Role of IgG subclass response to outer-membrane proteins in inhibiting adhesion of *Pseudomonas aeruginosa* to epithelial cells

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Summary. The IgG subclass response to the major outer-membrane proteins (OMPs) of *Pseudomonas aeruginosa* was investigated in 11 cystic fibrosis (CF) patients and 10 healthy controls. Inhibition of adhesion of *P. aeruginosa* to buccal epithelial cells by the IgG serum fractions from the CF patients has been established previously. The CF patients demonstrated marked heterogeneity in their individual IgG subclass response to pseudomonal OMPs. The predominant IgG1 and IgG4 responses were directed towards OMPs F, H2 and, with IgG1 only, to protein I. Proteins of 42 and 46 kDa primarily elicited an IgG2 response but some patients produced IgG4 antibodies. The IgG3 response varied from very weak in some patients to a strong reaction with proteins D2, E, G and I in others. The range of antigen-specific IgG subclass responses was similar in CF patients whose IgG fractions strongly inhibited the adherence of *P. aeruginosa* to epithelial cells and in those whose fractions gave only weak inhibition of adherence. There was no indication that an antibody response towards any particular OMP was implicated in the inhibition of bacterial adherence. Thus, the IgG subclass response to OMPs did not exert a significant effect on adherence when investigated in isolation, but may possibly play some role in combination with other processes.

Introduction

Two of the major components of the *Pseudomonas aeruginosa* outer membrane are lipopolysaccharide (LPS) and a series of outer-membrane proteins (OMPs). The primary functions of the OMPs include pore formation,¹ structure determination and maintenance,^{2,3} and specific substrate transport.⁴ The OMP profile of *P. aeruginosa* is highly conserved among serotype reference strains,⁵ clinical isolates⁶ and environmental isolates.⁷

The humoral immune response to the OMPs of *P. aeruginosa* in cystic fibrosis (CF) patients has already been studied. A correlation between the presence of antibody to mucoid exopolysaccharide and antibody to OMPs has been reported.⁸ Moreover, antibodies to OMPs have been detected, by immunoblotting, early in pseudomonal infection in CF patients.⁹ The prevalence of the immune response to the OMPs in CF has generated interest in their possible potential as vaccine candidates¹⁰ and the efficacy of the major OMPs F and I as vaccines in murine models has already been described.^{11,12}

It has been demonstrated that the adherence of an *Escherichia coli* strain to epithelial cells was inhibited

by outer membranes¹³ and that isolated outer membranes acted as competitive inhibitors of adherence.¹⁴ Moreover, we have shown that IgG antibody from CF patients' sera inhibits the adherence of *P. aeruginosa* to buccal epithelial cells and that each of the IgG subclasses contributed to this inhibition.¹⁵ In this report, we describe studies of the IgG subclass response to the OMPs of *P. aeruginosa* as related to the ability of these antibodies to inhibit bacterial adhesion.

Materials and methods

Bacterial strain and growth conditions

A non-mucoid clinical isolate of *P. aeruginosa* from a CF patient was used throughout. This strain was maintained at 4°C on tryptone soya agar. For isolation of OMPs, the organism was grown in tryptone soya broth at 37°C for 40 h, allowing the cells to reach stationary phase.

Patients

All CF patients investigated in this study were adults with chronic *P. aeruginosa* infection. Sera from age-matched, healthy individuals were used as controls.

Adhesion model system

The method used was similar to that described previously.^{15,16} Briefly, buccal epithelial cells from healthy volunteers were coated on to microtitration plates and incubated overnight at 37°C to dry. After fixing by treatment with glutaraldehyde 0.25% for 10 min at room temperature (RT), the cell monolayers were incubated with bovine serum albumin (BSA) 1% in phosphate-buffered saline (PBS) for 1 h at RT to block non-specific adherence. *P. aeruginosa* suspensions, pre-incubated for 1 h at 37°C with PBS or with IgG preparations from CF patients or controls, were then added to the buccal epithelial cell-coated wells for 1 h at 37°C. After fixing with glutaraldehyde 0.25%, and blocking with BSA 1%, bound bacteria were detected immunologically by the addition of a *P. aeruginosa*-specific monoclonal antibody (Serotec, Kidlington, Oxford) and incubation for 1 h at RT. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako, High Wycombe, Bucks) was added to the wells for 1 h at RT, followed by ortho-phenylene diamine substrate. The absorbance of each well at 450 nm was read after exactly 30 min with a Microplate Reader (Bio-tek, Winoski, VT, USA).

IgG isolation

The IgG fractions of CF and control sera were separated by Protein G-Sepharose (Pharmacia, Uppsala, Sweden) chromatography. The serum samples were applied to columns containing 1 ml of the gel, washed with 0.02 M sodium phosphate buffer, pH 7.8, and eluted with 0.1 M glycine, pH 2.8.

OMP isolation

Membranes were prepared essentially according to the method of Hancock and Nikaido.¹⁷ *P. aeruginosa* cultures were harvested by centrifugation at 12000 *g* for 15 min after growth under the conditions described above. After washing with 30 mM Tris-HCl buffer, pH 8.0, the combined cell pellets were resuspended in 25 ml of sucrose 20% in 30 mM Tris-HCl buffer, pH 8.0, containing 1 mg each of pancreatic DNAase and RNAase. The suspension was maintained at 4 °C for all subsequent steps. The cells were ruptured by sonication and hen egg lysozyme 2 mg was added to the mixture, followed, after 10 min, by 100 µM phenylmethylsulphonylfluoride. After removal of cell debris by centrifugation at 10000 *g* for 10 min, the supernate was decanted and diluted with 14 ml of the above Tris buffer. Approximate 7-ml volumes of the suspension were layered carefully on to a sucrose step gradient composed of a lower step of 3 ml of sucrose 70% w/v and an upper step of 18 ml of sucrose 15% w/v, both in the Tris buffer. After centrifugation at 183000 *g* for 1 h at 4 °C, the bottom 4-ml amounts were removed from each tube and pooled. A 3-ml

Fig. 1. SDS-PAGE profile of silver-stained OMPs of *P. aeruginosa*. Lane 1, molecular mass standards; 2, OMP fraction—major identified protein bands are listed on the right hand side; 3, an immunoblot developed with anti-protein F and anti-protein H2 MAbs, confirming identification of these components.

volume of this membrane fraction was layered on to a second sucrose density step gradient. The bottom step consisted of 4 ml of sucrose 70% w/v and successive upper steps of 7 ml each of sucrose 64%, 58%, 52% w/v, all in the Tris buffer. After centrifugation at 183000 *g* overnight at 4 °C, four bands were observed. Each was removed, dialysed against distilled water for 24 h, and concentrated by lyophilisation. The bottom two fractions contained the OMPs and the upper two contained mixed outer and inner membranes and purified inner membranes, respectively.

SDS-PAGE and Western blotting

OMPs were separated by SDS-PAGE by the method of Laemmli¹⁸ with a resolving gel 12.5% and stacking gel 3.0% in an Atto (Atto Corporation, Tokyo, Japan) mini-gel system. Samples were boiled for 10 min in a β -mercaptoethanol-containing sample buffer before application to the gel at a concentration of 5 µg of protein/well. Electrophoresis was at 25 mA and 150 V. An SDS-7 mol. wt marker kit (Sigma) was used for molecular mass estimations. This contained α -lactalbumin (14200), soybean trypsin inhibitor (20100), trypsinogen (24000), carbonic anhydrase (29000), glyceraldehyde-3-phosphate dehydrogenase (36000), egg albumin (45000) and bovine serum albumin (66000). Gels were either stained with silver,¹⁹ or electroblotted on to nitrocellulose according to the method of Towbin *et al.*²⁰ In the latter case, protein transfer to nitrocellulose was completed after 2 h in a

Fig. 2. Immunoblots demonstrating the reaction of IgG subclass antibodies from CF patients with OMPS of *P. aeruginosa*. Representative immunoblots from patients whose sera strongly inhibited adhesion of *P. aeruginosa* to buccal epithelial cells (nos. 1, 2, 3, 7) are shown in panel I and those from patients whose sera weakly inhibited adhesion (nos. 9, 10) are shown in panel II. Strips a, b, c and d illustrate the IgG1, IgG2, IgG3 and IgG4 subclass responses, respectively. The major protein bands are listed on the left hand side.

Pharmacia LKB Novablot semi-dry blotting box at a constant current of 250 mA. Visualisation of the proteins on the nitrocellulose was with reversible Ponsceau S stain. The nitrocellulose blots were cut into individual strips, each representing one lane from the original gel, and incubated with 0.1 M PBS, containing Tween 20 0.05%, (PBS-T), to block non-specific binding. PBS-T was used as diluent in all subsequent steps, which were performed at RT with gentle shaking. The IgG fractions from CF or control sera (0.7 mg/ml) were incubated with the strips for 1 h, after which the strips were washed in PBS, and incubated with either anti-IgG1 (Clone HP6001; Unipath, Basingstoke, Hants.), anti-IgG2 (Clone HP6014; Binding Site, Birmingham), anti-IgG3 (Clone HP6095; Janssen, Beerse, Belgium) or anti-IgG4 (Clone SK-44; Sigma) monoclonal antibodies (MAbs) for 1 h. Subsequently, the blots were washed

in PBS and incubated for 1 h with HRP-conjugated rabbit anti-mouse IgG (Dako). Protein-antibody interactions were visualised with 3-amino-9-ethyl-carbazole (AEC) substrate. OMPS F and H2 were identified with specific MAbs for these proteins, kindly donated by R. E. W. Hancock.

Results

OMP profiles of *P. aeruginosa*

The OMPS of *P. aeruginosa* were isolated by sucrose density centrifugation and separated by SDS-PAGE. The bands representing the major OMPS were assigned as D2 (45 kDa), E (42 kDa), F (40 kDa), G (24 kDa), H2 (19.5 kDa) and I (13 kDa) (fig. 1). The most prominent was protein F. Additional unidentified

bands were visualised by silver staining. The identification of proteins F and H2 was confirmed with specific MABs (fig. 1).

IgG subclass response to OMPs

The IgG subclass response of the CF patients and controls to the individual OMPs was investigated by immunoblotting. Representative blots, demonstrating the diversity in response observed amongst the CF patients are presented in fig. 2.

Panel I of this figure shows results for patients whose IgG fractions strongly inhibited adhesion of *P. aeruginosa* to buccal epithelial cells. IgG1 antibodies from each of these patients reacted with proteins F, H2 and I. A weak IgG1 antibody response to protein G was also observed, and a 29-kDa protein elicited an IgG1 response in two of the patients. The IgG1 response of Patient 2 was very weak, giving only faint reactions with proteins D2, E and F. The IgG2 response amongst these patients was predominantly directed towards the proteins putatively identified as D2 and E, and varied in intensity amongst the individual patients. In Patients 2, 3 and 7, a weak IgG response towards protein G was observed and a very faint reaction with protein F was observed for each of the CF patients. IgG3 antibody responses varied quite substantially amongst these patients, ranging from very weak in Patients 2 and 3, to a strong response to proteins D2, E, G and I in Patient 1. The strong reaction with proteins G and I was also observed with material from Patient 7 and a distinct 29-kDa band was visualised only with this fraction from Patient 1. Each of the patients whose IgG fractions strongly inhibited adhesion of *P. aeruginosa*, demonstrated a strong IgG4 antibody response to protein H2 but gave a weak IgG4 response to proteins D2 and E. The anti-protein F IgG4 response was strong in Patients 3 and 7, but weak in Patients 1 and 2.

Representative blots from two of the four CF patients whose IgG fractions had little ability to inhibit adhesion of *P. aeruginosa* to epithelial cells are presented in panel II of fig. 2. Heterogeneity of antibody response was also evident amongst these patients. IgG1 anti-F, -H2 and -I antibody responses were evident in each patient although those to the former two proteins were weak in Patient 10. An IgG1 response to protein G was also seen in Patient 10. IgG2 antibodies were directed primarily towards proteins D2 and E but those from Patient 10 also showed a very faint reaction with proteins F and G. The IgG3 antibody response varied considerably amongst the patients. Notably, Patient 10 had a strong IgG3 reaction towards proteins D2, E and G and an unidentified 55-kDa protein. The IgG4 response was quite uniform amongst these patients, being primarily directed towards proteins F and H2, with only weak reactions to proteins D2, E and G.

IgG preparations from the control sera did not react

with any of the OMP bands, except that a weak reaction was observed in four controls with proteins D2 and E.

Discussion

The IgG subclass response to individual OMPs of *P. aeruginosa* has not been characterised previously. The heterogeneity of the subclass response is striking but is not so surprising, perhaps, when one considers the complex and variable immune response of these patients.

The silver staining method used to visualise OMPs detected bands other than the major OMPs; nevertheless, the IgG subclass antibodies were observed to interact only with the major OMPs. The IgG1 and IgG4 responses were directed towards similar proteins, namely protein F and the lipoprotein H2 in most patients, although with varying intensity. This observation is consistent with previous reports of the highly-conserved and highly-antigenic nature of these proteins.^{8,10,21} Although each of the CF patients may have been infected with a different strain of *P. aeruginosa*, each manufactured antibodies that reacted with OMPs F and H2 of the isolate used here. Proteins F and H2 are thought to be surface exposed *in vivo*,²² a fact that may contribute to their strong antigenicity. Lipoprotein I also evoked an IgG1 response in each patient. Some sort of antibody response would have been expected here, as OMP I of *P. aeruginosa* is recognised as a vaccine candidate, and was found to cross-react with antisera raised against all serotype strains in an international antigenic typing scheme.¹²

The strong IgG2 and, in some cases, IgG4 responses to the protein bands at 42 and 46 kDa have not been commented upon previously. These bands appear to represent proteins D2 and E, based on molecular mass data. Molecular masses from 45 kDa to 53 kDa have also been reported for *P. aeruginosa* H-antigen flagellins²³ but the presence of flagellar protein is unlikely because of the exhaustive isolation protocol. Where detected in immunoblots, these bands always appeared as a doublet, with none of the patients having an antibody response to only one component of the pair. It is possible that the two proteins detected here may not be two distinct species, but that the one of lower molecular mass may have been a degradation product or oxidised form of the other protein.

The IgG2 response of the panel of patients studied was generally limited to proteins D2 and E. This restricted response might be due to the reputed weakly stimulatory nature of protein antigens for IgG2 antibodies.^{24,25} However, there is a lack of consensus regarding the nature of the antigenic stimulation required for IgG2 antibody production, with some authors suggesting that the IgG2 response is partially restricted to polysaccharide antigens,²⁶ whereas others have variation in response to these molecular species.^{25,27}

The IgG3 subclass response to OMPS demonstrated the greatest variation amongst patients. Low or undetectable levels of IgG3 and IgG2 antibodies have been reported in patients who were chronically exposed to protein antigens,²⁵ and this may account for the lack of IgG3 antibodies in some patients studied.

The immune response of one patient (no. 2) was limited primarily to an anti-protein H2 IgG4 and an anti-protein D2 and E IgG2. This patient also had low total levels of *P. aeruginosa*-specific subclass antibodies,¹⁵ nevertheless this individual's serum strongly inhibited adhesion of *P. aeruginosa* to buccal epithelial cells. This implies that the low levels of pseudomonas-specific IgG antibodies present in this patient may be directed towards some moiety which is involved in adhesion, but not one of the OMPS.

IgG antibodies from the sera of CF patients inhibit adherence of *P. aeruginosa* to buccal epithelial cells.^{15,16} However, the IgG subclass response to the OMPS of *P. aeruginosa* does not appear to influence the level of inhibition obtained, as these antibodies were detected both in sera that strongly inhibited the adhesion of *P. aeruginosa* and in sera that had little effect on the adhesion of the organism. An immune response to OMPS F and I would appear to provide some degree of protection against subsequent challenge, as has been shown in murine vaccination studies,²⁷ but this protection does not seem to involve an anti-adhesion role. Bacterial adhesion to epithelial cells is a complex process involving many components. The outer-membrane components investigated here might play some role in a multifactorial adhesion system.

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