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IgG subclass antibodies to *Pseudomonas aeruginosa* in sera from patients with chronic *Ps. aeruginosa* infection investigated by ELISA

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SUMMARY

ELISAs using subclass-specific monoclonal antibodies were developed for the quantification of human IgG1, IgG2, IgG3 and IgG4 antibodies to *Ps. aeruginosa*. We investigated the pattern of IgG subclass antibodies against *Ps. aeruginosa* in serum from patients with cystic fibrosis (CF), other patients with chronic *Ps. aeruginosa* infection, and healthy controls. Healthy controls and patients with CF but without *Ps. aeruginosa* infection showed no or very low titres of antibodies against *Ps. aeruginosa*. In the early stage of chronic *Ps. aeruginosa* infection, antibody titres in all four subclasses were significantly higher than either normals or CF patients without infection. Other patients with *Ps. aeruginosa* infection showed the same increased level of IgG subclass antibodies as CF patients in an early stage of infection. Sixteen patients (eight in good and eight in poor clinical condition) have been followed for an average of 13 years with multiple serum samples covering the pre-infection, early and late stages of chronic infection. Patients in a poor clinical condition showed significantly higher levels of IgG3 antibodies in the first year of infection and 2 years later also had significantly higher IgG2 antibody levels. We conclude that elevated levels of IgG2 and IgG3 antibodies to *Ps. aeruginosa* are a sign of poor prognosis in CF.

Keywords IgG subclass antibodies *Pseudomonas aeruginosa* cystic fibrosis

INTRODUCTION

Chronic *Ps. aeruginosa* lung infection is the leading cause of death in patients with cystic fibrosis (CF) (Wood, Boat & Doershuk, 1976). Many CF patients acquire a chronic bronchopulmonary infection with *Ps. aeruginosa* in the first decade of life (Høiby, 1974b) and despite intensive anti-microbial treatment this infection cannot be eradicated (Szaff, Høiby & Flensborg, 1983). Specific antibodies against *Ps. aeruginosa* antigens rise when the infection becomes chronic and a poor prognosis has been shown to correlate with a high number of anti-pseudomonas precipitins (Høiby, 1974a, 1977). It seems that the progressive pulmonary damage characteristic of this infection is a result of inflammatory reactions secondary to local immune complex formation (Høiby & Olling, 1977; Schiøtz *et al.*, 1979; Schiøtz, 1981). From a clinical point of view, the chronic *Ps. aeruginosa* infection runs a highly variable course in individual patients (Corey, Levison & Crozier, 1976). Some patients remain in good condition with only slight deterioration in pulmonary function over a period of many years, while other patients display a rapid downhill course with progressive pulmonary destruction.

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IgG subclass antibodies vary in their ability to promote phagocytosis and to activate complement (Schumaker *et al.*, 1976; Alexander *et al.*, 1978; Perelmuter, 1983). We have hypothesized that an imbalance of IgG subclass antibodies may explain individual differences in the clinical course of this chronic disease and we have in fact previously found an inverse correlation between the level of both total IgG2 and total IgG3 immunoglobulins and lung function in patients with CF (Pressler *et al.*, 1988).

Here we have investigated the pattern of appearance of specific IgG subclass antibodies to *Ps. aeruginosa* in CF and other patients with chronic *Ps. aeruginosa* infection. For this purpose we developed a specific method for quantitative determination of IgG subclass antibodies to *Ps. aeruginosa* in human sera by means of ELISA using monoclonal antibodies to human IgG subclasses.

MATERIALS AND METHODS

Patients and healthy subjects

Healthy subjects. In order to establish normal values for the assays, sera from 97 healthy controls with no history of *Ps. aeruginosa* infection were investigated. Median age (range) for 24 children was 12 years (1–16 years). For 73 adults it was 27 years (18–53 years).

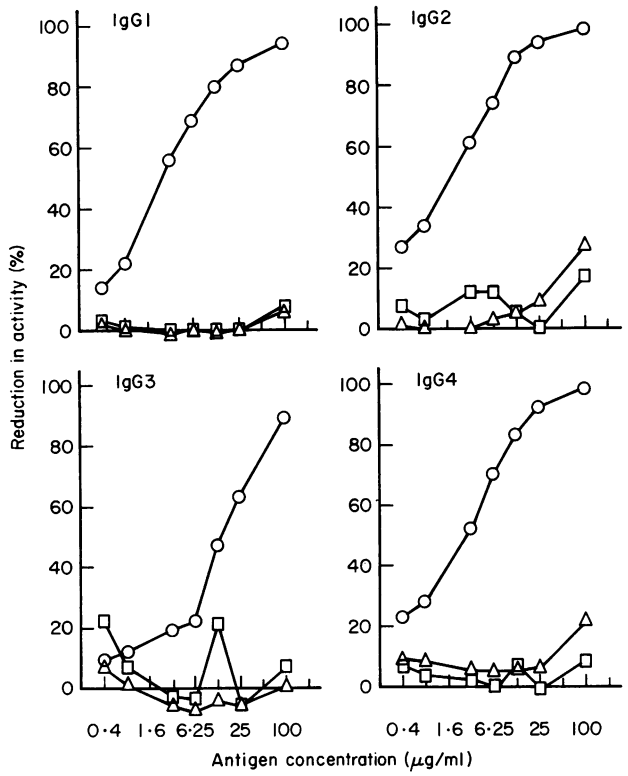

Fig. 1. Antigenic specificity of the assay expressed as reduction of activity after absorption of the standard serum with increasing amounts of antigen preparation of St-Ag:1-17 (○), *S. aureus* (△), and *H. influenzae* (□).

CF patients. Diagnosis of CF was established on the basis of abnormal sweat electrolytes and typical clinical features. All patients were controlled at the Danish CF Centre and were seen on a regular monthly basis. At each visit the clinical condition, pulmonary function and sputum bacteriology were examined and have thus been recorded prospectively since 1970. *Ps. aeruginosa* infection was regarded as chronic if the organism had been cultured from sputum at monthly intervals for 6 months. Pulmonary function was evaluated using an electronic spirometer (Spirotron, Dräger). All values were expressed as a percentage of expected values according to height and sex (Polgar & Promadhat, 1971; Quanjer, Dalhuisen & van Zomeren, 1983). The individual values in this study were the mean of all results in 1 year within the observation period. Decreased lung function was defined here as less than 70% of the expected values for either forced vital capacity (FVC) or forced expiratory volume in first second (FEV1).

Serum samples were taken at intervals of 3–4 months. All serum samples were stored at −20°C until analysis.

For the purpose of this study, groups of serum samples with the following characteristics were assayed: (i) cross-sectional study: a random group of 123 patients with CF were investigated. This group included 37 without (CF−P) and 86 with chronic *Ps. aeruginosa* infection (CF+P). The duration of chronic infection ranged from 0·4 to 15 years (median 6 years). The age of the patients was evenly distributed in the range 0·8–32 years (median 12 years). All serum samples used in this part of the study were collected during a period of 1 month. Data on pulmonary function were obtained from 64 patients with chronic *Ps. aeruginosa* who were old enough to cooperate; and (ii) longitudinal study: multiple serum samples from sixteen CF patients were obtained at intervals of 6 months–1 year. The samples covered the pre-infection period and the early and late phase of chronic *Ps. aeruginosa* infection. Ten to 14 serum samples were assayed for each of the patients, covering a period of 8 to 16 years (mean 13 years). Eight patients were in good clinical condition (Shwachman score >75) (Shwachman & Kulczycki, 1958) and eight were in poor condition (score <50).

Table 1. Distribution of IgG subclass antibodies to *Ps. aeruginosa* in patients with CF and controls

	IgG1	IgG2	IgG3	IgG4
Control children ($n=24$)				
Median	0	0	0	0
Range	0–1	0–1	0–1	0–1
Control adults ($n=73$)				
Median	2	0	0	0
Range	0–13	0–6	0–4	0–7
CF−P ($n=37$)				
Median	0	0	0	0
Range	0–74	0–13	0–4	0–33
CF+P <2 years ($n=17$)				
Median	36	2	3	14
Range	2–76	0–34	0–35	1–68
CF+P >2 years ($n=69$)				
Median	68	36	20	62
Range	0–126	0–106	0–183	0–251
Other Pts+P ($n=17$)				
Median	31	3	10	11
Range	5–96	0–95	0–106	0–137

P, *Ps. aeruginosa* infection; Pts, patients

Table 2. Correlation between IgG subclass antibody levels and lung function parameters in 64 patients with CF and chronic *Ps. aeruginosa* infection

	Lung function	
	FVC	FEV1
IgG1		
r	−0·21	−0·25
P	0·1	0·04
IgG2		
r	−0·31	−0·43
P	0·01	6×10^{-4}
IgG3		
r	−0·38	−0·46
P	2×10^{-3}	2×10^{-4}
IgG4		
r	−0·25	−0·27
P	0·05	0·03

Patients were selected after 5–8 years of chronic *Ps. aeruginosa* infection.

Other patients with chronic Ps. aeruginosa *infection.* This group included 10 patients with paraplegia and chronic or intermittent *Ps. aeruginosa* urinary tract infection, four patients with *Ps. aeruginosa* osteomyelitis, and three patients with primary cilia dyskinesia and chronic *Ps. aeruginosa* lung infection.

Preparation of Ps. aeruginosa *antigen*

Ps. aeruginosa strains representing the 17 serotypes of the International Antigenic Typing Scheme were obtained from Dr O. S. Mikkelsen (Mikkelsen, 1971) and from the Cross Infection Laboratory, London. From each strain water-soluble antigens were produced by sonication as described previously (Høiby, 1977). Equal volumes of each antigen were pooled and designated St-Ag: 1–17. Aliquots of St-Ag: 1–17 were stored at −20°C until use. The protein concentration was estimated as 22 g/*l* by the method described by Lowry *et al.* (1951). Crossed immunoelectrophoresis of St-Ag: 1–17 against rabbit antiserum raised against a previously described sonic extract showed 64 distinct lines of precipitation in crossed immunoelectrophoresis (Høiby, 1975).

ELISA

Irradiated 96-well polystyrene microtitre plates (Immunoplate no. 1; Nunc, Roskilde, Denmark) and reagent volumes of 100 μl were used. All incubation steps were carried out at 22°C. Between each step, plates were washed in phosphate-buffered saline (PBS) with 0·1% Tween 20.

Plates were coated with St-Ag: 1–17 (2·2 μg of protein per well), diluted in PBS, pH 7·2, and incubated for 1 h.

Additional binding sites in the wells were blocked by incubation with a buffer (A) of Na_2HPO_4 (8 mM), KCl (0·2 mM), and NaCl (0·5 M), pH 7·2, containing 1% (wt/vol) Triton X-100 (Sigma Chemical Co., St Louis, MO) and 1% (wt/vol) bovine serum albumin (BSA) (Sigma) for 1 h.

Serum samples were diluted 1/2000 in buffer A. The samples were allowed to react overnight (18 h). Horseradish peroxidase-labelled monoclonal anti-IgG subclass-specific antibodies (Janssen Biochimica, Belgium. Catalogue numbers 24.145.89, 24.144.88, 24.143.87 and 24.142.86) were added to each well and incubated for 1 h. Antibodies were diluted 1/10000 for IgG1; 1/3000 for IgG2; 1/1000 for IgG3; and 1/4000 for IgG4, in order to obtain the same optical density with a standard serum for all four subclasses.

Sodium citrate chloride (0·1 M, pH 5·0), containing 1,2-phenylenediamide-dihydrochloride (2·2 mM) (Sigma) and H_2O_2 (6·5 mM) was added per well, and enzyme reaction was stopped by addition of H_2SO_4 (1 M) after incubation in the dark for 60 min.

The optical density (OD) at 492 nm was read on an automatic plate reader.

The antigen concentration, serum and antibody dilutions and incubation times were chosen after preliminary investigations with serial dilutions of sera from non-colonized and chronic *Ps. aeruginosa*-infected patients with CF in plates coated with various concentrations of St-Ag: 1–17. All samples were tested in duplicate.

The assays were calibrated by using a standard of pooled serum from 10 chronic infected patients with CF. Results were expressed as ELISA units (EU) and calculated by dividing the mean OD value of the test sample by the mean OD of standard serum given an arbitrary value of 100 EU for all four subclasses. If the test sample had an OD value exceeding the upper limit of the reader the sample was diluted 1/8000 and retested. Units were calculated as before and multiplied by 2·3 for IgG1; 2·4 for IgG2; 2·9 for IgG3; and by 3·3 for IgG4. These correction factors were obtained from linear regression analysis of the EU obtained by serial dilution of standard serum and four serum samples.

Assessment of antigenic specificity of the assay

Besides *Ps. aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae* are the most common causes of pulmonary infections in CF (Høiby, 1974). To investigate whether the present assays would be influenced by antibodies to these organisms, standard serum diluted 1/2000 was absorbed with increasing amounts of sonicated preparations of *H. influenzae* and a protein A-deficient *S. aureus*, prepared as described previously (Schiøtz, Høiby & Hertz, 1979a, 1979b). A protein-A-deficient strain of *S. aureus* was chosen to avoid reaction with the Fc part of IgG (Forsgren & Sjøquist, 1966). The absorption was compared with similar absorptions performed with St-Ag: 1–17. Additionally, serum samples from six non-CF patients chronically infected with *H. influenzae* and/or *S. aureus* but without *Ps. aeruginosa* infection were tested.

Assessment of specificity of the monoclonal antibodies to human IgG subclasses

The microtitre plate was coated with IgG myeloma proteins of each subclass diluted in PBS at concentrations of 4–0·05 μg/ml. Incubation was overnight at 4°C. Two different IgG myeloma proteins were used of each subclass, except for IgG3, where only one myeloma protein was available.

The further procedures were performed as described for the IgG subclass antibody ELISA, with the monoclonal antibodies applied in a checkerboard fashion.

Reproducibility of the assays

Ten serum samples from CF patients with chronic *Ps. aeruginosa* infection were tested in duplicate in two different plates on two different days. The intraplate, plate-to-plate and day-to-day variations were determined using the formula $\text{s.d.} = \sqrt{(\Sigma d^2/2n)}$, where s.d. is the standard deviation, Σd^2 is the sum of squared differences of double determinations of the same sample, and n is the number of observations.

Statistical analysis

The Mann–Whitney *U*-test for non-parametric unpaired data and the Spearman rank sum correlation test were used. The level of significance was 5% (two-tailed).

RESULTS

Specificity and reproducibility of the assays

Antibodies against the different subclasses reacted only with the corresponding myeloma proteins. The OD values for corresponding myeloma proteins were between 0·99 and 1·40. Non-specific binding was 0·10–0·20.

Absorption with increasing amounts of St-Ag: 1–17 resulted in decreasing levels of antibody activity of all four subclasses.

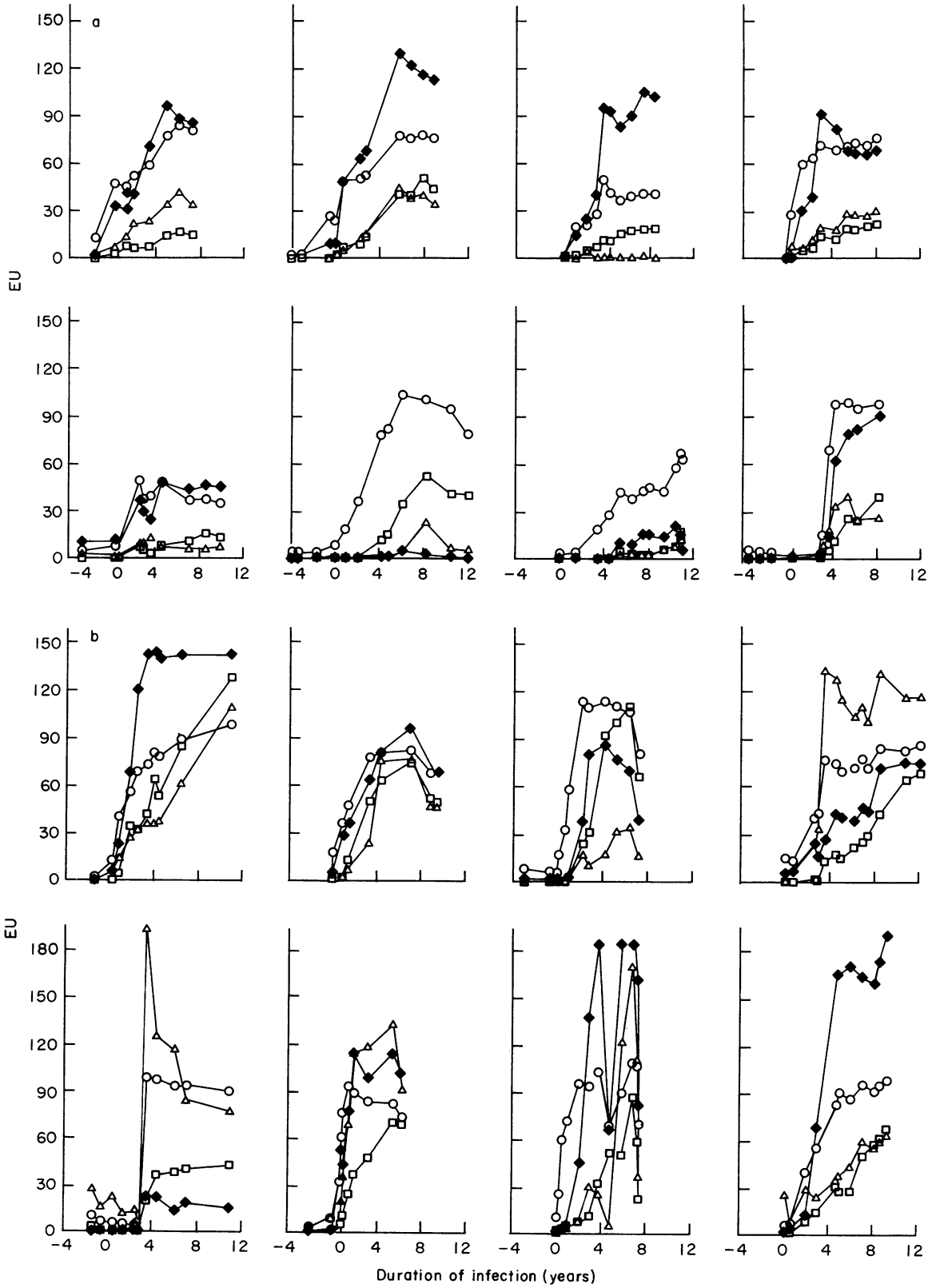

Fig. 2. The development of *Ps. aeruginosa* specific IgG subclass antibodies during chronic infection: (a) eight patients in a good clinical condition; (b) eight patients in poor clinical condition. ○, IgG1; □, IgG2; △, IgG3; ◆, IgG4.

Table 3. Development of *P. aeruginosa* IgG subclass antibodies in 16 patients with CF during chronic infection

	Clinical status							
	Good ($n=8$)				Poor ($n=8$)			
	IgG				IgG			
Duration of infection (years)	1	2	3	4	1	2	3	4
	9	0	1	4	8	1	6	2
0–1	26	3	3	17	40	5	14*	17
1–2	39	6	9	30	65	17	34*	56
2–3	46	7	12	50	81*	31*	79*	86
3–4	60	9	10	41	85	51*	60*	86
4–5	67	20	25	77	88	53	87	116
5–6	72	22	20	66	89	63*	88*	103
6–7	58	19	19	77	89*	53*	76*	87
>7	68	29	19	63	88	71*	86*	98

* $P<0.05$

Only minor reductions in OD values were seen after absorption with antigen preparations of *H. influenzae* and protein A-deficient *S. aureus* (Fig. 1). Samples from *H. influenzae*- or *S. aureus*-infected patients had EU slightly higher than normal controls but they did not differ from CF patients without *P. aeruginosa* infection (data not shown).

The intraplate variation was 4–6%, plate-to-plate variation 3–9% and day-to-day variation 12%, 19%, 8% and 21% for IgG1, IgG2, IgG3 and IgG4, respectively.

Measurements of IgG subclass antibodies to Ps. aeruginosa *in human sera*

Table 1 shows the level of subclass antibody response and the median and range in EU for each group of patients. Fifty-one out of 97 controls had detectable *Ps. aeruginosa*-specific IgG subclass antibodies of at least one subclass. No differences in subclass levels were found between normal adults and children.

The median antibody levels in sera from 37 noninfected children with CF was 0 EU for all four IgG subclasses. The median levels of 17 patients with CF in the early stage of *Ps. aeruginosa* infection (duration of infection <2 years, median 1 year) were 36, 2, 3 and 14, respectively, for IgG1–4. All values were significantly higher than in both normals and CF patients without *Ps. aeruginosa* infection ($P<0.001$). The antibody levels in sera from 69 patients with CF and chronic *P. aeruginosa* infection (duration of infection >2 years, median 7 years) were significantly higher than all other investigated groups ($P<0.005$) being 68, 36, 20 and 62, respectively, for IgG1–4 (Table 1).

Other patients with Ps. aeruginosa *infection*

Seventeen patients with chronic *Ps. aeruginosa* infection showed the same levels of IgG subclass antibodies as CF patients in an early stage of chronic infection, but significantly lower levels than patients in later stages ($P<0.05$, Table 1).

Correlation between pulmonary function and IgG subclass levels

Pulmonary function tests were carried out in 64 chronically infected CF patients. The correlations between IgG subclass antibody levels and pulmonary function are shown in Table 2. Decreased lung function correlated significantly to high levels of all IgG subclasses.

Longitudinal study

The development of *Ps. aeruginosa*-specific IgG subclass antibodies during the chronic infection in 16 patients (eight patients in good and eight in poor clinical condition) are shown in Fig. 2. The median values of *Ps. aeruginosa* subclass antibodies in these two groups of patients in relation to duration of infection are shown in Table 3. Patients in poor clinical condition showed significantly higher levels of IgG3 antibodies already in the first year of infection and 2 years later they had significantly higher IgG2 antibodies level.

DISCUSSION

The present assays had a good reproducibility, with analytical variation similar to other assays used for determination of *Ps. aeruginosa*-specific antiboddies (Høiby, 1977; Pedersen, Espersen & Høiby, 1987). The assays show good antigen and subclass specificity. In the present study we found only a few healthy controls with detectable *Ps. aeruginosa* subclass antibodies. Using the same antigen, similar results were obtained by means of crossed immunoelectrophoresis (Høiby, 1977). A previous ELISA study showed a larger proportion of controls with detectable IgG antibodies to St-Ag: 1–17 (Pedersen *et al.*, 1987). The present assays were calibrated with pooled high titre serum from CF patients who were chronically infected with *Ps. aeruginosa*. Because of this, and because of the dilutions used, low levels of subclass antibodies could not be detected. The subclass assays do not measure the concentration of specific subclass antibodies, but are valid for comparing changes in the occurrence and levels of each subclass in a patient or in groups of patients. The unusual susceptibility of CF patients to respiratory tract colonization and infection with *Ps. aeruginosa* remains poorly understood. The infection causes a pronounced specific antibody response and a poor prognosis has been associated with increasing numbers of anti-pseudomonas precipitins (Høiby, 1977). In a previous study we found that elevated serum levels of total IgG2 and total IgG3 immunoglobulins were associated with poor lung function and high titres of *Ps. aeruginosa* antibodies in patients with CF (Pressler *et al.*, 1988). We hypothesized that similar relationship would be found when *Ps. aeruginosa*-specific subclass antibodies were determined. In the present study we found that specific antibodies to *Ps. aeruginosa* of all four IgG subclasses were increased in the infected CF patients (Table 1). A similar pattern was seen in other patients with chronic *Ps. aeruginosa* infection (Table 1). More importantly, however, the present study confirms the association between elevated levels of specific IgG2 and IgG3 *Ps. aeruginosa* subclass antibodies and poor prognosis as evaluated here by lung function (Table 2) and by clinical score (Table 3).

Specific IgG subclass antibodies to *Ps. aeruginosa* have been determined in patients with CF by other investigators (Fick *et al.*, 1986; Moss *et al.*, 1987; Moss, 1987; Shryock *et al.*, 1986). In those studies *Ps. aeruginosa* lipopolysaccharide (LPS) was used as antigen. In our assays we used a sonicated extract of *Ps.*

aeruginosa, which is mainly composed of protein antigens (Høiby, 1977). However, the antigenic components in our assays are unknown, and need to be investigated. In particular, it would be important to study the subclass of antibody produced in response to protein and polysaccharide antigens separately, because the antibody response to certain antigens may result in immunoglobulins which are relatively or even completely restricted to a particular IgG subclass (Stevens *et al.*, 1983; Hammarström, Persson & Smith, 1985; Barrett & Ayoub, 1986; Weinberg *et al.*, 1986).

It has been suggested that patients with CF have impaired natural IgG2 antibody responses to *Ps. aeruginosa* LPS (Moss *et al.*, 1987). In the present study we found that patients with chronic *Ps. aeruginosa* infection reacted with increased antibody response in all four subclasses, and this was similar to other patients with infections due to this organism (Table 1). This indicates no major defects in these patients for development of IgG subclass antibodies against *Ps. aeruginosa*.

In our cross-sectional study of a group of patients with CF we found that poor lung function correlated with higher levels of *Ps. aeruginosa*-specific IgG2 and IgG3 antibodies. Furthermore, a longitudinal study of the antibody pattern in 16 patients with CF showed that in the initial stage of infection IgG1 and IgG4 antibodies dominated, and IgG2 and IgG3 antibodies showed a much more individual patterns. However, patients with a rapid downhill course of clinical condition and progressively increasing pulmonary destruction, leading to death in five out of eight patients, showed rapid increase of IgG2 and IgG3 *Ps. aeruginosa*-specific antibodies. A significant difference existed already within the first 2 years of infection. We therefore suggest that increased levels of IgG2 and IgG3 *Ps. aeruginosa*-specific antibodies are early markers of unfavourable course of disease.

It has been reported that CF whole serum interferes with the phagocytic function of alveolar macrophages (Fick *et al.*, 1981, 1984; Penketh *et al.*, 1983) and the presence of 'bactericidal blocking' antibodies has been suggested as an explanation for this selective inability. Fick *et al.* (1981) have reported that CF sera depressed bacterial uptake and once internalization of opsonized bacteria had occurred, intracellular killing was sluggish. Shryock *et al.* (1986) found that sera with high LPS-specific IgG2, IgG3 and IgG4 titres also showed phagocytosis-inhibitory activity. Fick *et al.* (1984) proposed that the phagocytosis-inhibitory activity of CF sera was due to a defect in the Fc portion of IgG, and that this prevents proper receptor attachment and internalization by macrophages. However, the same investigators also postulated that an alteration in the normal ratio of IgG subclasses may affect phagocytosis (Fick *et al.*, 1986). The necessary ratio of opsonic-to-non-opsonic subclasses for phagocytic enhancement or inhibition is unknown, and further work with combinations of individual subclasses will be required to explore this fully.

Furthermore, local immune complexes formation plays a pivotal role in the tissue damage in chronic *Ps. aeruginosa* infection (Schiøtz *et al.*, 1979, 1981); however, very little is known about the relative importance of different IgG subclass specific complexes in this aspect.

The pattern of antibody response is genetically determined (Morrell *et al.*, 1972). It is conceivable that genetic differences between the patients may explain the present differences in subclass patterns, and that the unfavourable clinical course seen in patients with high IgG2 and IgG3 levels may be a consequence of the particular biological properties of these subclasses (Alexander *et al.*, 1978; Perelmuter, 1983; Schumaker *et al.*, 1976).

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