Production and characterization of monoclonal antibodies to outer membrane proteins of *Pseudomonas aeruginosa* grown in iron-depleted media

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The iron uptake systems of pathogenic bacteria provide potential targets for immunological intervention. We have partially purified the high molecular mass, iron-regulated outer membrane proteins (IROMPs) from *Pseudomonas aeruginosa* and used them to prepare a panel of monoclonal antibodies (mAbs). Five mAbs reacted with an 85 kDa IROMP separated by SDS-PAGE, but gave only low-level binding to whole cells by immunogold electron microscopy. However, iodination of whole cells indicated that the 85 kDa IROMP is surface-exposed. The mAbs were only cross-reactive with clinical isolates representing eight of the 17 International Antigenic Typing Scheme serotypes of *P. aeruginosa*, suggesting significant heterogeneity with respect to this IROMP.

Introduction

It is widely recognised that the ability of an invading bacterium to adapt rapidly to the host environment is a key determinant of virulence and pathogenicity (Smith, 1990). Although there are a large number of environmental signals, the low availability of iron is acknowledged as a key determinant of virulence (Griffiths, 1987). Iron is predominantly located within the body intracellularly, as haem, haemosiderin and ferritin-type compounds; such extracellular iron is bound to the high-affinity iron-binding glycoproteins transferrin and lactoferrin. In the absence of specific uptake systems the level of free iron is too low to sustain bacterial growth. To obtain iron, many bacterial species secrete low molecular mass iron chelating compounds (siderophores) which compete successfully with transferrin and lactoferrin for iron. Other essential components of the iron acquisition

system are high molecular mass, iron-regulated outer membrane proteins (IROMPs), produced under conditions of iron deprivation, which act as receptors to facilitate the uptake of iron-siderophore complexes (Neilands, 1982). *Pseudomonas aeruginosa* expresses several high molecular mass IROMPs *in vivo*, in both chronic (Brown *et al.*, 1984; Cochrane *et al.*, 1988) and acute infections (Ward *et al.*, 1988). A 14 kDa outer membrane protein has been reported as the receptor for the *P. aeruginosa* siderophore ferripyochelin (Sokol & Woods, 1983). Antibodies directed against proteins associated with iron uptake may exert a bacteriostatic effect by blocking ferric siderophore-mediated uptake and therefore have considerable potential as immunotherapeutic agents. When considering outer membrane (OM) components as vaccine candidates, proteins are generally less serotype-specific than other components such as lipopolysaccharide (LPS) and should therefore be more appropriate for immunoprotective and immunodiagnostic studies. Antibodies directed against such proteins should be cross-reactive with strains of different serotypes. OM porin protein F, lipoprotein I and protein H₂ have been shown to cross-react immunologically between the different serotypes of *P. aeruginosa* (Mutharia *et al.*, 1982) and have been the subject of

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Abbreviations: IATS, International Antigenic Typing Scheme; IROMP, iron-regulated outer membrane protein; LPS, lipopolysaccharide; mAbs, monoclonal antibodies; OM, outer membrane; PBS, phosphate buffered saline; TBS, Tris-buffered saline.

investigations as to the protective potential of antibodies directed against them. Gilleland *et al.* (1984) reported that a purified protein F preparation successfully protected mice, after either passive or active immunization, from a subsequent challenge with a heterologous LPS immunotype of *P. aeruginosa*. Using a burned mouse model these workers demonstrated greater protection after immunization with a porin protein F preparation than with LPS immunization (Matthews-Greer & Gilleland, 1987). Hancock *et al.* (1985) have also demonstrated the immunotherapeutic potential of monoclonal antibodies (mAbs) to *P. aeruginosa* porin protein F. Reports of the use of IROMPs as vaccine components are scant. Bolin & Jensen (1987) have demonstrated passive protection against *Escherichia coli* septicaemia in turkeys using polyclonal antisera reactive against IROMPs. Sokol & Woods (1986*a*) demonstrated strain-dependent passive protection with polyclonal antisera raised against the 14 kDa ferripyochelin binding protein and showed that mAbs directed against this protein reduced ferripyochelin binding and promoted phagocytosis of *P. aeruginosa* by human polymorphonuclear neutrophils (Sokol & Woods, 1986*b*). Purified polyclonal antisera raised against an 80 kDa IROMP inhibited the uptake of ferripyoverdine by *P. aeruginosa* PAO (Cornelis *et al.*, 1989). Interestingly, in this study, pyoverdine-type specificity was noted (Cornelis *et al.*, 1989), wherein polyclonal antisera were directed only against IROMPs from strains producing structurally similar pyoverdine molecules. Three pyoverdine-specific groups were identified. To explore the immunotherapeutic potential of *P. aeruginosa* IROMPs, we have partially purified these proteins to circumvent the immunodominant response to LPS and used this preparation to raise mAbs. We have used immunogold electron microscopy to investigate the interaction between these antibodies and intact cells of *P. aeruginosa*.

Methods

Bacterial strains and culture conditions. *P. aeruginosa* PAO1 (ATCC 15692) and AK1282, an LPS defective mutant of PAO obtained from Dr A. M. Kropinski (Jarrell & Kropinski, 1981), were used. Representative strains of the 17 International Antigenic Typing Scheme (IATS) serotypes of *P. aeruginosa* were clinical isolates in the University of Aston culture collection. Strains were routinely maintained on nutrient agar (Oxoid) slopes at 4 °C and cultured in an iron-depleted chemically defined simple salts medium (CDM-Fe) comprising 5 mM-NaCl, 0.062 mM-KCl, 0.4 mM-MgSO₄, 3.2 mM-K₂HPO₄, 40 mM-(NH₄)₂SO₄, 50 mM-MOPS, pH 7.4, and 40 mM-glucose (Brown *et al.*, 1984). Iron-replete cells were grown in the chemically defined medium with 0.062 mM-FeSO₄·7H₂O (CDM+Fe). Bacteria were grown in CDM-Fe or CDM+Fe to early stationary phase (OD₄₇₀ 1.0) in an orbital shaking incubator at 37 °C, harvested by centrifugation at 10000 g for 10 min and washed once with saline.

Preparation of the antigen. OMs were prepared by the method of Filip *et al.* (1973). The washed bacterial pellet, containing approximately 2×10^{11} cells, was suspended in 20 ml distilled water and broken by 10×60 s pulses of sonication in an ice bath, with 60 s intervals for cooling. Unbroken cells were removed by centrifugation at 10000 g for 10 min. Sarkosyl (*N*-lauryl sarcosinate, sodium salt; Sigma) and EDTA were added to the supernate to final concentrations of 2% (w/v) and 10 mM, respectively. The mixture was incubated at room temperature for 1 h and then centrifuged at 100000 g for 1 h. The OM pellets were washed twice by suspending in distilled water and centrifuging at 100000 g for 1 h. The washed OM pellet was extracted with 5 ml solubilization buffer comprising 0.3% (v/v) Empigen BB (Albright & Wilson), 10 mM-EDTA, 1 mM-phenylmethylsulphonyl fluoride, 0.02% (w/v) sodium azide and 20 mM-triethanolamine, pH 7.5, for 1 h at room temperature. The supernatant was recovered after centrifugation at 100000 g for 1 h and stored at -20 °C.

Gel-filtration chromatography. A Superose 12 gel filtration column (Pharmacia) was used with a modified deoxycholate running buffer (Heckels, 1977) comprising 1% (w/v) sodium deoxycholate (Sigma), 10 mM-EDTA, 1 mM PMSF, 20 mM-triethanolamine, pH 9.5. A 500 µl sample of Empigen BB solubilized OM preparation (1 mg protein ml⁻¹) was injected onto the column. A flow rate of 0.5 ml min⁻¹ was used and 1 ml fractions were collected. Protein elution was monitored in-line by measuring *A*₂₈₀. The fractions were dialysed against 20 mM-triethanolamine, pH 9.5, for 24 h prior to concentration by freeze-drying. Aliquots of each fraction were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Fractions containing IROMPs were pooled and the protein content determined by a modified Lowry method.

Preparation of flagella. The method of Montie *et al.* (1982) was used. *P. aeruginosa* AK1282 was grown in 8 litres CDM-Fe and shaken at 50 r.p.m. for 48 h at 37 °C. The cells were harvested by centrifugation at 10000 g for 10 min and suspended in 20 ml saline. The flagella were sheared by treatment in a commercial blender for 10 s and isolated by differential centrifugation. The suspension was centrifuged at 10000 g for 10 min to remove whole cells and the supernatant centrifuged at 100000 g for 4 h to precipitate the flagella. This procedure was repeated until a clear pellet was obtained. The pellet was resuspended in 1 ml distilled water and stored at -20 °C.

Immunization schedule. An aliquot of pooled antigen equivalent to 50 µg protein was emulsified with an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously into F₁ (CBA × Balb/c) mice. Four weeks later, 50 µg antigen emulsified in Freund's incomplete adjuvant (Sigma) was injected intraperitoneally. After a further 4 weeks a final boost of 10 µg antigen was given intravenously. Blood samples were taken during the immunization schedule and immediately prior to fusion. Immunoblotting and ELISA assays were performed to determine the level and specificity of circulating antibody. Three days after the last injection the spleen cells of mice giving a strong antibody response by immunoblotting were fused with the NS1 myeloma cell line (Kohler & Milstein, 1976).

Selection and cloning of hybridomas. The hybridomas were screened for specific antibody production using an ELISA technique. Nunc microELISA plates (Gibco, UK) were coated overnight with 100 µl of iron-depleted *P. aeruginosa* AK1282 OMs 2 µg ml⁻¹ in 50 mM-carbonate/bicarbonate buffer, pH 9.6. Plates were washed in isotonic phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBS/Tween) and blocked with 200 µl 0.05% (w/v) fish gelatin (Sigma) in PBS for 1 h at 37 °C. After washing thoroughly, the plates were incubated at 37 °C for 1 h with 50 µl volumes of hybridoma supernatants, diluted 1:2 in PBS/Tween. Plates were washed three times in PBS/Tween and 100 µl 1:1000 anti-mouse F(ab')₂ fragments conjugated to horseradish peroxidase (Amersham) in PBS/Tween and

1% (v/v) sheep serum was added to each well and incubated at 37 °C for 1 h. Plates were washed and developed with 100 µl 0.0058% (w/v) 3,3',5,5'-tetramethylbenzidine in 0.1 M-acetate buffer, pH 5.2 for 10 min; the reaction was stopped with 50 µl 2 M-sulphuric acid. A_{450} was measured using a Multiscan microplate reader (Flow Laboratories). For the second screen the ELISA was repeated as above, but LPS from AK1282 (2 µg ml⁻¹, 100 µl per well), purified by the hot-phenol method (Westphal & Jann, 1965) was used as the antigen coat.

Determination of antibody isotype. Various specific murine Ig isotype antibodies were used in an ELISA isotyping assay. ELISA plates coated with AK1282 OMs as described above were incubated with hybridoma supernatants for 1 h at 37 °C. After washing, 100 µl 1:1000 biotinylated sheep anti-murine IgM, IgG1, IgG2a, IgG2b and IgG3 isotype conjugates (Amersham) in PBS/Tween were incubated at 37 °C for 1 h. The plates were washed and incubated with 100 µl streptavidin-horse radish peroxidase (Amersham) for 20 min at 37 °C. The colour reaction was developed as described above.

SDS-PAGE. This was performed on 12% (w/v) acrylamide gels (Laemmli, 1970) in a Mini-Protean apparatus (Bio-Rad). Gels were stained with 0.1% (w/v) Coomassie Brilliant blue R-250 in methanol/water/acetic acid (50:40:10, by vol.) and destained in water/methanol/acetic acid (83:10:7, by vol.). If LPS antigenic sites were to be investigated, OM preparations were incubated with 2.5 mg proteinase K (Sigma) ml⁻¹ in sample buffer comprising 50 mM-Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol for 1 h at 60 °C prior to electrophoresis. Silver staining was done by the method of Tsai & Frasch (1982).

Immunoblotting procedure. Proteins and LPS separated by SDS-PAGE were electroblotted to nitrocellulose by the method of Towbin *et al.* (1979) using a Transblot apparatus (Bio-Rad). Transfer of proteins was confirmed by staining with 1% (w/v) amido black in water/methanol/acetic acid (83:10:7, by vol.). The nitrocellulose was cut into strips and incubated at 37 °C for 1 h in 5% (w/v) skimmed milk in 10 mM-Tris-buffered saline (TBS), pH 7.4, to block non-specific antibody binding. The nitrocellulose strips were incubated for 4 h at 37 °C with hybridoma supernatant 1:50 in TBS/milk. The blots were washed three times in TBS and incubated with 1:1000 protein A (from *Staphylococcus aureus*) conjugated to horseradish peroxidase (Sigma) in TBS/milk for 2 h at 37 °C. The antigenic components were visualized with 0.0025% (w/v) 4-chloro-1-naphthol (Sigma) in 0.01% (v/v) H₂O₂ with 10 mM-Tris/HCl, at pH 7.4.

Immunogold electron microscopy. *P. aeruginosa* strains PAO1 and AK1282 were grown in CDM-Fe to the late exponential phase, harvested by centrifugation at 10000 g for 10 min and fixed in 0.1% (v/v) glutaraldehyde in saline. Aliquots (1 ml) of fixed cells (10⁸ ml⁻¹) were washed in PBS and suspended in 200 µl PBS. Aliquots (10 µl) were incubated with 90 µl hybridoma supernatant (diluted 1:2 in PBS) or 90 µl polyclonal serum (diluted 1:100 in PBS) for 1 h at 37 °C. The cells were then washed and suspended in 40 µl PBS. Aliquots (20 µl) were pipetted onto Parafilm (American Can Co.). Inverted electron microscope grids (Agar Aids, UK) were placed onto the samples. After 10 min the grids were blotted, washed and incubated with 1:10 goat anti-mouse IgG immunogold conjugate (15 nm particle size, BioCell Ltd) in a humidified box overnight at 4 °C. The grids were washed, counterstained in 0.5% (w/v) phosphotungstic acid for 1 min, washed again and examined under a Philips EM 300 electron microscope.

Cell surface iodination. Iodination was performed using Iodo-Beads (Pierce Chemical Co.) in accordance with the manufacturer's instructions. A 1 ml aliquot of bacterial cells (10⁸ ml⁻¹ in PBS) was added to a reaction vial containing two Iodo-beads. A 20 µl volume of ¹²⁵I (Amersham) diluted into PBS (10 mCi ml⁻¹; 370 MBq ml⁻¹) was added to the reaction vial to give 0.2 mCi ¹²⁵I per reaction vial. The

samples were swirled gently at room temperature for 5 min. The reaction was terminated by removal of the Iodo-Beads and addition of 1 ml 10 mM-sodium iodide/PBS (NaI/PBS). The radiolabelled cells were washed six times by centrifugation (10000 g for 5 min) and suspension in 1 ml NaI/PBS. After the last wash the cell pellets were suspended in 100 µl SDS-PAGE sample buffer, boiled for 10 min and then 20 µl aliquots were electrophoresed on 12% (w/v) SDS-polyacrylamide gels. After SDS-PAGE, the gels were dried onto filter paper by heating under reduced pressure. Dried gels were exposed to Kodak XAR film (Eastman Kodak Co.) for 4 days at -70 °C to obtain an autoradiogram of the labelled proteins.

Results

Purification of IROMPs

In order to produce anti-OM protein mAbs, it was necessary to purify the antigen preparation from LPS and so circumvent the immunodominant response to this antigen. The LPS-defective mutant, *P. aeruginosa* AK1282, reported to have LPS comprising only lipid A and core components (Jarrell & Kropinski, 1981), was grown in an iron-limited chemically-defined medium which has been shown previously to induce OM protein profiles with similar characteristics to those isolated from cells *in vivo* (Brown *et al.*, 1984; Anwar *et al.*, 1984). An IROMP-enriched preparation was obtained by selective solubilization of OMs from the LPS-defective mutant *P. aeruginosa* AK1282 in 0.3% (v/v) Empigen BB. With selective solubilization, the IROMPs represented 52% of total protein compared with 22.5% for OM preparations as judged by densitometric scanning of Coomassie blue-stained SDS-polyacrylamide gels (results not shown). Preliminary studies raising polyclonal antisera to IROMPs prepared by excision and elution from SDS-polyacrylamide gels indicated that this method of purification yielded an antigen preparation with a high LPS content (results not shown). The IROMPs were purified further by gel-filtration chromatography with a deoxycholate separation buffer. Analysis of column fractions by SDS-PAGE and silver staining after treatment with proteinase K revealed the presence of LPS contamination in the protein fractions (results not shown). Immunoblotting using polyclonal rabbit antisera specific for *P. aeruginosa* AK1282 OMs was employed to study the protein profile of the column fractions (Fig. 1*a*), and, after proteinase K digestion, to reveal LPS antigenic sites (Fig. 1*b*). Whilst the IROMPs present in fractions 6 to 15 were separated from lipid A, they were contaminated with low amounts of the O-antigenic moiety of LPS, indicating that AK1282 is not completely O-antigen deficient.

Fig. 1. Immunoblot analysis of fractions from gel-filtration chromatography of Empigen BB-solubilized OM of *P. aeruginosa* AK1282. Fractions 1 to 29 (lanes 1 to 29) and extract (lane 30) for protein (a) and LPS (b) were probed with serum raised to OM of *P. aeruginosa* grown in CDM-Fe and developed with protein A-peroxidase. Fraction 30 shows the profile of the extract prior to gel filtration.

Isolation of mAbs

After fusion of NS1 myeloma cells with spleen cells of mice immunized with the partially purified IROMP preparation, hybridomas producing antibodies specific for *P. aeruginosa* AK1282 OM were cloned by limiting dilution until a single clone in each well was obtained. To eliminate hybridomas secreting anti-LPS antibodies, a second ELISA screen was used employing purified AK1282 LPS as the antigen coat. Only three out of 46 hybridomas reacted with LPS. Fourteen of the selected hybridomas were stable in culture. These were classified in terms of immunoglobulin isotype and ELISA titre against AK1282 OM (Table 1). Nine of the mAbs were of the IgG2a subclass, four were IgG2b and one was IgG1. There was no correlation between ELISA titre and antibody isotype.

Interaction of mAbs with *P. aeruginosa* AK1282 OM

Immunoblotting was performed to determine the antigen specificity of the mAbs listed in Table 1. mAbs C14, C16, C19, C103 and C108 reacted strongly with the 85 kDa IROMP; however, the reactions were not completely

Table 1. Classification of mAbs

mAb	Immunoglobulin isotype	Titre in ELISA*
C9	IgG1	12000
C12	IgG2a	7000
C13	IgG2b	10000
C14	IgG2a	9500
C15	IgG2a	8500
C16	IgG2b	6500
C19	IgG2a	8000
C23	IgG2a	12500
C24	IgG2a	10500
C28	IgG2a	13000
C31	IgG2b	10000
C32	IgG2a	12500
C103	IgG2a	9000
C108	IgG2b	8000

* Purified AK1282 OM were used at a concentration of 2 µg per well in the ELISA.

specific since cross-reactivity with OM components of lower molecular mass was noted (Fig. 2a). mAbs 9, 12, 13, 15, 23, 24, 28, 31 and 32 reacted against a 54 kDa antigen which was barely detectable on an amido-black-

Fig. 2. Strip immunoblots of OM proteins (a) and flagella (b) prepared from *P. aeruginosa* AK1282. The strips were probed with hybridoma end supernatants (C9 to C108) and developed with protein A-peroxidase. The leftmost lane (a and b) shows a nitrocellulose strip stained with amido black.

stained nitrocellulose strip. Similar strong reactions were noted against a purified preparation of flagella which migrated with the same apparent molecular mass (Fig. 2b), suggesting that these mAbs were directed against flagellar proteins. LPS immunoblots were performed to

determine if the observed cross-reactivity was attributable to anti-LPS activity against co-migrating LPS. None of the mAbs reacted against LPS (results not shown), indicating that the screening strategy had resulted in a panel of anti-OM protein mAbs.

Fig. 3. Interaction of mAb C108 with OMs prepared from clinical isolates representing the 17 IATS serotypes grown in CDM – Fe. (a) Coomassie blue-stained SDS-PAGE profile; (b) immunoblot probed with mAb C108.

Interaction of mAb C108 with the 17 P. aeruginosa IATS serotypes

Clinical isolates representing the 17 IATS serotypes were grown in CDM – Fe. The OMs were purified, separated by SDS-PAGE and electroblotted onto nitrocellulose. The Coomassie blue-stained SDS-PAGE profile is shown in Fig. 3(a) and the immunoblot probed with mAb C108 is shown in Fig. 3(b). Only the 85 kDa IROMP from IATS serotypes 2, 5, 8, 11, 13, 15, 16 and 17 was detected. Faint cross-reactivity with a 50 kDa antigen was noted with serotypes 16 and 17. Interestingly, the 85 kDa IROMP from these two serotypes gave the strongest reaction with mAb C108. Similar results were noted with the other anti-85 kDa mAbs (results not shown).

Interaction of mAbs with intact cells

Immunogold electron microscopy was used to assess the surface exposure of the 85 kDa IROMP. When mAb C108 was reacted with cells of strain AK1282 grown in

CDM – Fe there was negligible binding of gold particles to the cell surface (Fig. 4a). Preliminary experiments with cells fixed after addition of the mAbs did not show any differences in the level of binding. Although AK1282 is an LPS-defective mutant, treatment with anti-O antigen and anti-core LPS mAbs resulted in significant binding (results not shown). Preliminary attempts to remove LPS by washing in trichloroacetic acid (Hackstadt, 1988) were not successful. No reduction in binding of either of the anti-LPS mAbs was noted in an ELISA system using treated versus non-treated cells. Figure 4(b) shows cells of AK1282 treated with mouse polyclonal antisera taken immediately prior to fusion of the spleen cells. Treatment of AK1282 cells with a control antibody, mouse anti-human luteinizing hormone, did not result in any binding (results not shown). Two of the anti-85 kDa IROMP mAbs, C19 and C103, agglutinated cells of AK1282 grown in low-iron medium, but not in high-iron medium. Figure 5(a) shows the reaction of low-iron-grown AK1282 cells with mouse polyclonal antiserum taken prior to fusion and the agglutination with mAb C19 is shown in Fig. 5(b).

Fig. 4. Immunogold detection of surface antigenic sites of *P. aeruginosa* AK1282. Iron-depleted whole cells of AK1282 were reacted with anti-85 kDa IROMP mAb C108 (a) and mouse polyclonal antiserum raised to purified IROMPs (b). Bars, 0.5 μ m.

Cell surface iodination

Since the reaction of anti-85 kDa IROMP mAb 108 with whole cells of low-iron-grown AK1282 was not conclusive, cell surface iodination was attempted to determine if the 85 kDa IROMP was surface exposed. The autoradiograph of AK1282 cells grown in both CDM+Fe and CDM–Fe is shown in Fig. 6, together with the corresponding Coomassie blue-stained SDS-

PAGE profiles. A strongly-labelled band corresponding to the 85 kDa IROMP was present in CDM–Fe cells but absent in those grown in CDM+Fe.

Discussion

The major obstacle in the purification of Gram-negative OM proteins for immunological studies is the difficulty

Fig. 5. Agglutination of *P. aeruginosa* AK1282 revealed by immunogold electron microscopy. Iron-depleted whole cells were reacted with (a) mouse polyclonal antiserum raised against the purified IROMP preparation taken prior to fusion of spleen cells or (b) anti-85 kDa IROMP mAb C19, which caused agglutination. Bars, 1 µm.

of separating them from the LPS with which they are intrinsically associated. Deoxycholate gel-filtration chromatography, noted for breakdown of LPS into monomeric subunits (Heckels, 1977), effected separation of the IROMPs from the lipid A component of LPS and enabled us to produce a panel of anti-OM protein mAbs. In this work we have characterized a panel of nine anti-flagella mAbs and five mAbs which react against the 85 kDa IROMP of *P. aeruginosa* AK1282. The isolation of anti-flagella mAbs is not surprising since flagella are recognized as dominant *P. aeruginosa* antigens (Ward *et al.*, 1988) with protective capability (Holder & Naglich, 1986).

Fig. 6. Cell surface iodination of *P. aeruginosa* AK1282. Whole cells of *P. aeruginosa* were radiolabelled with ^{125}I and electrophoresed on an SDS-polyacrylamide gel. The gel was dried and subjected to autoradiography. Lane 1, molecular mass markers; lanes 2–4, Coomassie blue-stained SDS-PAGE profiles of Sarkosyl-insoluble OM fractions of low-iron grown AK1282 (lane 2) and iodinated whole cells grown in high- (lane 3) and low-iron (lane 4) media. Lanes 5 and 6, corresponding autoradiogram of lanes 3 and 4.

The anti-IROMP mAbs demonstrated low-level cross-reactivity with lower molecular mass OM protein components of strain AK1282; however, this was either not seen or greatly reduced when other clinical strains were examined. Whilst this may be due either to proteolytic breakdown of the IROMPs or to conserved epitopes, it is not clear why mAb C108 should be specific for the 85 kDa IROMP in one strain but not in another. The pattern of cross-reactivity amongst the five mAbs against OM fractions prepared from AK1282 grown in CDM-Fe is different, suggesting that different epitopes are being recognized. Cross-reactivity among the IROMPs (72–115 kDa) was noted by Cornelis *et al.* (1987), but not with other OM protein components. The pattern of cross-reactivity amongst the 17 IATS serotypes was surprising; the IROMPs have been postulated to be involved in uptake of iron-siderophore complexes and so it would be expected that such a system would be common to *P. aeruginosa* strains. Our immunoblotting

data support the findings of Cornelis *et al.* (1989) that *P. aeruginosa* has immunologically distinct IROMPs of a similar molecular mass. It is not clear whether the approximately 85 kDa IROMP in this study is the same as the 80 kDa pyoverdine receptor reported by Cornelis *et al.* (1989), nor, as is suggested by these authors, is it clear why proteins having the same molecular mass and apparent biochemical function should exhibit antigenic variation and be immunologically distinct. It is noteworthy that Christoffersen & Cox (1989) have described heterogeneous forms of pyoverdine, varying in the composition of the amino acid side-chain, both within the same strain and from different strains of *P. aeruginosa*, although only a portion of these molecules appear to have importance in pathogenesis through mobilization of iron. Interestingly, Leong *et al.* (1990) have demonstrated strong receptor specificity between an 86 kDa OM receptor, the PupA gene product, in the potato-growth promoting pseudomonad *Pseudomonas putida* WCS358 and the ferric complex of its native siderophore, pseudobactin 358. Whilst not explaining why we observe interaction of the mAbs in this study with some *P. aeruginosa* strains but not others, these data contribute to the notion of strains having structurally different IROMPs depending on the structure of the pyoverdine-type siderophore they produce. An investigation of the chemical relatedness of the pyoverdines produced by these strains would be valuable to demonstrate this correlation.

From the immunogold results, it is not possible to conclude unequivocally whether the 85 kDa IROMP is surface exposed. Only low-level gold binding was noted using the anti-85 kDa IROMP mAb C108 and the LPS-defective strain AK1282. Moreover, it cannot be ruled out that this was attributable to binding to the lower molecular mass OM components with which it exhibited cross-reactivity in immunoblot studies. A larger study using more strains would be useful in this respect. The strong iodination of a protein migrating at 85 kDa suggests surface exposure. This would be consistent with the postulated role of IROMPs as receptors for iron-siderophore complexes. However, it should be noted that this interpretation is based on the relative labelling of the 85 kDa IROMP since many more proteins were weakly radiolabelled than are determined to be OM proteins by Sarkosyl solubilization studies. It has been reported that the lactoperoxidase method described by Lambert & Booth (1982) was not specific and labelled other non-OM proteins (Sullivan & Williams, 1982). From this study it would appear that the method based on Iodo-beads falls into a similar category.

Possible reasons for the low-level binding of the anti-85 kDa IROMP may be (1) reactivity against a primary rather than a conformational epitope, or (2) masking by

LPS. It is well recognized that antisera raised against a protein in a denatured form often react poorly or not at all with native protein (Benjamin *et al.*, 1984; Gabay *et al.*, 1985), the epitopes being buried within the three-dimensional structure of the protein. The mAbs in this study were raised against IROMPs denatured by detergent solubilization. They reacted strongly with OM proteins separated by SDS-PAGE, but gave only low titres against whole cells in an ELISA assay (1:40 to 1:280) and low-level binding by immunogold electron microscopy. We believe that the low level of binding of the anti-85 kDa IROMP mAb in this study is attributable to masking of binding sites by LPS. Although the study was carried out using an LPS defective strain we found strong immunogold labelling with a mAb directed against a 'ladder-like' component of LPS on SDS-PAGE, typical of O-antigen. When an anti-core LPS/lipid A mAb was used, the binding was lower than when the anti-O antigen mAb was used, even though it would be expected that the surface density of core LPS would be much greater than O-antigen in the LPS-defective mutant. These data suggest some masking of LPS components, possibly including IROMPs, from interaction with immunoglobulins, by LPS O-antigen. Smaller molecules such as pyoverdine would not be inhibited from gaining access to these proteins. Mutharia & Hancock (1983) reported a similar finding with mAbs directed against *P. aeruginosa* OMP H₂. The mAb showed no fluorescence on intact, smooth cells, but was able to interact with a rough, LPS-deficient mutant. A similar phenomenon has been reported for the 74 kDa ferric-aerobactin receptor in *E. coli* by Roberts *et al.* (1989). These workers also demonstrated that polyclonal antisera did not protect against infection with a smooth strain, suggesting that this receptor is not a good target for immunotherapy. Whilst a similar argument could be made against the IROMP described in this study, these IROMPs are dominant antigens in acute human infections (Ward *et al.*, 1988), being immunologically recognized very early in the course of infection and prior to other OM components. It is therefore likely that they play a role in immune protection, but this is perhaps mediated or enhanced by LPS. The degree to which antibodies directed against the high molecular mass IROMPs might be involved in immune protection against *P. aeruginosa* infection is relatively unknown, but the ability of some of the mAbs to agglutinate cells may prime the complement cascade or facilitate opsonization. Our finding does, however, confirm an interaction with surface components.

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