Quantitative analysis of immunoglobulin G subclass responses to *Pseudomonas aeruginosa* antigens in cystic fibrosis

E. LIKAVCANOVA and J. LAGACÉ

Department of Microbiology and Immunology, Faculty of Medicine, University of Montreal, PO Box 6128, Station A, Montreal, Quebec, Canada H3C 3J7

Summary. The four subclasses of IgG have different structures, functions and implications in the antibody response. IgG subclass reactions to individual *Pseudomonas aeruginosa* structural antigens in 22 adolescents and young adults with cystic fibrosis (CF) were studied qualitatively and quantitatively by densitometric analysis of Western blot assays. These patients had been infected by *P. aeruginosa* for 7 years or longer and were divided into two groups according to their pulmonary status: Group 1 comprised 11 patients with relatively good pulmonary status; Group 2 consisted of 11 patients with poor pulmonary status. There was a relative decrease of IgG1 and a relative increase of IgG2 and, especially, of IgG3 and IgG4 antibodies against *P. aeruginosa* antigens in the CF patients. Comparison of the two CF patient groups showed a significant increase in the proportion of IgG3 in the Group 2 patients. This could be a potential cause or effect in the deterioration of their pulmonary function. Densitometric analysis of Western blots revealed more than 24 *P. aeruginosa* antigens and indicated those that were the targets of the isotype antibody response(s) that were apparently most harmful. Thus, there was a significant increase of IgG2 or IgG3 reactivity (or of both) against proteins F, H (H1 and H2), and I in the Group 2 patients. One other striking observation of this study was the high reactivity of IgG4 antibodies to protein H. IgG4 was the major antibody to this protein in seven of the 11 Group 1 patients compared to two of the 11 in Group 2. We hypothesise that IgG4 antibodies may antagonise IgG2 antibodies, helping to preserve stable pulmonary function.

Introduction

Chronic broncho-pulmonary infections with *Pseudomonas aeruginosa* occur in 70–90% of patients with cystic fibrosis (CF).1,2 Despite intensive antimicrobial treatment, the bacteria are difficult to eradicate and most patients ultimately succumb to respiratory failure. Nevertheless, these infections take a highly variable course in individual patients.

The variability and severity of the clinical features of this infection probably reflect not only its duration and intensity but also the degree and character of the various immunological responses to the bacteria. Generalised immune deficiency does not occur in CF.3,4 Although specific anti-*pseudomonas* antibody titres usually increase progressively during the infection, the bacteria are rarely eliminated and a high humoral anti-*P. aeruginosa* response is associated with a poor prognosis.5,6,7 Many attempts have been made to explain this behaviour in terms of inflammatory reactions secondary to local immune complex formation and the production of blocking antibodies.9–11

In the search for a better understanding of the humoral immunity of CF patients, some studies have examined the subclasses of IgG produced in response to *P. aeruginosa* lipopolysaccharides (LPS), exoenzymes and polysaccharide antigens.12–18 Although the results are not entirely consistent, it has generally been found that IgG2, IgG3 and IgG4 antibodies to these antigens are elevated in the sera of CF patients with chronic *P. aeruginosa* infections. Because different IgG subclasses vary in their ability to promote phagocytosis and to activate complement,19–21 in their protective effect against bacteria20,22–24 and in other immunoregulatory roles,25,26 we hypothesised that the subclass distribution of IgGs against *P. aeruginosa* structural antigens might explain why some patients who have been colonised with *P. aeruginosa* remain clinically stable for long periods whereas others rapidly deteriorate.

In the present study, we attempted to identify IgG subclass responses to *P. aeruginosa* infection in two groups of CF adolescents who had been infected for 7 years or longer by this bacterium, but who varied in their clinical status. To dissect the IgG subclass
Materials and methods

Patient selection and serum collection

Twenty-two CF patients were selected. They were of both sexes, and were followed by the CF Clinic at St Justine’s Hospital. All had been chronically colonised with *P. aeruginosa* for at least 7 years. Most underwent clinical assessment every 3 months. At the time of blood collection for the present study, the patients were clinically scored by the Shwachman system, which is based on a perfect score of 100 points, with a perfect score being a good 71–85; mild 56–70; moderate 41–55; severe ≤40). Chest radiology scores (CRXSc) were determined independently by radiologists following the Brasfield system, with 25 points being a perfect score (good 20; midpoint 15; feeble 10). Blood samples were obtained from these patients at their regular clinic visits; none had acute exacerbation of respiratory symptoms at the time of collections. Blood samples from these patients and from normal volunteers (some of whom were laboratory workers who regularly handled *P. aeruginosa*) were separated at room temperature within 6 h of collection, and the sera were stored in divided small volumes at −70°C until used.

The CF patients were divided into two groups based on the summed values of the Shwachman and Brasfield scores. Group 1 comprised 11 patients (four males, seven females; mean age 16.4, SD 2.3 years, range 13–21) who had a relatively good clinical status with mean summed scores of 99.8, SD 16, raged 119–77). Group 2 comprised seven males and four females (mean age 17.0, SD 24 years, range 12–20) who had poor pulmonary condition, as demonstrated by a mean score of 56.3, SD 14.6, range 75–36. Sera from 24 healthy adolescents and young adults (mean age 19.6, SD 4.6 years, range 14–24) were used to evaluate the normal IgG subclass distribution; sera from 10 of these individuals were also used as controls in Western immunoblot analyses.

P. aeruginosa strains and cultural conditions

In a preliminary study, the cellular composition of 19 isolates of *P. aeruginosa* from the sputum of CF patients, and of ATCC strains 25619 and 27853 was analysed by SDS-PAGE. Identification of the clinical strains was by culture on a selective medium containing phenanthroline (Sigma Chemical Co., St Louis, MO, USA) and 9-chloro-9-(4-diethylamino)phenyl]-9,10-dihydro-10-phenylacrine hydrochloride (C-390, kindly donated by Norwich Eaton Pharmaceuticals, Norwich, NY, USA). SDS-PAGE protein profiles of these strains, and Western blots performed with sera from CF patients were compared to select a representative organism in terms of the number and quality of resolution of the protein bands. *P. aeruginosa* strain ATCC 25619 was selected on this basis and used for subsequent parts of the study. A *P. aeruginosa* mutant that was deficient for protein F was generously provided by R. E. W. Hancock, and was utilised to confirm the identification of this porin in SDS-PAGE profiles.

Whole-cell antigens and outer-membrane preparations

Cultures were grown overnight at 37°C with constant agitation in a medium containing tryptone (Difco) 0.8% w/v, yeast extract (Difco) 0.5% w/v and NaCl 0.5% w/v, harvested by centrifugation for 10 min at 6000 g at 4°C, and then washed once with saline. The bacteria were collected in phosphate-buffered saline (PBS, pH 7.2) and stored at −70°C until used. Outer-membrane proteins were purified with sodium-lauroyl sarcosinate (Sarkosyl), as described by Ward et al.

Monoclonal antibodies against human IgG subclasses

Mouse monoclonal antibodies (MAbs) to human IgG1 (clone SRT5) and IgG2 (clone HP-6014) were purchased from Serotec (Oxford). MAbs to IgG3 (clone HP-6050) and IgG4 (clone HP-6025) were supplied by ICN Biomedicals (Costa Mesa, CA, USA). These IgG MAbs are of World Health Organization reference quality and displayed the desired specificity in immunoassays. We selected them from amongst others that are available after preliminary studies confirmed that each was specific to its respective IgG subclass and did not cross react in ELISA tests with heterologous human myeloma cells producing IgG1 (ICN Biomedicals) or IgG2–IgG4 (Calbiochem Co., La Jolla, CA). This ELISA test also allowed us to determine the appropriate dilution factor for each of the four subclass-specific antibodies so as to give equivalent reactivity.

ELISA was used to confirm that the reactivity of the mouse MAbs to human IgG subclasses in the sera of the 24 normal adolescents and young adults was in general agreement with previous data.

ELISA

Purified outer membranes (OM) and whole cells of *P. aeruginosa* ATCC 25619 were suspended in 0.5 M carbonate buffer, pH 9.6, and adsorbed to the wells of polystyrene plates (Immulon; Dynatech Laboratories, Inc., Chantilly, VA, USA) at a concentration of 3 mg/L (300 ng/well) and processed as described previously with slight modifications. The plates were blocked with PBS containing Tween 20 0.05% v/v and skimmed milk 1% w/v (PBS-M). In preliminary experiments, serum samples were diluted 1 in 100 and 1 in 1000. It was found that these gave identical results and in later experiments, only the 1 in 1000 dilution was used.
used. After incubation for 1 h at 37°C, the wells were washed with Tween-20 0.05% v/v in PBS (PBS-T), then incubated with the MAbs to IgG1, IgG2, IgG3 or IgG4 for 1 h at 37°C. Finally, the plates were developed with goat anti-mouse IgG conjugated to horseradish peroxidase (Dimension Zymed, San Francisco, CA, USA). The colour reaction was allowed to develop for 30 min and then read in a microplate reader (Boehringer ELISA Processor II, Hoechst, Marburg, Germany). The tests were performed in triplicate on three occasions, with completely reproducible results.

**SDS-PAGE and immunoblotting**

The electrophoresis method of Laemmli was used with a separating gel of acrylamide 14%: N,N'-methylene bis acrylamide 0.38% and a stacking gel of acrylamide 4%: N,N'-methylene bis acrylamide 0.11% for SDS-PAGE. Whole bacteria or purified OMs were suspended at a protein concentration of 0.5 mg/ml (as measured with a BioRad protein assay kit) in 62:5 mM Tris-HCl buffer, pH 6.8, containing SDS 2% w/v, β-mercaptoethanol 5% v/v, glycerol 10% v/v and bromophenol blue 0.001% v/v (BioRad Laboratories, Richmond, CA, USA). The samples were heated for 2 min at 95°C and then electrophoresed at 300 V at 9°C for 4 h. The following mol. wt standards (Sigma) were electrophoresed in parallel: bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20000), and bovine milk α-lactalbumin (14000). Subsequently, the gels were either stained with silver, as described by Morrissey, or transferred, by electrophoresis at 50 mA for 17 h at 9°C, to nitrocellulose membranes (NC) (BioRad), by the method of Towbin and Gordon. The quality of protein separation and the transfer efficacy were monitored by staining two strips of NC paper containing reference mol. wt markers with a Biotin-Blot Total Protein Detection Kit (BioRad) by the method recommended by the manufacturer. Elution was monitored by staining the gel with Coomassie Blue to ensure that minimal residue of protein was left.

NC sheets containing the separated antigens were cut vertically into 3 mm × 145 mm strips, which were blocked by incubation for 30 min at 37°C in PBS-M in slotted incubation trays (BioRad) on a slow orbital-shaking platform which was also used for all subsequent steps. The strips were washed with PBS-T and incubated for 60 min at 37°C with a 1 in 100 dilution of patient’s serum in the blocking buffer. They then were washed three times for 5 min each with PBS-T and further incubated with MAbs to IgG1, IgG2, IgG3 or IgG4 for 60 min at 37°C. After incubation, the NC strips were again washed with PBS-T, and then immersed in a solution of [3H]-rabbit anti-mouse IgG antibody (0.25 mCi/L; DuPont Canada, NEN Mississauga, Ontario, Canada) in blocking buffer for a further 60 min at 37°C. Afterwards, the strips were washed three times for 15 min each with PBS-T, then allowed to dry and exposed to Cronex 4 film (DuPont) in an intensifying screen (Kodak) at -70°C for 24 h.

**Densitometry**

Autoradiograms were examined with a high-resolution laser densitometer (LKB 2202-020 UltraScan XL) linked to “Gelscan XL” software (Pharmacia). The trace of absorbance versus distance generated for each strip was corrected for background absorbance by subtracting the tracing given when non-reactive human control serum was treated with the same anti-subclass MAb on the same Western blot. The integrator identified each peak (i.e., antibody bound to individual antigen) by its distance from the origin of the trace and determined the area under each peak.

**Analysis of data**

Data obtained from densitometry of autoradiograms (integrals of absorbance unit* peak width) were collected for each IgG subclass response to each P. aeruginosa antigen. These values were also summed to give a measure of the total specific antibody response. The responses in Group 1 and 2 patients were compared statistically by analysis of variance followed by Student’s t test. Data obtained by ELISA tests were evaluated similarly.

**Results**

**Reproducibility of measurements**

The reproducibility of band intensity depended on the use of standardised reagents and protocols. Equal amounts of proteins were loaded on to each gel. Elution of proteins from the gel and their efficient binding to the immobilising membrane were carefully optimised so that only negligible amounts of protein remained in the resolving gel. Only blots where transfer was very efficient were analysed in this study. The reproducibility was also controlled by treating each blot with the same positive serum. As a further control, ELISA tests were used to confirm the data obtained in Western blots.

The IgG subclass distribution found in the 24 normal control sera (IgG1: 67.6 SD 13.9%; IgG2: 27.3 SD 12.1%; IgG3: 4.3 SD 4.0%; IgG4: 1.1 SD 5.3%) were in conformity with accepted values, further confirming the reliability of our methods.

We assessed the levels of anti-P. aeruginosa IgG1, IgG2, IgG3 and IgG4 antibodies in the sera of 22 CF adolescents and young adults who had been colonised by this bacterium for long periods. Control sera from 10 normal individuals of similar ages were examined in parallel. Whole cells or purified OMs of P. aeruginosa ATCC 25619 were used as the antigen in both the ELISA and Western blots tests. Table I lists the results
Table I. Subclass distribution of anti- \( P. \) aeruginosa IgG antibodies (total summed reactivity) determined by densitometric analysis of Western blot and ELISA for Group 1 and 2 CF patients

<table>
<thead>
<tr>
<th>P. aeruginosa ATCC 25619 antigens and assay</th>
<th>IgG of specified subclass as percentage of total IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>Whole bacteria Western blot</td>
<td>Group 1</td>
</tr>
<tr>
<td>39.5</td>
<td>32.7</td>
</tr>
<tr>
<td>ELISA Purified OM Western blot</td>
<td>40.8</td>
</tr>
<tr>
<td>36.3</td>
<td>35.2</td>
</tr>
<tr>
<td>38.1</td>
<td>33.0</td>
</tr>
</tbody>
</table>

* Student's \( t \) test.
NS: Not significant: \( (p > 0.05) \).

Table II. Levels of IgG subclass antibodies to individual \( P. \) aeruginosa ATCC 25619 antigens in relation to clinical status

<table>
<thead>
<tr>
<th>P. aeruginosa antigens</th>
<th>Mean integral* of antibodies of subclass</th>
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<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>Total reactivity (all bands)</td>
<td>Group 1</td>
</tr>
<tr>
<td>75.5</td>
<td>143.5</td>
</tr>
<tr>
<td>Protein H</td>
<td>94</td>
</tr>
<tr>
<td>Protein F</td>
<td>91</td>
</tr>
<tr>
<td>Protein I</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Integral of optical density and areas under curves drawn following densitometric analysis of Western blots (arbitrary units).
†Student's \( t \) test; NS, not significant.

for each IgG subclass. Compared to the IgG subclass distribution in normal individuals, the concentration of specific anti-\( P. \) aeruginosa IgG1 was relatively low in the sera of CF patients whereas the relative concentrations of IgG2, and especially of IgG3 and IgG4 against \( P. \) aeruginosa antigens were elevated. Statistical comparison of results obtained with sera from Group 1 versus Group 2 patients revealed a significant \( (p < 0.005) \) increase in the percentage of IgG3 in the latter. This difference and its significance were confirmed by ELISA. No measurable immune reactions to \( P. \) aeruginosa antigens were noted by ELISA for the sera of 10 non-colonised controls; only a few faintly reactive bands were observed in Western blots with the serum of one of these individuals.

Densitometric analysis of the immunoblots revealed a significant increase of IgG2 and IgG3 reactivity against protein F \( (p < 0.005 \) and \( p < 0.005) \) and protein H \( (p < 0.001 \) and \( p < 0.01) \), respectively, in the Group 2 patients (table II). The difference between the Group 1 and 2 patients in IgG1 reactivity against proteins H, F and I was not significant (table II). A significant elevation in the level of anti-protein I IgG3 was demonstrated in Group 2 patients \( (p < 0.001) \). Total IgG2 and IgG3 reactivity levels measured from Western blots were significantly higher in Group 2 than in Group 1 patients \( (p < 0.001 \) and \( p < 0.005) \) respectively). These conclusions were supported by the ELISA data \( (p < 0.005) \). Concordant data were obtained when whole bacterial antigens were replaced by OM material (data not shown) but at lower levels of significance \( (p < 0.025 \) and \( p < 0.05 \) for IgG2 and IgG3 respectively).

The serum reactivity against individual \( P. \) aeruginosa antigens is illustrated in fig. 1. Generally, with whole bacterial antigens, strong antibody binding was observed to proteins of approximate mol. wts 36000 (protein F), 20000 (protein H), 56000, 52000 and 14000 (protein I). When OM preparations were used as the antigens, the strongest antibody binding was noted to protein H followed, in rank order, by that to proteins F, I, D, G and E (not shown). Identification of proteins H (H1 and H2) was confirmed with MAbs produced in our laboratory. The identity of protein F was confirmed by comparison with a \( P. \) aeruginosa mutant strain lacking this protein. The identity of the bands was further confirmed from their mol. wts. The protein nomenclature follows that of Hancock and Carey.38

One striking observation was the strong binding of IgG4 antibodies to protein H in most of the sera from CF patients (fig. 1). This proportion generally was higher in Group 1 patients, where the level of anti-protein H IgG4 exceeded 40% of total IgG activity.
Fig. 1. Qualitative assessment of the reaction of IgG1, IgG2, IgG3 and IgG4 anti-\textit{P. aeruginosa} antibodies with whole-cell antigens in sera from six CF patients from Group 1 (nos. 1, 3, 4, 6–8), one patient from Group 2 (no. 5) and two non-colonised controls (CF control no. 2; normal control no. 9). The background reactivity with bacterial antigens can be assessed from results with normal human serum (far right). For each patient, the four tracks, from left to right, show IgG1, IgG2, IgG3 and IgG4 antibody binding, respectively. Approximate mol. wt values are indicated on the right.

Fig. 2. Qualitative assessment of the reaction of IgG1, IgG2, IgG3 and IgG4 anti-\textit{P. aeruginosa} antibodies to whole cell antigens with the sera from three CF patients from Group 1 (nos. 13, 15 and 16), four from Group 2 (nos. 10–12, 14), and two non-colonised controls (CF control no. 17; normal control no. 18). The format is an indicated for fig. 1.

against this protein in four of the 11 cases. Anti-protein H IgG4 never exceeded 40% of total IgG activity against this protein amongst Group 2 patients and IgG4 was the dominant subclass in only two individuals from this group. Finally, the anti-protein H IgG4: IgG2 antibody ratio was significantly higher in Group 1 than Group 2 patients. (p < 0.005).

\textbf{Discussion}

Most CF patients suffer from chronic recurrent pulmonary infection with \textit{P. aeruginosa}. Because of sustained antigenic stimulation, hypergammaglobulinaemia is common. The nature of the patient's immune response may critically determine the clinical prognosis. In an attempt to identify those immune responses that correlate with the preservation of good clinical status, we examined IgG subclass reaction to \textit{P. aeruginosa} antigens in CF patients with either good or poor clinical status following prolonged pseudomonas colonisation. Sera from normal adolescents served as controls.

The antibody responses to \textit{P. aeruginosa} antigens revealed by Western blots are complex, with about 28 antigenic bands being detected. Nevertheless, qualitative and quantitative analysis of the clinical status of CF patients is possible with the development of densitometric reading techniques for the blots. The method demands considerable care. The intensity of the bands in Western blots depends on the consistency of technique, the use of standardised reagents,
and on the antigen concentration. The efficacy of antigen transfer must be controlled. A 100-fold dilution of patient serum was used here to obtain high sensitivity and to ensure antigen excess. The inclusion of a positive control on each blot allowed strict control of the appropriate antigen, MAb and conjugate. Parallel ELISA further demonstrated the reliability of our Western blot data.

It was found that IgG1 was reduced relative to IgG2-IgG4 in both CF patient groups (table I) compared to normal individuals, where the following subclass distribution is reported: IgG1, 60.3–71.5%; IgG2, 19.4–31%; IgG3, 5.0–8.4%; IgG4, 0.7–4.2%. A relative increase of IgG3 and IgG4 antibodies was significant in all CF patients (table I) but only the relative rise in IgG3 antibodies was significantly different between patients of Groups 1 and 2. This increase in IgG3 reactivity could be of potential significance as a cause or effect in the deterioration of pulmonary function in CF. Several other differences in IgG subclass response were noted between the Group 1 and Group 2 patients. Significant increases in IgG2 to proteins H and F were seen in the Group 2 patients as were significant increases in IgG3 to proteins F, H and I. The high levels of anti-P. aeruginosa IgG2 and IgG3 in the Group 2 patients were confirmed against P. aeruginosa whole cell antigens by ELISA and from total reactivity on immunoblots.

Our results thus confirmed, generally, that a high prevalence of IgG antibodies to P. aeruginosa antigens was associated with a poor prognosis in CF,41,42 and the densitometric analysis of Western blots allowed us to identify those antigens that are the targets of the apparently most harmful isotype antibody responses. Thus, elevated IgG2 or IgG3 antibodies, or both, to proteins H and F and, at a lesser degree, protein I were significantly related to poor pulmonary status.

The reactivity of IgG4 antibodies to protein H was striking, particularly in the Group 1 patients in whom IgG4 was the major isotype in 64% of individuals as compared to 18% in Group 2. It was also demonstrated that the anti-protein H IgG4: IgG2 antibody ratio was elevated in Group 1 patients suggesting "blocking" of IgG2 antibodies by IgG4 antibodies. This is not surprising because IgG4 antibodies are exceptional in several respects. IgG4 antibodies to various antigens appear to be functionally monovalent and thus produce small, non-precipitating immune complexes whereas other IgG isotype antibodies cross-link comparable antigens. Moreover, IgG4 cannot fix complement, and so may perhaps reduce complement-mediated pathology.61 Although IgG4 normally occurs at the lowest concentration of any of the IgG subclasses and its normal biological function is not known, IgG4 deficiency has been associated with recurrent pulmonary infections in non-CF patients.62 There is also an association between IgG4 antibodies and allergy and it has been suggested that IgG4 antibodies are pathogenic.63-65 This claim should be considered with caution for many reasons. Some evidence indicates that IgG4 antibodies may modulate IgE-mediated allergic responses,66 possibly by competing with anaphylactic IgE antibodies for antigens67,68 or by acting as anti-IgE antibodies.69 Moreover, IgG4 levels are increased in successful desensitisation treatments for allergic disorders,70 and virtually all beekeepers with more than 3 years experience have an IgG4-restricted response without adverse effects.71 Previous work has shown that IgG4 antibodies become prominent during chronic antigenic stimulation72 whereas rising IgG2 levels characterise chronic infections13-15,18 and IgG1 antibodies often predominate in individuals with limited antigenic stimulation.73 One can speculate that a high level of IgG4 antibodies is a marker of hyperimmunoreactivity; their presence could be harmful or important in suppressing the immunopathology caused by other antibodies, via antagonistic or competitive mechanisms. In the present case, protein H may have a special quality for inducing so strong an IgG4 response. It is known to be a lipoprotein containing covalently-linked fatty acids and it is non-covalently associated with peptidoglycan.55,56

High IgG2 and IgG3 levels were significantly related to poor pulmonary status in the Group 2 patients. IgG2 in these patients was particularly directed against proteins H and F. The biological functions of IgG2 are controversial. The antibody has generally weak complement-fixing activity and binds to monocyte membranes.74-76 However, some authors have demonstrated a strong capacity of human IgG2 monoclonal antibodies to P. aeruginosa LPS to fix complement and to initiate opsonophagocytic killing activity.77 Elevated IgG3 levels may mediate tissue damage primarily in type III immunological reactions.78,79 IgG1 and IgG3 are the most efficient activators of complement and can cross-link antigens; hence they favour immune complex formation with the contingent risk of complement-mediated tissue damage.80 Such a pathogenic picture is consistent with the type of tissue damage observed in CF patients, in whom circulating immune complexes are common.5,7,81

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