Introduction

Although widely distributed in the environment, *Pseudomonas aeruginosa* rarely infects man in health. However, it is a major opportunist pathogen when normal immune mechanisms are impaired. Chronic *P. aeruginosa* lung infection is at present the major cause of death in cystic fibrosis (CF) patients because progressive destruction of infected lung leads to respiratory insufficiency. Several enzymes and toxins of *P. aeruginosa* are thought to be responsible for this destruction during the early stages of infection. Prolonged antigenic stimulation caused by the continuous presence of bacteria in the lungs exacerbates the direct damage because neutrophils and macrophages recruited to the site of infection break down, with local release of lysosomal enzymes and inflammatory mediators. Evidence is accumulating that immune complexes formed from antibodies and persisting bacteria can also mediate hypersensitivity reactions which further increase the lung damage. Although antibiotics rarely eradicate the bacteria from the lungs, regular courses of intensive treatment with antipseudomonal antibiotics improve lung function, patient well-being and long term survival. Therefore, it is important to identify the onset of chronic lung infection so that treatment can begin before tissue damage becomes extensive.

The serum antibody response to *P. aeruginosa* may be monitored by crossed immuno-electrophoresis (CIE) with water soluble extracts of cell sonicates as antigen (standard antigen, StAg) when up to 64 precipitating antibodies to *P. aeruginosa* have been detected. Results from CIE studies indicate that a substantial antibody response is mounted in CF lung infection, that an increase in the number of precipitin peaks shown by CIE correlates with clinical deterioration and that this may be used as an indication for antibiotic treatment. CIE shows the number of antigen-antibody reactions at different stages of the infection but has rarely been used to identify the antigens involved.

The cell envelope of *P. aeruginosa* contains a number of macromolecules which may interact with host cells to produce an immune response. Outer-membrane proteins (OMPs) have been shown to be highly conserved in strains of *P. aeruginosa* of different O-serotypes and the presence of antibodies to OMPs has been demonstrated in CF serum. However, these studies were not done with clinical isolates grown under iron-limited conditions such as prevail in vivo. Iron is essential to the growth and mechanisms to acquire it are virulence factors in pathogenic and potentially pathogenic bacteria such as *P. aeruginosa*. Iron-regulated outer-membrane proteins (IRMPs), induced in iron-limited conditions,
are thought to function as cell-surface receptors in high affinity uptake systems for iron-siderophore complexes. Antibodies to IRMPs have been reported in the serum of an infected CF patient. Our aim in this study was to investigate CF patients for antibodies to IRMPs by immunoblotting, and to determine how the chronological appearance of antibodies to OMPS and to IRMPs correlates with the duration of infection and the patient's clinical condition. Immunoblotting has been compared with CIE with P. aeruginosa StAg.

Materials and methods

Patients and treatment

Diagnosis of CF was made on the basis of abnormal sweat electrolytes and exocrine pancreatic insufficiency. All patients in the study attended the outpatient clinic at the Danish CF Centre at Rigshospitalet at monthly intervals for bacteriological investigations and physical evaluation. The progress of the disease was charted by following the patient's records, including the results of sputum bacteriology, weight gain and lung function, and was correlated with changes in CIE precipitins. Since 1976, all patients with chronic P. aeruginosa infection have been admitted to the Centre every 3-4 months for a 2-week course of intravenous antibiotics.

Before 1976, antipseudomonal chemotherapy was given only when there was acute pulmonary exacerbation or clinical deterioration. Serum samples not used immediately were stored at 20°C. Precipitating antibodies against P. aeruginosa were determined at least once a year by CIE. P. aeruginosa was identified in sputum samples by the method of Lowry et al. Bacteria grown to early stationary phase at 35°C in CDM were harvested by centrifugation, resuspended in distilled water and broken by sonication in an iced bath. Unbroken cells were removed by centrifugation at 5000g for 10 min. Sarkosyl (N-lauroylsarcosine, sodium salt; Sigma), was added to the supernate to a final concentration of 2%. The mixture was incubated for 30 min at 20°C then centrifuged at 38 000g for 1 h. The membrane pellets were washed twice with distilled water and stored at -20°C. Protein determinations on the isolated OMPS were by the method of Lowry et al.

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

OM preparations were separated by SDS-PAGE with an acrylamide 14% running gel and a 5% stacking gel as described previously. Gels were stained with silver or with Commassie Brilliant Blue (Sigma) 0.1%. Molecular weights (M, s) of separated proteins

| Table I. Strains of P. aeruginosa isolated from CF sputum samples |
|---------------------------------|-----------------|-----------------|-----------------|
| Strain no. | Phenotype | O serotype | Phage type |
| 6680       | Mucoid    | 3            | 21/44/F8/109/1214/Col2/Col21n |
| 1118       | Non-mucoid| 3            | 7/21/F7/M4/Col2/Col11+ |
| 8815       | Mucoid    | 10           | 21/68/73/F8/109/352/1214+ |
| 1222       | Non-mucoid| 3/6/9/10     | 7/109/352+ |
were estimated by comparison with the M, of known protein standards (Bio-Rad, Richmond, CA, USA). Gels for immunoblotting were cast without a comb to form a flat top as described previously. The loading of OM was 15 µg of protein/0.6 cm of the top surface.

**Immunoblotting**

Antigens were transferred from polyacrylamide gels to nitrocellulose (NC) paper (Schleicher and Schuell, Dassel, Germany) by a modification of the method of Towbin. Blotting was done in a Trans-Blot apparatus (Bio-Rad) at 90 V for 2 h with a cooling coil, then reduced to 30 V and continued overnight. The high initial voltage was found to be necessary to transfer the high M, IRMPs and did not adversely affect the transfer of other OMPs. After transfer of proteins the NC paper was cut into strips and antigenic sites were visualised as described previously. To show complete qualitative transfer of proteins from the polyacrylamide gel, some strips of NC paper were stained with colloidal gold (AuroDye, Jansen, Beerse, Belgium) immediately after transfer. M,s of blotted proteins were determined by comparison with the M, of pre-stained protein standards (Bio-Rad).

**Preparation of P. aeruginosa standard antigen (StAg)**

Strains representing the 17 serotypes of *P. aeruginosa* were obtained from Mikkelsen and from the Cross Infection Laboratory, Public Health Laboratory Service, Colindale Avenue, London. Strains were grown on plates of agar containing peptone 5%, glucose 0.2% and NaCl 0.5%, pH 7.5 (Truche Agar; State Serum Institute, Copenhagen) for 18 h at 35°C. The cells were harvested with distilled water and centrifuged at 10,000 g for 10 min at 4°C. The bacterial pellet was resuspended in five volumes of distilled water and cells were broken by sonication at 20 KHz for three 45-s bursts in an ice bath. The sonicate was centrifuged at 38,000 g for 1 h. The supernate containing soluble antigens (StAg) was stored at -20°C until use.

**Crossed immuno-electrophoresis (CIE)**

Precipitating antibodies against *P. aeruginosa* were determined by CIE. StAg samples were applied to wells punched in agarose 1% gels (Indubiose A 37; L’Industrie Biologique Francaise S.A., France) and separated in the first dimension at 10 V/cm for 30 min. Second dimension electrophoresis was at 1-2 V/cm for 18 h into agarose 1% containing patient’s serum at a concentration of 15 µl/cm². Immunoprecipitates were stained with Coomassie Brilliant Blue and the number of precipitin peaks (precipitins) was counted.

**Immune sera**

Polyclonal antisera to StAg and to the OM of strain 6680 were prepared by hyperimmunisation of rabbits according to the method of Harboe and Ingild.

**Results**

**Outer membrane of *P. aeruginosa* strain 6680**

The Coomassie Blue-stained gel of OMPs and core lipopolysaccharide (LPS) of strain 6680 after separation by SDS-PAGE (fig. 1a) conforms largely to the scheme described by Hancock and Carey. Proteins at 17, 19, 30 and 52 Kda in the OM of this strain are not characterised by letters in the Hancock and Carey scheme. In addition, iron deprivation induces four high-M, proteins, two major at 74 and 83 Kda and two minor at 71 and 76 Kda; these could be repressed completely by inclusion of 0-12 mM iron in the growth medium (data not shown). The more sensitive silver stain of the same preparation (fig. 1b) shows more clearly the protein bands at 17 and 19 Kda and reveals a number of minor proteins not visible in the Coomassie Blue-stained gel. Both Coomassie Blue and silver stains show the amount of protein H1 in the OM of this strain to be greater than that of H2. All the protein bands visualised by the silver stain of the OM could be seen in a direct gold stain of the NC paper after electrophoretic transfer (not shown) and after reaction with serum from rabbits immunised with the OM preparation (fig. 1c) and StAg (fig. 1d).

**Fig. 1.** The OM of *P. aeruginosa* strain 6680 separated by SDS-PAGE and stained with Coomassie Brilliant Blue (a) and silver (b), or electrophoretically blotted to NC paper and incubated with rabbit antisem to OM (c) or to StAg (d). Numbers in heavy type indicate the M, (Kda) of proteins not characterised by a letter in the scheme of Hancock and Carey.
CIE precipitins and clinical condition

Serum samples from the nine infected CF patients encompassed the progress of the disease from a time before infection through to the late stages of chronic broncho-pulmonary disease. The mean age of the patients at the time they became chronically infected with \textit{P. aeruginosa} was 10.7 years (range 3.8–19.1 years). In all nine patients there was an increase in precipitin numbers during the first year of chronic infection. Four of the patients had a rapid increase (table II), which was defined as a rise of more than five precipitin lines a year. In another two patients the increase in precipitin numbers was rapid in the first year and then levelled off, in both cases at 14–17 precipitin lines; the numbers remained steady for the rest of the study period. The rapid rise in precipitin numbers was associated in all cases with isolation of mucoid strains of \textit{P. aeruginosa} (table II) which was associated in these patients with a deterioration of lung function to 50–75% of normal predicted values and with stunting of growth. The clinical state of four of the six patients improved with intensive antibiotic treatment but deteriorated between treatments.

Three patients showed a slower overall rate of increase in precipitin numbers and in these patients normal weight gain and lung function were maintained with the onset of infection. In this group, infection was initially with non-mucoid strains, which were replaced by mucoid strains after a period of 1–4 years (table II). A rapid increase in the number of precipitin lines was seen at the same time as isolation of the mucoid form, but in only one of the three patients in this group was this associated with deterioration in lung function and weight loss.

Longitudinal study of antibodies to OMPs by immunoblotting

Sequential serum samples from the nine patients were allowed to react with OMs after immunoblotting. The earliest anti-OMP antibodies to appear in most patients were directed against proteins H1 and G (table II). In two patients, both in the group with a rapid initial rise in precipitins, antibodies to H1 appeared alone. In another five patients, antibodies to H1 were amongst the first to appear, most often in combination with antibodies to proteins G and I (table II). In seven of the patients, immunoblotting showed the presence of antibodies to protein H1 in serum before the appearance of CIE precipitins or the constant presence of \textit{P. aeruginosa} in sputum. In two patients, antibodies to protein G also appeared at this early stage. The appearance of antibodies to other OMPs was co-incident with an increase in precipitins in the first year of chronic infection. Serum samples with eight or more precipitin peaks in CIE contained antibodies that reacted with all the major OMPs.

| Table II. Antibodies to OMPs in the early stages of chronic \textit{P. aeruginosa} lung infection in CF |
|-------------------------------------|----------------|
| Increase in CIE precipitins | Phenotype | First OMP antigen recognised |
| Rapid Patient a | M | I | 1 |
| b | M | H1 | H1 |
| c | M | All together | 17 Kda, G, 30 Kda, F, E, D, 52 Kda, 74 Kda, 83 Kda |
| d | M | H1 | 19 Kda, I, H1, F, E*, D*, 52 Kda |
| Initially rapid, then slow Patient e | Mixed M & NM | I, H1*1, G*, E, D, 52 Kda |
| f | NM then mixed M & NM | I, H1, 30 Kda*, F, E, D, 74 Kda*, 83 Kda* |
| Slow Patient g | NM, becomes mixed M & NM after 1 year | H1*1 | I, H1, 52 Kda, 74 Kda*, 83 Kda* |
| h | NM, becomes M after 4 years | H1, G | I, H1, G, 30 Kda |
| i | NM, becomes M after 2 years | All together | I, H1, F, E, D |
| Anti-OMP antibodies at first isolation of \textit{P. aeruginosa} | Number of precipitins (CIE) |
| | Anti-OMP antibodies |
| Rapid Patient a | I, 17 Kda, 19 Kda, H1, G*, F, E, D, 52 Kda |
| b | I, 17 Kda, 19 Kda, H2, H1, G, F, D*, 52 Kda, 74 Kda, 83 Kda* |
| c | I, G, 30 Kda, F, E, D, 52 Kda, 74 Kda, 83 Kda |
| d | 19 Kda, I, H1, F, E*, D*, 52 Kda |
| Initially rapid, then slow Patient e | I, H2, H1, G, E, D, 52 Kda, 74 Kda*, 83 Kda* |
| f | 19 Kda, I, H1, G, 30 Kda, F, E, D, 52 Kda, 74 Kda, 83 Kda |
| Slow Patient g | I, H1, 52 Kda, 74 Kda*, 83 Kda* |
| h | I, H1, 30 Kda, F*, D, E, 52 Kda, 74 Kda*, 83 Kda* |
| i | I, H1, 30 Kda, F* |
| Anti-OMP antibodies at onset of chronic infection |
| | |
| Rapid Patient a | 19 Kda, I, H1, G, F, E, D, 52 Kda, 74 Kda*, 83 Kda* |
| b | 19 Kda, I, H1, G, 30 Kda, F, E, D, 52 Kda, 74 Kda, 83 Kda |
| c | I, H1, F, E* |
| d | I, H1, F, E, D |

M, mucoid; NM, non-mucoid. * Indicates a faint reaction.
The pattern of appearance of antibodies to OMPs differed in patients with slow or rapid increases in precipitin numbers. Figs 2, 3 and 4 show reactions of OM antigens with sera from three of the patients. Patient i (fig. 2) was from the group with a slow initial increase in precipitin numbers (table II). In this patient's sera, no antibodies to OMPs were detected by immunoblotting before the presence of CIE precipitins (fig. 2, strips 1 and 2). By the time that three precipitins were detected by CIE, immunoblotting showed antibodies to OMPs D, E, F, H1 and I. Thereafter, antibodies appeared to the 17-Kda protein (fig. 2, strip 5). Antibodies to IRMPs appeared weakly when there were three precipitins (fig. 2, strip 4). It was not possible to distinguish the four individual IRMPs due to their closely similar Mₐs and they are labelled with the Mₐ of the two major proteins. The number of antibodies and the intensity of the reaction (as judged by the density of the colour reaction) did not increase greatly with the increase in precipitins, nor with the isolation of the mucoid form. In patients g and h, however, there was a marked increase in the intensity of the reaction to core-LPS and to the 52-Kda protein with isolation of the mucoid form (fig. 3, patient h). In this patient, antibodies to proteins H1 and G were recognised by immunoblotting before there was a CIE precipitin response. Antibodies to the other major OMPs appeared as precipitin numbers increased and all were visualised by immunoblotting of the serum sample with nine precipitins (fig. 3, strip 5). Antibodies to IRMPs were present from this time onwards but did not react strongly. This was the only patient in the present study whose serum precipitins consistently decreased after reaching a maximum. The decrease did not correlate with a decrease in the number of blotted bands (fig. 3, strips 11–14). However, the patient did improve clinically.

The number of antigens recognised and the intensity

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**Fig. 2.** Immunoblots of separated OM components of strain 6680 after reaction with serum from an infected CF patient (i) in stable clinical condition. P.a indicates the first isolation of *P. aeruginosa* from patient's sputum; mP.a indicates isolation of mucoid *P. aeruginosa*. The interval in months between subsequent serum samples is given below the blots (†). The lower line shows precipitin numbers in each serum sample (pptns).
Fig. 3. Immunoblots of separated OM components of strain 6680 after reaction with serum from an infected CF patient (h). Serum antibody levels increased in this patient with isolation of the mucoid form.

of the reaction was greater in those patients with a rapid increase in serum precipitins (fig. 4, patient d). In this patient, antibodies to protein H1 were present in the earliest serum sample available, some 3 years before there was a response detected by CIE. At the time when precipitin numbers started to increase (fig. 4, strip 4) there were antibodies in serum that reacted with all the major OMPs. The reaction with protein F was most pronounced. Thereafter the intensity of the reaction with all OMPs increased considerably with the increase in precipitin numbers. Antibodies to IRMPs were present from the time when there were four precipitins (fig. 4, strip 4) but they reacted only weakly until precipitin numbers in the high 30s were reached (fig. 4, strip 8). After this time the reaction with the 83-Kda protein became pronounced. This patient subsequently died as a result of chronic pulmonary infection.

Reaction of patient sera with OMPs from P. aeruginosa strains of different serotype and phenotype

Fig. 5 shows the reaction of the most recent serum samples from patients d and i with OMPs of mucoid and non-mucoid organisms of O-serotypes commonly isolated in the Danish CF Centre. The pattern of reaction of both sera with all four strains was similar to that seen with strain 6680 in the longitudinal study (figs. 2 and 4).

Reaction of control sera with OMPs

None of the sera obtained from young CF patients reacted with OMPs of strain 6680. Three of the adult sera showed a reaction with protein H and one with protein F; these reactions were very faint.

Discussion

The determination of precipitating antibodies by CIE is a well established method for the diagnosis and monitoring of P. aeruginosa lung infection in CF. An increase in the number of CIE precipitins with time, with the duration of infection and with the isolation of the mucoid form is confirmed in the present study. Antibodies in sera raised against the StAg preparation used in CIE react with all the major OMPs of P. aeruginosa (fig. 1d). However, it is not known which CIE precipitins represent individual OMPs. Our aim was to see how the onset of infection and the clinical condition of the patient were related to the appearance of antibodies to OMPs. The study was continued for periods up to 15 years in individual patients, but the later findings may not represent the natural history of
the lung infection because all patients were treated with anti-pseudomonal antibiotics after the diagnosis of chronic lung infection was made.

In the nine patients studied by immunoblotting, the appearance of antibodies to most OMPs was co-incident with the appearance of CIE precipitins. Immunoblotting showed antibodies to some OMPs before the appearance of CIE precipitins; this may reflect the greater sensitivity of the immunoblotting technique. Proteins G, HI and I in particular were likely to appear in immunoblotting before CIE precipitins and may thus be useful markers for the onset of infection. In the early stages of infection an increase in precipitin numbers was accompanied by an increase in the number of antibodies to OMPs detected by immunoblotting. There was, however, no direct correlation between the number of antibodies against OMPs detected by immunoblotting and CIE precipitin numbers (table II). This is probably because the StAg used in CIE is a whole cell preparation. We have previously shown a good correlation between precipitin numbers and anti-LPS antibody concentrations determined by ELISA. A rapid increase in antibodies to OMPs detected by immunoblotting paralleled a rapid increase in precipitins which is associated with a poor prognosis. This study shows that a pronounced antibody response to OMPs of P. aeruginosa does not lead to the resolution of CF lung infection. Persistent infection and a continuous antibody response may lead to immune-complex formation and is consistent with the theory that immune complexes are implicated in the pathogenesis of CF lung disease.

A previous study of P. aeruginosa isolated directly from the lungs of a patient with CF showed the cells to be growing in conditions of iron limitation. Antibodies to IRMPs were detected in the patient's serum. In the present study, antibodies to IRMPs did not appear early in the infection in any of the patients studied and were detected at high concentrations only in seriously ill patients. This is in agreement with the report of Kubesch et al. and that of Brown et al. whose CF patient had had chronic lung infection for 8 years. This was an unexpected observation if it is assumed that the IRMPs are necessary for iron uptake and hence bacterial growth.

It is not known why the antibody response to IRMPs should appear late in P. aeruginosa lung infection. The level of free iron in the lung is well below that required for bacterial growth. IRMPs are certainly immunogenic, because immunisation of rabbits with OMs isolated from cells grown in iron-limited conditions causes formation of antibodies to IRMPs (fig. 1c) within a few weeks. It is possible that the mucoid exopolysaccharide characteristic of strains infecting the CF lung, or the microcolony mode of
growth, masks the surface of the bacteria or alters the dynamics of iron uptake. The pH in the CF lung appears to be neutral, but acid metabolic products and a local inflammatory response may lower the pH in the mucoid microcolony to the optimum for iron uptake by pyoverdin and pyochelin. However, this explanation is in conflict with the observation that infection in CF is initially with classical non-mucoid strains which become mucoid only later. IRMPs may, in fact, not be expressed until late in the infection. Protease enzymes secreted by P. aeruginosa have been shown to cleave transferrin and make iron available to the bacteria. Transferrin has been shown to be present in CF lung. The production of proteases is known to be stimulated by growth in iron-limited conditions. Proteases are neutralised by antibody later in the infection and by a 2-macroglobulin in serum transudates. At this stage it may become necessary to make use of the high affinity uptake system and express IRMPs. The appearance of high concentrations of antibodies to IRMPs could be a marker of more advanced illness.

The OM of the mucoid clinical isolate 6680 contains some atypical proteins. Immunoblotting with CF serum shows them to be antigens that stimulate a considerable antibody response in vivo. A low M, (14 Kda) OMP of P. aeruginosa has been reported to bind the iron-siderophore complex, ferripyochelin. Surface expression of the ferripyochelin-binding protein is required for virulence and we are investigating the low M, OMPs of mucoid strains for iron binding activity. The proteins at 17 and 19 Kda are very similar in M, to that reported for pili in immunoblots of P. aeruginosa OM. Similarly the protein at 52 Kda could be flagella. However, strain 6680 is non-motile and electronmicroscopy shows very few cells to be flagellate. We have isolated three mucoid strains from CF lung with a protein at 52 Kda in their OM but it is absent in others. A 54-Kda protein has been reported in the OM of strains of P. aeruginosa resistant to norfloxacin. Given the practical difficulty in comparing M,s determined in different systems this may be the 58.5-Kda protein reported by Fernandes to cause an antibody response in CF serum. Proteins at 23 and 30 Kda in the OM of strain 6680 produced an antibody response which increased in the later stages of infection (figs. 3 and 4). A 32-Kda protein has been reported previously in the OM of antibiotic-susceptible mucoid strains and has also been shown to be associated with serum sensitivity. This may be the protein we see at 30 Kda. It is possible that these unusual OMPs are selected by intensive antibiotic treatment of the lung infection. We are currently investigating the antibiotic sensitivity of mucoid strains with these atypical proteins in the outer membrane. The antibody response to these unfamiliar OMPs emphasises the importance of investigations with clinical isolates grown in iron-limited conditions because laboratory strains grown in iron-rich complex media may not express surface antigens characteristic of cells growing in vivo.

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References


