigenic components of LPS. Due to the importance of LPS as a virulence factor

(Cryz *et al* 1984b) and as a major protective antigen (MacIntyre *et al* 1986a; 1986b), much effort has been expended in the production of monoclonal antibodies against LPS antigens. Several other workers have characterised antibodies specific for O-antigens of *P. aeruginosa*, for example a monoclonal antibody that recognised an epitope on the O side chain of *Pseudomonas aeruginosa* immunotype-1 LPS was prepared by Stoll *et al* (1986). The antibody bound immunotype 1 LPS in ELISA, immunodiffusion and immunoblotting assays and agglutinated and opsonised bacteria. All of these activities were found to be immunotype specific.

Antibodies similar to A2, but showing less cross-reactivity than that discussed in section 5.3, may be useful in immunodiagnostic kits. Ojeniyi *et al* (1989) carried out studies to determine if the unique specificity of O-specific monoclonal antibodies could reduce the amount of cross-reactivity observed with polyclonal sera, and therefore improve the efficiency of serotyping. These workers found monoclonal antibodies to be particularly effective for typing of cystic fibrosis isolates as many of these were polyagglutinable, auto-agglutinable or non-typable with conventional sera (Pitt *et al*, 1986; Hancock *et al*, 1983). These abnormal typing patterns were found by Hancock *et al* (1983) to correlate with a deficiency in the amount of high molecular weight LPS, which usually constitutes the outer surface of the bacterial cells, thereby allowing more of the common core region epitopes of LPS to be exposed and to take part in agglutination reactions. Strickland *et al* (1988) compared polyclonal rabbit antisera with monoclonal antibodies for serological typing of *P. aeruginosa*; they found that the monoclonal antibodies gave clear results and were highly comparable with and as specific as those obtained with rabbit antisera.

Antibody A2 was used to probe LPS immunoblots of proteinase K-digested OM preparations of representative strains of the seventeen serotypes of *P. aeruginosa*. The antibody bound to determinants expressed by serotypes 2, 4, 5, 6, 8, 10, and 11, which was an unexpected result as *P. aeruginosa* is serologically heterogeneous and the basis of the

serotyping system, is the differences between the O-antigenic side chains (Liu *et al*, 1983). It has been found that cross-genus-reactivity is usually associated with lipid A-specificity (Bogard *et al*, 1987; Pollack *et al*, 1989), however, other workers have demonstrated that the anti-O-antigen monoclonal antibodies they produced were not specific for one serotype. This is discussed in section 5.8, but additional evidence indicating that certain epitopes on the O-antigen side chains of *P. aeruginosa* may be shared, includes that presented by Zweerink *et al* (1988) who generated human IgM isotype monoclonal antibodies specific for the LPS of *P. aeruginosa*. Three of the cell lines (RM5, FDD7 and 9H10) produced antibodies that reacted specifically with *P. aeruginosa* Fisher immunotypes 2, 4 and 5 respectively (Fisher *et al*, 1969). A fourth cell line produced a single antibody species that recognised *P. aeruginosa* immunotypes 3, 6 and 7. Additionally, Lam *et al* (1987b; 1987c), produced a panel of monoclonal antibodies against O-antigens of thirteen out of the seventeen serotypes of *P. aeruginosa*, and found significant strong cross-reaction between serotypes 07 and 08. The cross-reactivity suggested that 07 and 08 strains probably have common epitopes on their O-antigens.

The structure of the O-specific carbohydrate determinants of several immunotypes and serotypes has been reported (Knirel *et al*, 1982; 1986; 1987; Stanislavsky *et al*, 1989; Wilkinson, 1983) and it has been shown that some of the carbohydrate components are shared by different serotypes. Serotype-specific antibodies such as those produced by cell lines RM5, FDD7 and 11F9 (Zweerink *et al*, 1988) recognise determinants other than these shared carbohydrates, or may recognise two or more determinants that have to be presented together for recognition. Other factors contributing to antibody specificity include differences in adjacent sugars and/or the nature of the glycosidic bonds. Similarities between certain O-antigen components may constitute a shared antigenic site and thus explain the multiple reactivity of monoclonal antibodies 9H10 (Zweerink *et al*, 1988) and A2.

In summary, the evidence from previous studies suggests that it should be possible to produce both serotype-specific and cross-reactive monoclonal antibodies against the O-antigenic side chains of most serotypes of *P. aeruginosa*.

Notable work on the production of monoclonal antibodies specific for OM components has been presented by Lam *et al* (1987 a; 1987b; 1987c). These workers have produced a panel of monoclonal antibodies against the 17 serotype strains of *P. aeruginosa* described by the IATS, and examined the cross-reactivities, isotypes, titres and epitope specificities displayed. The antibodies secreted were found to be reactive with both formalin-fixed whole cells and purified LPS of homologous strains in ELISA assays. The work carried out by Lam *et al* (1987 a; 1987b) was a systematic approach to produce monoclonal antibodies against each serotype strain.

Several of the monoclonal antibodies generated were able to agglutinate whole bacterial cells (see section 5.6); this indicated that the epitopes that they reacted with were surface accessible. Lam *et al* (1987b) produced monoclonal antibodies that could be divided into 2 groups based on their ability to agglutinate whole cells of homologous strains. The agglutinating antibodies were found to be reactive with the O-antigen chains of homologous LPS, while the non-agglutinating monoclonal antibodies were found to be directed towards the outer membrane protein-associated LPS. In addition, Hancock *et al* (1982) produced a monoclonal antibody (MA1-8) directed against the O-antigenic portion of PAO1 LPS, which was selected out of 15 clones that produced antibodies specific for OM antigens of *P. aeruginosa*. This antibody, which failed to interact with the OMs of 3 independently isolated rough mutants derived from PAO1 (AK1160, AK1188 and H223), was able to agglutinate heat killed bacterial cells and sheep erthrocytes (coated with PAO1 LPS, but not with rough AK1160 LPS, lacking O-antigen). The anti-O-antigen antibody generated here however, was unable to agglutinate whole cells of *P. aeruginosa* strain PAO1, while some of the anti-IRMP and protein D-specific antibodies were able to participate in

agglutination reactions.

A bank of highly serotype-specific monoclonal antibodies could act as immunological probes to provide highly accurate typing sera for the classification of clinical *P. aeruginosa* strains, and help in the study of the surface properties of bacteria. The antibodies generated could help to identify common epitopes of *P. aeruginosa* which are key antigens in the infection process (Mutharia *et al*, 1985b); these antigens would be examined for their potential to act as a cross-protective, effective vaccine for *P. aeruginosa* infections. Monoclonal antibodies have obvious advantages over polyclonal sera, including their availability, specificity and reproducibility and are gradually replacing rabbit antibodies as the immunological reagent of choice for serotyping and the analysis of bacterial cell surface antigens. The generation of the monoclonal antibodies has enabled further study of the complexity and differential expression of the IRMPs.

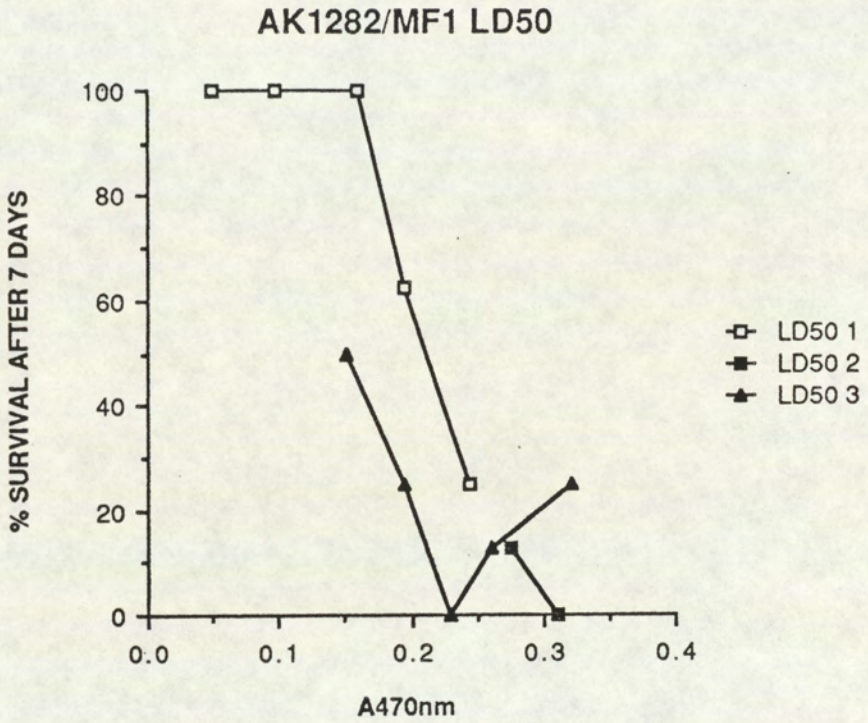
7 Protective activity of antibodies directed towards IRMPs of *P. aeruginosa.*

7.1 Introduction.

IRMPs have been shown to be key antigens *in vivo* and it is therefore likely that they also play a key antigenic role in immune protection against *P. aeruginosa* infection. In fact, there have been recent demonstrations of the protective potential of IRMPs (Bolin and Jensen, 1987; Sokol and Woods, 1986). If these reports are confirmed, the epitopes involved would appear to be ideal candidates for inclusion in immunising preparations, especially for those species of micro-organism that have multiple serotypes such as *P. aeruginosa.* Polyclonal and monoclonal antibodies raised to the IRMPs of *P. aeruginosa* were therefore analysed to extend evidence on the antigenic roles played by these proteins in protection against infections with *P. aeruginosa.*

7.2 LD_{50} determinations.

The LD_{50} values for various bacterial challenge/mouse strain combinations were determined to enable calculation of appropriate challenge doses. Four to five week old, 20-25g Hsd/Ola;MF_1 (MF_1) mice were used for polyclonal protection studies. F_1 hybrid mice of a CBA/Ca/Ola/Hsd x BALB/c/Ola/Hsd (F_1) cross were used for the production of monoclonal antibodies, and therefore passive protection studies with ascitic fluid was performed using this strain, at 18-22g, 6-7 weeks old. Bacterial challenges were *P. aeruginosa* strains PAO1, Type 1 and AK1282. PAO1 and AK1282 were selected as these were the strains that the polyclonal serum had been raised to and Type 1 was selected as this serotype expressed LPS which was not immunologically cross-reactive, and IRMPs



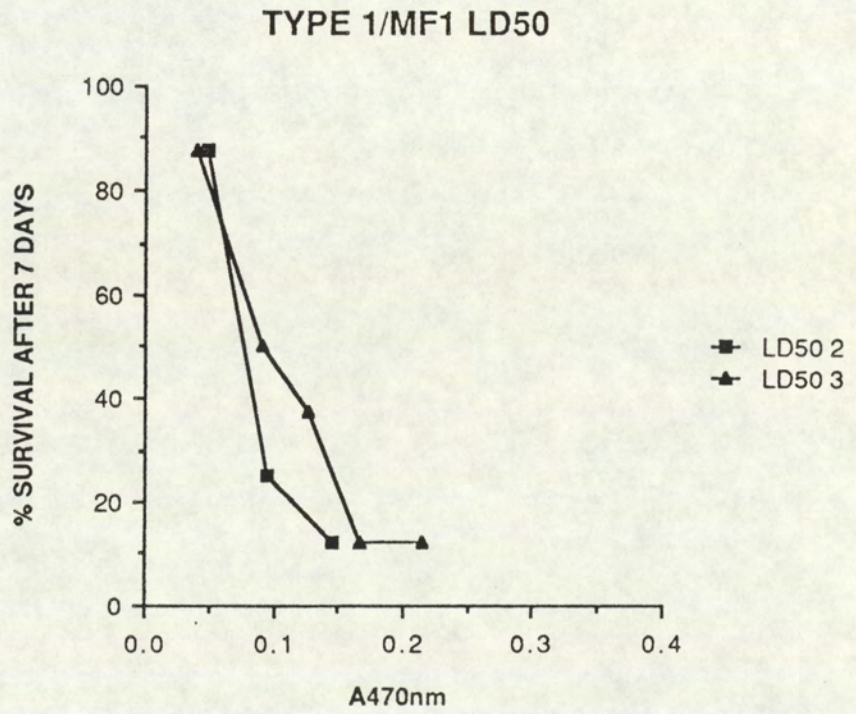
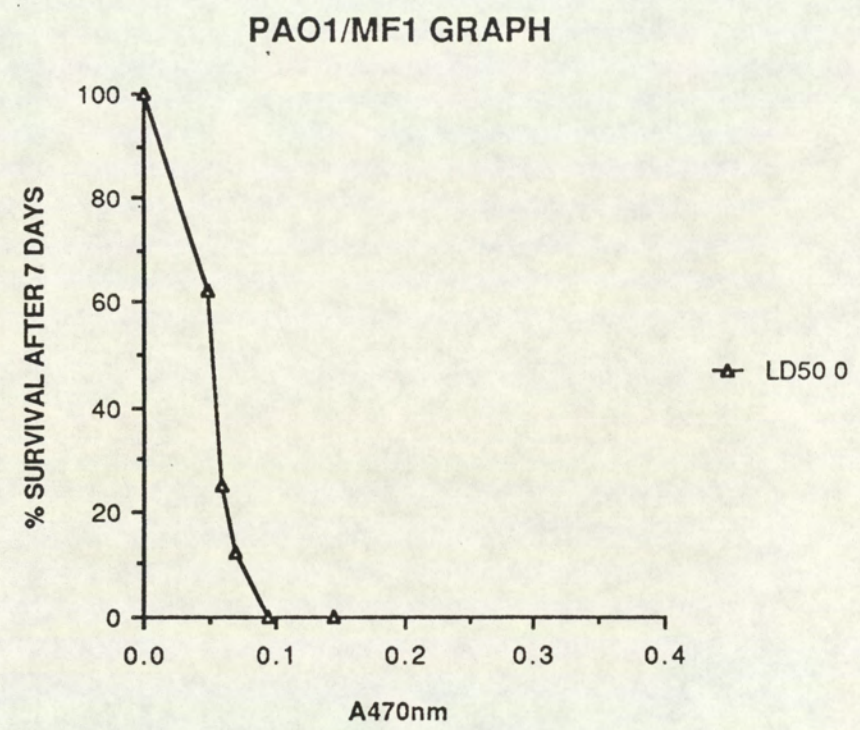


Fig. 7.2.1. LD_{50} determinations of AK1282 and Type 1 with MF_1 mice, shown as A_{470nm} of the bacterial challenge administered against % survival after 7 days. 8 mice/group. (The LD_{50} number represents the determination number).



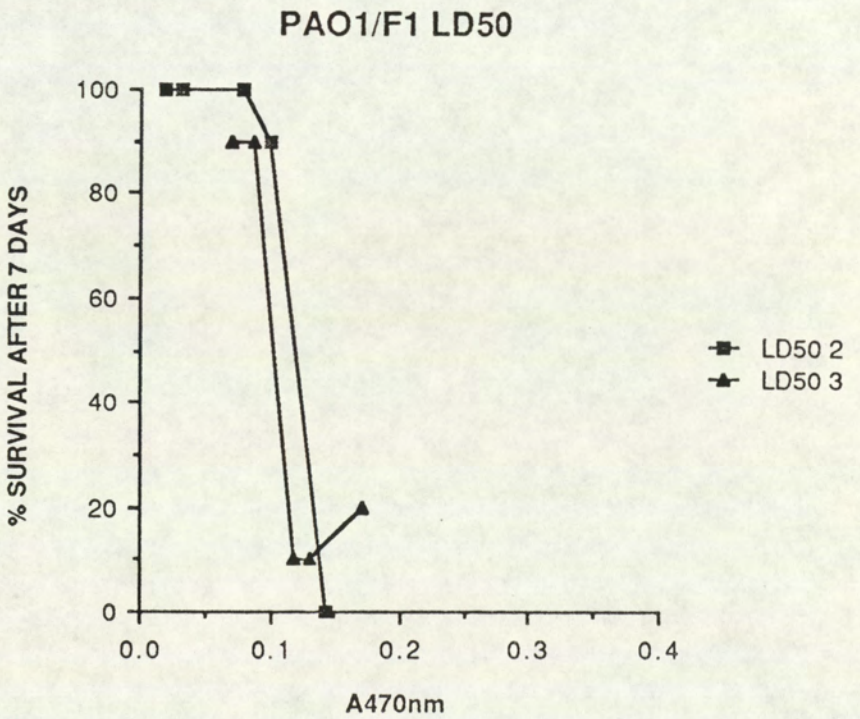


Fig. 7.2.2. LD_{50} determinations of PAO1 with MF_1 and F_1 mice, shown as A_{470nm} of the bacterial challenge administered against % survival after 7 days. The graph showing MF_1 results had 8 mice/group and that showing F_1 results had 10 mice /group. (The LD_{50} number represents the determination number).

which were immunologically cross-reactive with serotype 5 organisms (see chapter 5).

The growth of bacterial cell cultures could be monitored spectrophotometrically, and a calibration curve of A_{470nm} to viable count/ml was constructed. This enabled the rapid estimation of the bacterial cell number in a challenge prior to administration to the mice; more accurate determinations were carried out by viable count analysis. The results from the LD_{50} determinations are shown in figs. 7.2.1 and 7.2.2. Each of the data represents the percentage survival of a group of mice challenged with live bacteria, the MF_1 mice being in groups of 8 and F_1 mice being in groups of 10. It was found that the bacterial challenge had to be prepared from cells in the logarithmic phase of growth, in order for the cells to be virulent.

The average (of 1, 2 or 3 determinations) LD_{50} values (expressed as the viable count/ml, of which a 0.5ml dose killed 50% of the mice) were as follows: AK1282/MF_1 = 5.96 x 10^8; Type 1/MF_1 = 1.36 x 10^8; PAO1/F_1 = 2.75 x 10^8 and PAO1/MF_1 = 6.2 x 10^7.

7.3 Polyclonal protection.

A series of studies was undertaken to ascertain the protective potential of polyclonal antibodies directed towards IRMPs in an experimental intraperitoneal challenge model. Antibodies were raised in MF_1 mice to two antigen preparations:

1. IRMPs of *P. aeruginosa* PAO1, purified by the gel-excision method detailed in section 3.1.6.1. This serum was used in passive protection studies 1 and 2, and the titre against whole cells of strain PAO1 was greater than 1 in 81,920. This serum is termed IRMP in

tables 7.3.1 and 7.3.2.

2. IRMPs of *P. aeruginosa* AK1282, purified by selective solubilisation followed by 1% deoxycholate gel-filtration FPLC described in sections 3.1.2 and 3.2.5. This serum was used in passive protection studies 3 and 4 and half of it was absorbed with LPS purified from AK1282. The titre of the absorbed serum against whole cells of AK1282 was 1 in 20,000. This serum is termed LPS-ABS and NON-ABS in tables 7.3.3 and 7.3.4.

Forty MF_1 mice were used for each of the two antigen preparations, and serum was collected after 5 weeks (see section 2.2 for details of the immunisation schedule). Sera were analysed using both ELISA and immunoblotting techniques; it was found that serum contained antibodies directed mainly towards IRMPs, although anti-O-antigen antibodies were present in serum 1. Normal mouse serum or saline was used as a control, and the results from these two studies are expressed in graph format in figs. 7.3.1 and 7.3.2 and in tables 7.3.1 and 7.3.2.

Table 7.3.1. Passive protection study 1. MF_1 mice were challenged ip with 15.3 and approximately 30 x the LD_{50} doses of strain PAO1, and 19.1 and approximately 50 x the LD_{50} doses of strain Type I. 100µl aliquots of serum 1 or NMS were administered iv 1.5hrs prior to challenge.

Number of mice/group	Serum administered	Challenge	Number of mice surviving after 7 days
4	IRMP	No challenge	4
8	NMS	15.3 X LD_{50} PAO1	1
8	IRMP	15.3 X LD_{50} PAO1	4
8	IRMP	30 X LD_{50} PAO1	0
8	NMS	19.1 X LD_{50} TYPE1	0
8	IRMP	19.1 X LD_{50} TYPE1	0
8	IRMP	50 X LD_{50} TYPE1	0

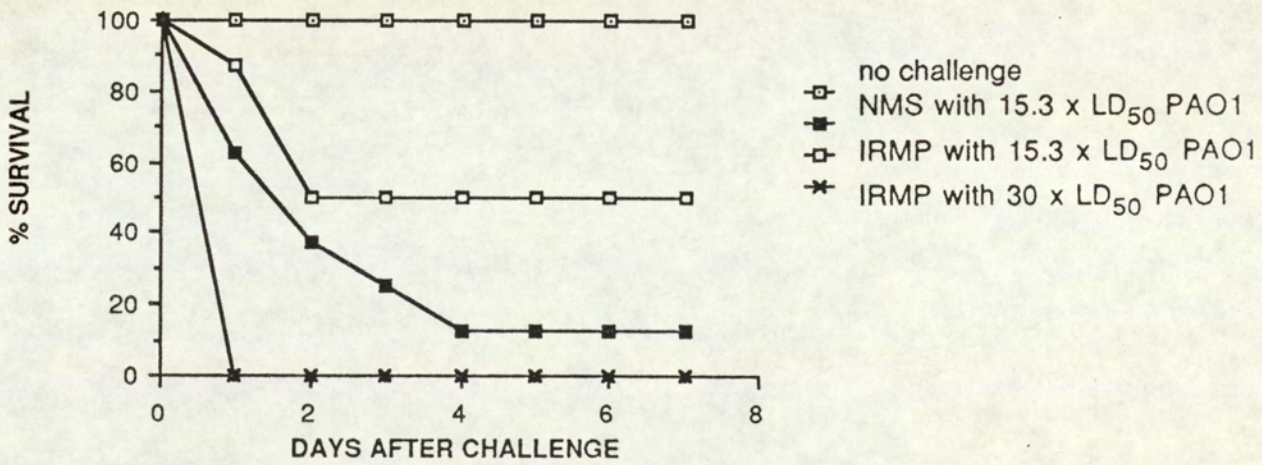


Fig. 7.3.1. Passive protection study 1. 8 MF_1 mice/group were administered 100µl polyclonal anti-IRMP antibodies (the antigen being prepared by excision of *P. aeruginosa* PAO1 IRMPs from SDS-PAGE)(IRMP) or normal mouse serum (NMS) and challenged ip with 15.3 and approximatly 30 times the LD_{50} doses of *P. aeruginosa* PAO1. The *P. aeruginosa* serotype 1 challenge was too high and so this result is not shown.

Table 7.3.2. Passive protection study 2. MF_1 mice were challenged ip with 6.45 and 19.35 x the LD_{50} doses of strain PAO1 and 0.074 and 15.44 x the LD_{50} doses of strain Type I. 100µl aliquots of serum 1, NMS or saline were administered ip 1.5hrs prior to challenge.

Number of mice/group	Serum/saline administered	Challenge	Number of mice surviving after 7 days
4	Saline	6.45 X LD_{50} PAO1	0
8	NMS	6.45 X LD_{50} PAO1	0
8	IRMP	6.45 X LD_{50} PAO1	8
8	NMS	19.35 X LD_{50} PAO1	0
8	IRMP	19.35 X LD_{50} PAO1	7
4	Saline	0.074 X LD_{50} TYPE 1	4
8	NMS	0.074 X LD_{50} TYPE 1	8
8	IRMP	0.074 X LD_{50} TYPE 1	8
8	NMS	15.44 X LD_{50} TYPE 1	0
8	IRMP	15.44 X LD_{50} TYPE 1	0

It was demonstrated that the polyclonal serum conferred protection to 6.45, 15.3 and 19.35 x LD_{50} challenges of the homologous strain of *P. aeruginosa*, but not to the (approximately) 30 x LD_{50} challenge. The protection demonstrated could not be attributed directly to the IRMPs, as antibodies specific for other OM proteins and O-antigen components were also present in the serum. Unfortunately, the approach in the experiments designed to analyse the contribution of LPS antibodies to the protection demonstrated failed, due to the challenges of Type 1 organisms being either being to high, or too low. It was important that the contribution of LPS to the protection afforded was taken into account in the design of future studies and a second set of passive protection studies was carried out using polyclonal antibodies raised to the IRMPs of *P. aeruginosa* strain AK1282. The antigen used to raise the antibodies was of higher purity than that

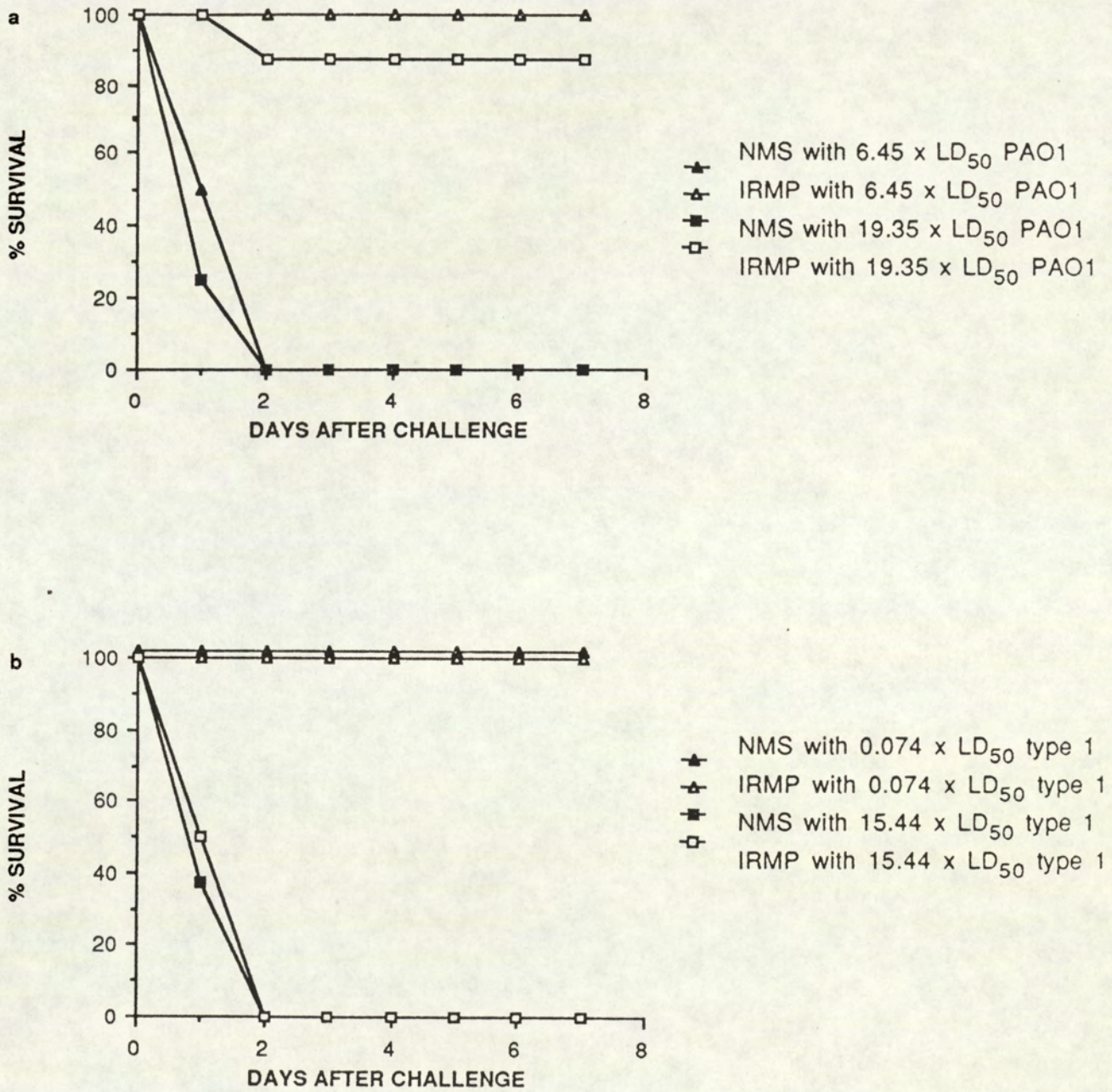


Fig. 7.3.2. Passive protection study 2. 8 mice/group were administered 100µl polyclonal anti-IRMP antibodies (the antigen being prepared by excision of *P. aeruginosa* PAO1 IRMPs from SDS-PAGE)(IRMP) or normal mouse serum (NMS) and challenged ip with 6.45 or 19.35 x the LD_{50} doses of *P. aeruginosa* PAO1 (a) or 0.074 and 15.44 x the LD_{50} doses of *P. aeruginosa* serotype 1 (b).

used for the generation of serum 1: IRMPs had been separated from other OM components by selective solubilisation and gel-filtration chromatography, rather than by excision from SDS-PAGE. To further eliminate the presence of anti-LPS antibodies, the serum was absorbed with purified LPS to immunoprecipitate anti-LPS antibodies. A bacterial challenge of another serotype was again included in passive protection studies 3 and 4, and the results from passive protection study 3 are shown in table 7.3.3.

Table 7.3.3. Passive protection study 3. MF_1 mice were challenged ip with 2.5 and 3.1 x the LD_{50} doses of strain AK1282, and 17.5 and 21.65 x the LD_{50} doses of Type I. 100µl aliquots of serum 2 (LPS-absorbed or non-absorbed) or NMS were administered ip 1.5hrs prior to challenge.

Number of mice/group	Serum administered	Challenge	Number of mice surviving after 7 days
4	LPS-ABS	No challenge	4
8	NMS	2.5 X LD_{50} AK1282	2
8	LPS-ABS	2.5 X LD_{50} AK1282	2
8	NON-ABS	2.5 X LD_{50} AK1282	0
8	LPS-ABS	3.1 X LD_{50} AK1282	0
8	NON-ABS	3.1 X LD_{50} AK1282	0
8	NMS	17.5 X LD_{50} TYPE 1	1
8	LPS-ABS	17.5 X LD_{50} TYPE 1	1
4	NON-ABS	17.5 X LD_{50} TYPE 1	1
8	LPS-ABS	21.65 X LD_{50} TYPE 1	2
8	NON-ABS	21.65 X LD_{50} TYPE 1	0

The antiserum used in passive protection study 3 did not demonstrate protection. To promote the potential of the protective activity of serum 2, the challenge dose was lowered, and the volume of antiserum was increased to 250µl/mouse. The results from passive protection 4 study are shown in table 7.3.4. Again, however, no protective activity of the antiserum was demonstrated.

Table 7.3.4. Passive protection study 4. MF_1 mice were challenged ip with 2.01 and 3.69 x the LD_{50} dose of strain AK1282. 250µl aliquots of serum 2 (LPS-absorbed or non-absorbed) or NMS were administered ip 1.5hrs prior to challenge.

Number of mice/group	Serum administered	Challenge	Number of mice surviving after 7 days
8	NMS	2.01 X LD_{50} AK1282	5
8	LPS-ABS	2.01 X LD_{50} AK1282	3
8	NON-ABS	2.01 X LD_{50} AK1282	1
8	LPS-ABS	3.69 X LD_{50} AK1282	4
8	NON-ABS	3.69 X LD_{50} AK1282	1
8	NMS	3.69 X LD_{50} AK1282	3

7.4 Monoclonal protection.

To explore the potential for monoclonal immunotherapy, hybridomas derived from mice immunised with the IRMPs purified from OMs of *P. aeruginosa* strain PAO1 were prepared (see chapter 6). Several monoclonal antibodies, selected for their ability to specifically bind to the IRMPs and protein D or to O-antigen, to show cross-reactivity with a panel of organisms and to bind to intact cells in immunogold assays were evaluated for protective activity by inclusion in the protection studies. The results from this study are shown in table 7.4.1. The challenge was 0.5ml of a 6.7 x 10^8/ cells/ml suspension of *P. aeruginosa* strain PAO1, which was only a 2.4 x the LD_{50} dosage, however, even at this low level of challenge, no protection was demonstrated.

Table 7.4.1. Monoclonal passive protection study. F_1 mice were challenged ip with 2.4 x the LD_{50} dose of strain PAO1. 100µl aliquots of ascitic fluid or NMS were administered ip 1.5hrs prior to challenge.

Number of mice/group	Ascites administered	Challenge	Number of mice surviving
10	B112.2.1	2.4 x LD50 PAO1	0
10	B8.1	2.4 x LD50 PAO1	0
10	B118.1	2.4 x LD50 PAO1	0
10	A2	2.4 x LD50 PAO1	0
10	cocktail of above	2.4 x LD50 PAO1	0
10	NMS	2.4 x LD50 PAO1	1

7.5 Discussion.

The rough *P. aeruginosa* strain AK1282 was found to be less virulent than the smooth strains (PAO1 and Type 1) for MF_1 mice. This is in accordance with the findings of several other workers (Cryz *et al*, 1984b; Kropinski and Chadwick, 1975 and Williamson, 1956). Spontaneous rough mutants were first described by Williamson (1956), when it was reported that they were less virulent than the parental smooth type for mice. Cryz *et al* (1984b) found that the virulence of several strains of *P. aeruginosa* was directly related to the dispersion of LPS into either the phenol or water phase formed during LPS extraction; virulence decreased as the proportion of LPS recovered from the phenol phase increased and was therefore associated with the length of the O-polysaccharide side chain. The contribution of LPS to virulence was studied further using the smooth LPS-specific bacteriophage E79 (obtained from A.M. Kropinski) to select mutants altered in LPS structure. One mutant (PA220-R2) was characterised and found to be completely deficient in high molecular weight polysaccharide. This rough mutant was serum sensitive and comparatively nonvirulent, with a 50% lethal dose more than 1,000-fold higher than that of it's parent strain for burned mice. Kropinski and Chadwick (1975) investigated the relative virulences of rough and smooth wild-type

cells of *P. aeruginosa* for the larvae of the moth *Galleria mellonella*, and found that the rough strains were 8-62-fold less pathogenic.

Several lines of evidence suggest that LPS contributes substantially to *P. aeruginosa* virulence. These include the toxic nature of LPS (Pennington *et al,* 1975), the fact that LPS may confer serum resistance (Young, 1972) and the fact that antibodies to LPS have been shown to be highly protective in experimental models (Collins and Roby, 1984; Cryz *et al,* 1983 a; 1984a; Pier *et al,* 1978). Rough mutants lack O-specific polysaccharides and express only portions of the core lipopolysaccharide (which contains antigenic determinants shared by most Gram-negative bacteria). They appear to retain their exoenzymes and pigment-synthesising capacities: this makes them useful in the study of the role of LPS in pathogenesis, and it is suggested that it is their deficiency in O-antigen that renders them less pathogenic by making them susceptible to phagocytic engulfment.

As vaccines are developed to combat bacteria by raising specific antibodies that will react with and neutralise the pathogens concerned, it is clear that the antigens involved will have to be expressed by the invader *in vivo* for the vaccine to be effective. For these reasons, it is essential to take into account the plasticity of the cell envelope and to investigate the cell surface of a pathogen, as it actually is in each infection situation, in the design of vaccines. Brown *et al* (1988) reviewed the importance of antigens expressed *in vivo* in vaccine development, and the potential of such immunogens to act as an effective vaccine. The recent work examining the properties of bacteria extracted directly from infected tissues (Anwar *et al*, 1984 and Ward *et al*, 1988) has indicated that certain OM components are different in bacteria grown *in vivo* from those grown *in vitro.* The whole range of virulence determinants expressed by organisms growing *in vivo* can be explored to generate information which can be used in the rational design of vaccines. Evidence acquired indicating the cross-reactivity and importance *in vivo* of IRMPs has lead to recent investigations of the protective potential of anti-IRMP antibodies.

It was demonstrated here that intravenous or intraperitoneal administration of polyclonal antibodies to MF_1 mice 1.5 hrs prior to intraperitoneal homologous bacterial challenge resulted in significant protection. The data indicated that polyclonal antibodies raised to the IRMPs of *P. aeruginosa*, if at a high titre, were able to protect mice against ip challenge with up to a 19.35 x LD_{50} challenge. It was also shown that serum 2 was unable to confer passive protection, and this was due to the considerably lower titre (more than 4-fold) of this serum compared to serum 1. Demonstrations of the protective potential of IRMPs have also been reported by Bolin and Jensen (1987) and Sokol and Woods (1986); the work of these authors is discussed in chapter 1.

Determination of the contribution of anti-LPS antibodies to the protection demonstrated was approached in two ways. The first involved the use of a challenge of a different serotype, an approach that has been taken by several other workers (such as Gilleland, 1984; Lieberman *et al* 1986; Matthews-Greer and Gilleland, 1987 and Sawada *et al*, 1984). The second approach was through the immunoprecipitation of antibodies directed towards LPS (as used by Bolin and Jensen, 1987 and Loeb, 1987). Unfortunately, the contamination of the IRMP antigen preparation with LPS still caused problems in the interpretation of results presented, as the challenges with the heterologous strain type 1 were either too high or too low, and the polyclonal serum which had been incubated with LPS to immunoprecipitate LPS antibodies was not at a high enough titre to confer protection.

Other workers have also encountered difficulties in the determination of the contribution of anti-LPS antibodies to the protective activity of a sample of serum. Apart from the two methods mentioned above, the LPS factor has additionally been taken into account by comparing the protection afforded by OM protein preparations with known amounts of LPS contamination with that afforded by LPS alone (Isibasi *et al* (1988); Matthews-Greer and

Gilleland, 1987; Moreno *et al*, 1985). For example, Matthews-Greer and Gilleland (1987), who found that it was "extremely difficult if not impossible" to extract an OM protein from *P. aeruginosa* that was completely free of LPS, circumvented the problem in their protection studies by using a protein F preparation that contained a high concentration of PAO1 LPS (7μg/10μg protein); they compared results obtained with this preparation with those of a control preparation of 7μg of LPS. Additionally, Isibasi *et al* (1988) investigated the protective activity of the OM proteins from *Salmonella typhimurium* by comparing the protection obtained with OM proteins contaminated with 4% LPS, with that obtained with LPS alone.

The antigen preparation appeared on Coomassie blue stained SDS-PAGE gels to contain only IRMPs, however, a variety of specificities was generated in animals immunised with these 'pure' preparations. This problem was also described by Loeb (1987), who prepared an antiserum to a highly purified preparation of protein a, and found that the polyclonal antiserum reacted with other cell envelope antigens, demonstrated in immunoblot analysis. Loeb (1987) suggested that either these antibodies were reacting with multimers of protein a, or with degradation products of this protein. An additional suggestion was that the antibodies may represent those specific for protein impurities present in the inoculum, or that they might have been produced non-specifically during the immune response to protein a. Loeb (1987) used this serum in protection studies and suggested that the levels of antibodies specific for OM components other than the proteins of interest are an unavoidable consequence of experiments carried out using polyclonal sera.

In the work presented here, the titre of serum preparation 2 was not high enough to protect mice from even low bacterial challenges (2.01-3.69 x the LD_{50} doses). Due to this, the protective activity of anti-IRMP antibodies could not be determined with

sufficient accuracy. This emphasises the point that large quantities of antibody were required to study protection in the passive transfer model, and to identify the antigenic determinants toward which protective responses were directed. The development of hybridoma technology has provided a means to generate the quantities of antibodies necessary to dissect the antibody response to a complex bacterial antigen. To investigate the potential for monoclonal immunotherapy, antibodies B112.2.1, B8.1, B118.1 and A2 were evaluated for their abilities to confer passive protection. Ascitic fluid containing these antibodies, however, failed to demonstrate any protective activity. It is suggested that the antibodies generated from fusion C, as they demonstrated a higher avidity for OM antigens as determined in the immunoblot and ELISA screens and immunofluorescence assay, may demonstrate protective activity. Additionally, antibodies may need to be purified by affinity chromatography before protective analyses are undertaken.

Previously there have been several demonstrations of protection mediated by monoclonal antibodies specific for OM antigens. These are discussed in chapter 1 (Colwell *et al*, 1984; Sawada *et al*, 1984; Stoll *et al*, 1986; Zweerink *et al*, 1988). Most of the demonstrations of protection, however, involve antibodies directed towards O-antigenic determinants. LPS is a potent, effective serotype antigen and it has been shown in animal models that antibodies against the O-specific carbohydrates on the LPS molecule are important mediators of serotype-specific protection against *P. aeruginosa* infection (Cryz *et al*, 1983; 1984a; MacIntyre *et al*, 1986; Pennington *et al*, 1986; Sawada *et al*, 1984, Stoll *et al*, 1986; Tegtmeier and Anderson, 1983). These serotype-specific antibodies may be of some use as the majority of nosocomial infections are associated with relatively few serotypes (McManus *et al*, 1985), and in some cases, it should be noted, the activity of anti-O-antigen monoclonal antibodies has been shown to be not strictly serotype-specific (Hancock *et al*, 1982; Lam *et al*, 1987b; Zweerink *et al*, 1988). Cross-reactivity could lead to the mediation of cross-protective activity. Additionally, other possible mechanisms to account for cross-protection against heterologous immunotype strains, other than that

afforded by cross-reactive anti-protein antibodies, include polyclonal B cell activation by LPS, which would stimulate the production of antibodies that react with heterologous O-antigens (Siber *et al*, 1985), or the stimulation of non-specific resistance.

The overall aim in the development of a vaccine to combat *P. aeruginosa* infections is that it should offer protection against a broard spectrum of isolates (Gilleland and Matthews-Greer, 1987); this was the basis of evaluating protein components of the bacterial OM for their potential to provide protection, as these should be less serotype-specific than LPS candidates. It was demonstrated that there was cross-reactivity between the IRMPs expressed by the seventeen serotypes of *P. aeruginosa* (see chapter 5).

The effectiveness of polyclonal or monoclonal antibodies as prophylactic therapeutic agents indicates their potential and importance for clinical use. The preliminary results presented here demonstrated that the polyclonal antibodies raised to IRMPs excised from SDS-PAGE gels were able to protect against a challenge with a homologous serotype challenge. The protection studies need to be extended to determine the contributions of the various antibodies present in the polyclonal serum. In addition, monoclonal antibodies could be purified by affinity chromatography from ascitic fluids and used in protection studies. Passive immunotherapy is particularly applicable to burn patients, who can not usually be classified as at risk of infection before they are burnt. Active immunisation of these patients would be ineffective at protecting against infection, due to the inability of these immunocompromised patients to produce specific antibodies, and additionally, the time required to develop the immune response. Passive immunotherapy of patients, however, shows potential (Neely and Holder, 1987) and although the intraperitoneal challenge model used here may only partially mimic the clinical disease situation, the potential use of passive immunotherapy as an adjunctive therapy in immunocompromised patients at risk of *P. aeruginosa* infection is promising.

8 Concluding remarks.

P. aeruginosa continues to be a problem as an opportunist pathogen partly due to it's lack of susceptibility to antibiotic treatment (Brown, 1977a; Hancock, 1986). Much effort has therefore been invested in the design of vaccines which can protect high-risk groups from *P. aeruginosa* infections. Ruling out LPS as a protective vaccine, due to the toxicity associated with the lipid A moiety and the serotype-specific response generated, two other main groups of potential vaccines have been investigated. Polysaccharide components that *P. aeruginosa* presents to it's host have been investigated by many workers, notably Cryz *et al*, (1984; 1986; 1987a and b), Pier (1983), Pier *et al* (1978; 1983) and Woods and Bryan (1985). Outer membrane proteins are the other group of potential vaccines. Porin protein F has been investigated as this protein has previously been shown to cross-react immunologically between the seventeen serotypes of *P. aeruginosa* (Mutharia *et al*, 1982). Protein F has been demonstrated to stimulate the production of protective antibodies (Gilleland *et al,* 1984; Matthews-Greer and Gilleland, 1987), and recently, the gene encoding porin protein F has been cloned and characterised in detail (Duchene *et al*, 1988; Woodruff *et al*, 1986). The cloning and characterisation of the gene encoding for *P. aeruginosa* lipoprotein I has very recently been reported (Duchene *et al*, 1989) and these workers intend to go on to characterise the immunogenic and protective activities of liporotein I.

The use of molecular and immunological techniques to identify bacterial virulence factors provides a powerful approach. These methods are often limited, however, by the frequently inappropriate expression of virulence factors by bacteria during laboratory cultivation and analysis. It is therefore of great importance to attempt to mimic the growth enviroment that the pathogen encounters *in vivo*, before genetic analysis is performed. Iron-limitation has been shown to be an important factor *in vivo.* The work presented here reinforces the importance of iron-limitation in the growth of bacterial

pathogens *in vivo*. IRMPs, which are integral components of the high affinity iron-uptake system were shown to be expressed *in vivo* in a pleural cavity infection and an infection of *Otitis externa*. These important cell surface immunogens show potential to be included in a cross-protective vaccine as they are surface exposed and conserved among different serotype strains of *P. aeruginosa*.

There are two ways to demonstrate the potential of OM proteins without the problems of distinguishing the protective effect of the proteins from the protective effect of the LPS encountered during the polyclonal protection studies: use of monoclonal antibody technology or molecular cloning techniques. The panel of monoclonal antibodies produced in this study has been useful in the investigation of the expression of IRMPs. These antibodies represent a bank of invaluable immunological reagents which have applications in serotyping, epitope mapping, LPS and protein structural determinations and studies of protection against *P. aeruginosa* infections.

The cloning and expression of the genes coding for the IRMPs will be the next phase of the investigation of the IRMPs of *P. aeruginosa*. Cornelis *et al*, (1987) attempted to clone the genes responsible for the synthesis of the siderophore receptors of *P. aeruginosa*, using antibodies to screen a phage bank in *E. coli*. Several DNA fragments were identified and analysed, however the protein that they encoded had a molecular weight of 55K: this is unlikely to be an IRMP. The use of recombinant *P. aeruginosa* IRMPs in protection studies will permit the testing of whether this antigen is protective, without contamination of *P. aeruginosa* LPS. Ideally, the most important IRMPs, as regards their protective abilities and their involvement in iron-uptake, will be cloned in a foreign host eg *E. coli*, to enable further investigation of the immunogenicity of these critical surface antigens. This work is forming the basis of the continuation of the project.

In summary, the production of a useful vaccine for *P. aeruginosa* may include removal of LPS to reduce toxicity, removal of antigens which elicit antibodies which are not protective (which may compete with neutralising antibodies and depress host defences) and determination of key antigens which are antigenically important *in vivo.* A vaccine providing the most effective immunotherapy against *P. aeruginosa* may infact be a conjugate vaccine including key OM proteins, perhaps conjugated to high molecular weight polysaccharide. IRMPs appear to be ideal candidates for inclusion in immunisation preparations as they are key surface antigens in the infection process.

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