Inhibition of the adherence of *Pseudomonas aeruginosa* to epithelial cells by IgG subclass antibodies

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**Summary.** A beneficial role of the antibody response to *Pseudomonas aeruginosa* seen in cystic fibrosis (CF) patients has not been established. We investigated a possible role for these antibodies as inhibitors of the adherence of *P. aeruginosa* to mammalian cells. An adhesion model system was used, employing buccal epithelial cells in an enzyme-labelled immunoperoxidase procedure on microtitre plates. Total levels of IgG, IgA, IgM and the four IgG subclasses were estimated in 11 CF patients and 10 healthy controls. Most of the CF patients demonstrated increased levels of all these immunoglobulin types. Sera from seven patients with elevated serum IgG were observed to cause greater inhibition of the adherence of *P. aeruginosa* to buccal cells than were the sera from four CF patients with low serum IgG and from ten healthy controls. Nevertheless, the levels of individual anti-*P. aeruginosa* IgG subclass antibodies varied amongst the patients and did not correlate with the degree of inhibition of bacterial adherence. Negative affinity chromatography was used to obtain antibody fractions enriched for IgG1, IgG2 or IgG4 and protein A-Sepharose chromatography was used to isolate IgG3 antibodies from CF patients. The IgG1-, IgG2-, or IgG4-enriched fractions similarly inhibited the adherence of *P. aeruginosa* in the test system, whereas three of five IgG3-enriched fractions from CF patients had no greater effect on adhesion than did IgG from control individuals.

**Introduction**

Despite aggressive antibiotic therapy, chronic infection with *Pseudomonas aeruginosa* remains the major source of morbidity and mortality amongst patients with cystic fibrosis (CF). Bacterial adherence is a pre-requisite to colonisation in this infection, as in any other, and both the receptors and the adhesins have been studied. Specific disaccharides that are commonly found in mucins1 have been shown to bind *P. aeruginosa*, as have the neutral glycosphingolipids of epithelia.2,3 Binding of *P. aeruginosa* to mucins from CF sputum has been demonstrated recently to be non-specific, contrary to what was once believed.4 The role of pili has been investigated by many groups. Purified pilin and monoclonal antibodies (MAbs) directed against peptide sequences within pilin have both been shown to inhibit adherence of *P. aeruginosa* to human buccal epithelial cells5,6 and a human epithelial cell-binding domain has been identified in the C-terminal region of the pilin protein.7 Nevertheless, free pilin fail to inhibit entirely the adhesion of *P. aeruginosa* to human cells in competition experiments, indicating the participation of other bacterial adhesins in the binding process. Data supporting a role for muco-exopolysaccharide in adherence of mucoid *P. aeruginosa* have been reported8 and recent evidence suggests that exo-enzyme S may be a major adhesin, as it binds to a series of glycosphingolipids with the same specificity as to whole *P. aeruginosa* cells.9 In support of this view, it was noted that addition of free exo-enzyme S and antibodies to exo-enzyme S inhibited adherence of *P. aeruginosa*.9

Although a hyperimmune response is characteristic seen in CF patients, this is ineffective in eliminating pseudomonal infection. Indeed, and paradoxically, high IgG titres to *P. aeruginosa* antigens correlate with worsening pulmonary disease.10 Thus, a beneficial role for the anti-pseudomonal antibody response has not been established. The IgG subclass response to *P. aeruginosa* has formed the basis of many investigations. The relative amounts of the different isotypes have been correlated with pulmonary function and stage of infection,11 deficiency in opsonophagocytosis12,13 and efficacy in immunoprotection.14

This present study describes the use of negative affinity chromatography to obtain enriched fractions of each of the individual IgG subclasses and the subsequent assessment of the ability of each subclass to inhibit adhesion of *P. aeruginosa* to buccal epithelial cells.
Materials and methods

Bacterial strain and growth conditions

A non-mucoid clinical strain of *P. aeruginosa* isolated from a CF patient was used throughout the investigation. This strain has been shown previously to bind strongly to buccal epithelial cells in an adhesion model system. Cultures were maintained on tryptone soya agar at 4°C.

Patients

All CF patients were adults, aged from 16 to 38 years, with chronic *P. aeruginosa* infection. Sera from age-matched healthy individuals were used as controls.

Quantification of immunoglobulin isotypes

Wells in microtitration plates (Dynatech M129B) were coated overnight at 4°C with anti-human IgG, IgA or IgM MAbs (Cappell, Organon Teknika, Westchester, PA, USA), applied as 100 µl amounts at concentrations to 10 mg/L in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Subsequently, the plates were washed with 0.1 M phosphate-buffered saline, pH 7.2, containing Tween 20, 0.05% (PBS-T). Either sera from patients or standard IgG, IgA or IgM preparations (Binding Site, Birmingham), were then added. After incubation for 1 h at room temperature (RT) followed by washing, horseradish peroxidase (HRP)-conjugated anti-human IgG, IgA or IgM MAb (Tago, Burlingame CA, USA) was added to the appropriate wells and the plates were incubated for 1 h at RT. The plates were then washed, after which ortho-phenylene diamine (OPD) substrate was added. After further incubation for exactly 30 min at RT, the absorbance at 450 nm was read with a Microplate Reader (Bio-tek, Winskosi, VT, USA). The concentrations of IgG, IgA and IgM in the sera from patients were estimated from the reference curves constructed from the data obtained with the standard IgG, IgA and IgM preparations.

IgG subclass estimation

An ELISA procedure was developed for the quantification of IgG subclasses. Subclass-specific MAbs as follows were used, at concentrations of 10 mg/L in 0.1 M carbonate-bicarbonate buffer, pH 9.6, to coat the wells of microtitration plates. IgG1 (Clone 6001; Unipath Ltd, Basingstoke, Hants); IgG2 (Clone 6014; Binding Site); IgG3 Clone 6050; Unipath); IgG4 (Clone 6023; Binding Site). After incubation overnight at 4°C, the plates were washed with PBS-T, and non-specific binding was blocked by incubation with bovine serum albumin 1% in PBS-T for 3 h at 37°C. IgG-subclass standard (Binding Site) or sera from patients diluted in PBS-T were then added to the plates, which were incubated for 1 h at 37°C. Subsequently, HRP-conjugated anti-human IgG MAb (Cappell) was added and binding reactions were detected with OPD substrate as described above.

IgG subclass fractionation

Purification and coupling of subclass-specific antibodies. The four MAbs used in the affinity purification of IgG immunoglobulin subclasses were obtained as ascites fluids from Recognition Sciences Ltd, Birmingham, and comprised Clones TM-10 (anti-IgG 2, 3, 4), HP6019 (anti-IgG1, 3, 4), OF3 (anti-IgG1, 2, 3) and HP6025 (anti-IgG4). The method followed for their purification from ascites fluid was essentially that of Persson, with some modifications. Briefly, the preparation samples were dialysed against three changes of 0.02 M Tris-HCl buffer, pH 7.6, before purification of the IgG fraction on a DEAE-Affi Gel Blue column (BioRad, Hemel Hempstead, Herts), with gradient elution at 30 ml/h. The elution buffer was 0.02 M Tris-HCl, pH 7.6, applied as steps containing 0, 20, 60, 100 and 500 mM NaCl. The monoclonal IgG eluted at a NaCl concentration of 60 mM. The appropriate fractions were then concentrated by ultrafiltration (Centricon Units, Amicon Ltd) and washed several times with 0.1 M sodium bicarbonate buffer, pH 8.1, containing 0.5 M NaCl. Each purified antibody was then coupled to CNBr-activated Sepharose 6B (Pharmacia LKB, Milton Keynes, Bucks) according to the manufacturer's instructions. The coupling ratio was c. 7 mg of antibody/ml of gel.

IgG isolation and IgG subclass fractionation. The IgG fractions of patient and control sera were isolated by protein G-Sepharose chromatography. Columns containing 1 ml of the pre-made gel (Pharmacia) were equilibrated with 20 mM sodium phosphate buffer, pH 7.6. Serum samples (1 ml) were then applied. After washing with the equilibration buffer the bound IgG was eluted with 0.1 M glycine, pH 2.8. Fractions were neutralised with 2 M Tris-HCl, pH 8.0, and were used directly in the adhesion model or were purified further by negative affinity chromatography as described below.

For isolation of individual IgG subclasses, columns containing 1 ml of sepharose-linked anti-IgG subclass antibody were prepared as described above, and were equilibrated with 0.02 M Tris-HCl, pH 7.8, containing BSA 0.01% 0.5 M NaCl and sodium azide 0.01%. Patient or control serum IgG fractions were applied, and the unbound subclass of interest was eluted with the equilibration buffer. The purifications of IgG1 and IgG4 were achieved in single-step procedures. A column substituted with anti-IgG4 from Clone MP6025 (anti-IgG4) was incorporated as a preliminary step in the IgG2 procedure, as large amounts of contaminating IgG4 otherwise remained. Yields from negative affinity columns tended to be quite low (20-40%) but the extent of enrichment was high, with > 90% purity attained as assessed by ELISA.
IgG3 fractions were obtained with a protein A-Sepharose column which had poor affinity for IgG3. The IgG preparation was applied and the unbound IgG3 was collected with 20 mM sodium phosphate buffer, pH 7.8, as the eluent. The column was washed with 0.1 M glycine, pH 2.8, and then re-equilibrated. The yields were low (20%) but over 80% purity was attained.

Adhesion model system

Buccal epithelial cells were collected on sterile cotton swabs from healthy adult volunteers and suspended in phosphate-buffered saline (PBS). The cells were pooled, washed five times (1000 g, 5 mins), and resuspended in PBS at a concentration of approximately 10^3/ml. Samples (100 µl) of this suspension were added to a microtitration plate, left for 10 min at RT, and then centrifuged at 1000 g for 10 min. The supernates were aspirated carefully with a multichannel pipette and the remaining monolayer of epithelial cells was incubated overnight at 37°C to dry, then fixed by treatment with glutaraldehyde 0.25% for 10 min. After this and all subsequent steps, the wells were washed three times with PBS. The epithelial cells were treated with BSA solution 1% for 1 h at RT to block non-specific binding of bacteria. *P. aeruginosa* suspensions (100 µl of 10^9 cfu/ml) were then added to the buccal epithelial cell-coated wells and incubated for 1 h at 37°C. After washing, the bound cells were fixed by treatment with glutaraldehyde 0.25% for 10 min at room temperature and blocked with BSA 1% for 1 h at RT. Bound bacteria were detected immunologically with a pseudomonas-specific MAb (Serotec, Kidlington, Oxford) which was used at a 1 in 10000 dilution in PBS and allowed to react for 1 h at RT. HRP-conjugated rabbit anti-mouse IgG (Dako, High Wycombe, Bucks) was used for detection of the bound complexes, with OPD as substrate. The absorbance at 450 nm was measured after 30 min at RT with a Biotek Microplate reader. All samples were assayed in triplicate.

To assess the ability of antibodies to inhibit adhesion, *P. aeruginosa* suspensions were incubated for 1 h at 37°C with the appropriate IgG or IgG subclass fractions at a protein concentration of 0.7 mg/ml. The suspensions then were washed once in PBS at 10000 g for 5 min, resuspended in PBS and added to the wells coated with buccal epithelial cells. Adhesion of the bacteria to the buccal epithelial cells was then assayed as described above. The percentage inhibition of adhesion was defined as:

\[
\left(1 - \frac{A_{\text{ads, IgG-treated sample}}}{A_{\text{ads, control}}} \right) \times 100
\]

Estimation of *P. aeruginosa*-specific IgG subclass levels

The *P. aeruginosa* suspensions used in the adhesion assays were coated directly onto microtitration plates in 100-µl amounts and incubated for 1 h at 37°C. After fixing and blocking, as in the adhesion assay (above), a panel of subclass specific MAbs at "equipotent dilutions" was used to assess the amount of each subclass bound to the *P. aeruginosa* cells.
“Equipotent” dilutions were those that yielded comparable absorbances in binding to solid-phase-adsorbed homologous purified human IgG subclass myelomas and were: 1 in 1000 for anti-IgGl; 1 in 2000 for anti-IgG2; 1 in 2000 for anti-IgG3; 1 in 5000 for anti-IgG4. The antibodies used were Clones HP6001 (Unipath; anti-IgGl), HP6014 (Binding Site; anti-IgG2), HP6010 (Unipath; anti-IgG3) and SK-44 (Sigma; anti-IgG4). Two other IgG3-specific MAbs, Clones HP6095 (Janssen, Beerse, Belgium) and HP6050 (Binding Site) were also used to confirm levels of P. aeruginosa-specific antibody. After incubation with these antibodies for 1 h at 37°C, the plates were washed, and HRP-conjugated rabbit anti-mouse IgG antibody (Dako) was added. After incubation for a further 1 h at RT, the plates were washed, OPD substrate was added and absorbance was recorded at 450 nm.

Arbitrary ELISA units were assigned in order to compare the relative responses within a set of data thus:

\[ \text{ELISA unit} = A_{450} \text{(sample)} \times 10 \]

These units we used only to compare data within one assay and not to compare data obtained from different assays.

**Statistical analysis**

Data from the immunoglobulin isotype studies were analysed by the Mann-Whitney U test, with levels of significance set at $p < 0.05$.

**Results**

**Immunoglobulin isotype levels**

To establish whether the hyperimmune response usually observed in CF patients was evident in the
INHIBITION OF P. AERUGINOSA ADHESION BY IgG ANTIBODY

Inhibition of adhesion by purified serum IgG

IgG was isolated from CF and control sera, and incubated with P. aeruginosa cells, and the adherence of the bacteria to buccal epithelial cells was then measured. The extent of inhibition of adherence obtained with IgG from the 11 CF patients and the 10 healthy controls is shown in fig. 3. Following incubation of P. aeruginosa with the IgG fractions, the levels of anti-P. aeruginosa IgG subclass antibodies were estimated with subclass-specific MAbs. High degrees of inhibition of adherence (30–50%) were observed with IgG from seven of the CF patients. IgG from the other four CF patients demonstrated lower inhibition levels, similar to those observed with two of the control samples. The remaining control samples gave little inhibition of pseudomonal adherence.

Estimation of P. aeruginosa-specific subclass antibodies identified major variation amongst patients. The levels of P. aeruginosa-specific IgG1, IgG2 and

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Fig. 3. Inhibition of adherence of P. aeruginosa to buccal epithelial cells observed after incubation with IgG from each of (a) 11 CF patients and (b) 10 healthy controls: ●; LH scale. The estimates of P. aeruginosa-specific subclass antibody (in ELISA units) are also shown: IgG1 □, IgG2 ▼, IgG3 △ and IgG4 ○; RH scale.

present study population, the total concentrations of IgG, IgA and IgM in the CF patients and controls were estimated (fig. 1a, b and c). The levels of IgG and IgM were significantly higher in the CF group than in the control group (p < 0.05) and the difference in IgA concentrations was even more marked (p < 0.01). The concentration of each IgG subclass was estimated in patients and controls (fig. 2). The subclass most significantly elevated in the CF population was IgG3 (p < 0.001) but levels of IgG4 were also significantly raised (p < 0.01) as were those of IgG1 (p < 0.05). The IgG2 concentrations did not differ significantly (p > 0.05) between the CF and control groups.
IgG4 antibodies also varied from patient to patient, with some whose samples strongly inhibited the binding of _P. aeruginosa_ yielding low levels of _P. aeruginosa_-specific antibodies (e.g., CF patient 2) whereas others whose samples had little effect on the binding of _P. aeruginosa_ to epithelial cells had high levels of specific antibody (e.g., CF patient 9). Thus, there was no obvious association between the inhibition of bacterial adherence and the levels of _P. aeruginosa_-specific IgG subclass antibodies. Very low levels of _P. aeruginosa_-specific subclass antibodies were detected in all of the healthy controls. The levels of anti- _P. aeruginosa_ IgG3 were uniformly low in both patient and control samples, as confirmed with the three different IgG3-specific MAb5s tested.

**Comparison of IgG subclasses in inhibition of adhesion**

Fractions enriched for each of the four IgG subclasses were obtained by negative affinity chromatography and used in the adhesion model system. Similar degrees of inhibition, in the range of 2–3 inhibition units, were obtained with the fractions enriched for IgG1, IgG2 or IgG4 from all five CF patients (fig. 4). The IgG3-enriched fractions from two patients inhibited the adhesion of _P. aeruginosa_, whereas those from the other three were much less active.

**Discussion**

As a preliminary to this study it was appropriate to establish the overall immunoglobulin profile of the CF and control populations and, in particular, the IgG subclass levels, so as to eliminate the possibility that any variation in specific anti- _P. aeruginosa_ antibody levels was attributable merely to a variation in the overall immune response. The CF patients investigated in this study demonstrated the variation in immune responses commonly observed in such populations, with some having markedly elevated serum immunoglobulin levels. IgG3 was particularly elevated and levels of IgG4 were also raised compared to controls. Much previous attention has been focused on the relevance of the isotypic variation in IgG subclass response to _P. aeruginosa_ infection in CF. It has been suggested that elevated IgG2, a subclass with a low affinity for Fc receptors on pulmonary macrophages, could reduce clearance of _P. aeruginosa_ via impaired opsonophagocytosis. This theory is supported by the reported correlation between elevated serum IgG2 and decreased lung function. Although the IgG2 levels found in this study did not differ significantly between the CF and control groups, the sample size was small.

We showed that IgG from the sera of patients with CF inhibited the adherence of _P. aeruginosa_ to buccal epithelial cells. Nevertheless, low degrees of inhibition were observed for some CF sera that contained high levels of anti- _P. aeruginosa_ antibodies. The immunological experience with pseudomonal infection in CF is that patients become hyperimmune to a wide spectrum of microbial antigens, including those implicated in adhesion, e.g., flagella, mucopolysaccharide and pili. These antibodies are responsible for the ability of the IgG fraction from CF patients to inhibit adhesion of _P. aeruginosa_. Those patients whose sera had little effect in the adhesion of _P. aeruginosa_ and yet had levels of anti- _P. aeruginosa_ antibodies similar to those of “high inhibition” patients are postulated to have had a poor immune response directed towards the particular _P. aeruginosa_ antigens involved in adhesion. Those patients whose IgG fraction inhibited pseudomonal adherence only weakly were those who had low total concentrations of IgG, IgA or IgM and the IgG subclasses. Continuous steroid treatment in CF is known to be accompanied by a decrease in antibody levels. Even if steroid treatment was responsible for the low total antibody levels measured in the “low inhibition” patients, it still does not explain their failure to inhibit adhesion, as the concentration of IgG used in the adhesion assay was the same for each patient and the levels of _P. aeruginosa_-specific subclass antibodies were similar for both “high” and “low” inhibition groups.

The levels of _P. aeruginosa_-specific subclass antibodies recorded were quite diverse within the group of CF patients. It has been suggested previously that the IgG2 response to microbial polysaccharide antigens is defective in CF patients but this was not apparent in the present study. High levels of LPS-specific IgG4 antibodies have been postulated to inhibit optimal pulmonary clearance of _P. aeruginosa_. The levels of anti- _P. aeruginosa_ IgG4 recorded in this study varied amongst patients but generally were high and may have actually been detrimental rather than beneficial to the patients. Levels of anti- _P. aeruginosa_ IgG3 were
very low compared to the other subclasses, suggesting a poor IgG3 response to \textit{P. aeruginosa}, or at least to the antigens exposed in the adhesion assay. Chronic lung disease has been reported to be associated with patient groups who have combined serum deficiencies for IgG1 and IgG3.\textsuperscript{25} Other reports have associated high anti-\textit{P. aeruginosa} IgG3 titres with poor prognosis.\textsuperscript{24} These previous studies\textsuperscript{24} used a standard \textit{P. aeruginosa} antigen preparation and a different IgG3-specific MAb, and so are not directly comparable with our data. In the present study, we used whole \textit{P. aeruginosa} cells, as the emphasis was on investigating whether the nature of the IgG subclass antibodies bound to the bacteria could be correlated to the extent of inhibition of bacterial adhesion to buccal epithelial cells.

When fractions enriched for IgG1, IgG2 or IgG4 were applied in the adhesion assay, similar levels of inhibition of bacterial adherence were obtained. The enriched fractions were isolated, were chosen because the inhibition of bacterial adherence were obtained. The bound to the bacteria could be correlated to the extent of inhibition of bacterial adhesion to buccal epithelial cells.

When fractions enriched for IgG1, IgG2 or IgG4 were applied in the adhesion assay, similar levels of inhibition of bacterial adherence were obtained. The five CF patients, from whose sera the subclass-enriched fractions were isolated, were chosen because of the high degree of inhibition of pseudomonal adhesion observed with their IgG antibodies. If, as has been suggested,\textsuperscript{25,26} particular immunoglobulin subclasses are produced in response to the protein or polysaccharide nature of the inducing antigens, it is conceivable that individual subclasses might inhibit adherence to a homogeneous adhesin in an adhesion system. Because each of the three subclasses was capable of inhibiting adherence when used alone, albeit at a lower level than that obtained with a combination, it would suggest that the adhesin was not exclusively protein or polysaccharide in nature, or more likely, that more than one adhesin was involved. Components implicated, in the literature, as playing a part in adhesion are diverse, ranging from pili\textsuperscript{27,28} to lectins\textsuperscript{29} to muco-exopolysaccharide\textsuperscript{3} to exo-enzyme S.\textsuperscript{9,30} The low inhibition of adherence obtained with IgG3 preparations suggests that the surface components that elicit the IgG3 response are not involved in adhesion.

It is generally accepted that the initial contact between patient and \textit{P. aeruginosa} occurs in the upper respiratory tract, with colonisation by the non-mucoid form of the organism. Prevention of colonisation by this phenotype is probable more dependent on protection against adhesion of pili or outer-membrane adhesins than muco-exopolysaccharide. It is at this pre- or early stage of infection that anti-adhesion antibodies might contribute to minimising \textit{P. aeruginosa} infection. Unfortunately, the anti-adhesion antibodies investigated here were isolated from patients with chronic infection and thus were probably not produced at a sufficiently early stage of infection to combat the huge burden facing the immune system in CF.

References

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