

Immune complexes and *Pseudomonas aeruginosa* antibodies in cystic fibrosis

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SUMMARY Serum samples from 57 patients with cystic fibrosis were tested for the presence of IgG, IgA, IgM, IgE, and circulating immune complexes containing IgG, IgA, and IgM. Titres of class specific antibodies to *Pseudomonas aeruginosa*, and class specific antibodies to *Ps aeruginosa* in circulating immune complexes, were also measured. According to the Shwachman score the patients were divided into three clinical groups: group 1 – moderate and severe disease, group 2 – mild disease, and group 3 – well. The results of the immunological investigations were correlated with the clinical state of the patients as assessed by the Shwachman score. Serum concentrations of IgG, IgA, and IgM were inversely correlated with the Shwachman score, but the differences between the groups were only significant for IgG and IgA. The same correlations were found for circulating immune complexes containing IgG and IgA. Antibodies to *Ps aeruginosa* could be detected in most of the patients' serum samples. IgA antibody specific to *Ps aeruginosa* was the most often raised, even in patients in group 3. It is therefore suggested that IgA antibody specific to *Ps aeruginosa* could be an early marker of colonisation by *Ps aeruginosa* and a sensitive measurement of infection with *Ps aeruginosa* in young children with cystic fibrosis. Moreover, in the circulating immune complexes, class specific antibodies to *Ps aeruginosa* were found in nearly half the patients. The highest titres of IgG and IgA antibodies specific to *Ps aeruginosa* in the circulating immune complexes were detected in the patients with the worst clinical state (group 1).

Chronic bronchial infection with *Pseudomonas aeruginosa* is a major complication of cystic fibrosis leading to respiratory failure and ultimately death. Though lung injury can be directly caused by bacterial invasion, the role of the host defence system in permitting infection is still not clear. Circulating immune complexes have been found in serum,¹ respiratory secretions,² and various tissues³ of patients with cystic fibrosis, and there is some evidence that tissue damage by circulating immune complexes could be a critical factor in the pathogenesis and progression of the disease. These complexes include antigens and antibodies to *Ps aeruginosa* as well as to other respiratory pathogens.⁴⁻⁶

In this study we looked for disorders of humoral immunity that correlated with clinical state and the results of lung function tests. We therefore measured IgG, IgA, IgM, IgE, and circulating immune complexes containing IgG, IgA, and IgM in a group

of patients with cystic fibrosis. We also measured titres of class specific antibodies to *Ps aeruginosa*, and class specific antibodies to *Ps aeruginosa* in circulating immune complexes.

Patients and methods

Serum samples were obtained from 57 patients attending a cystic fibrosis clinic (Zeepreventorium, De Haan, Belgium). The 31 boys and 21 girls were aged from 4 to 26 years (mean 14.1). In all patients the diagnosis of cystic fibrosis was confirmed by a positive sweat test. They all had pulmonary and pancreatic disease. Overall clinical state was assessed by the Shwachman score.⁷ A maximum of 25 points was given for each of the following: chest radiograph, nutritional state, general activity, and physical examination. The lower the score, the worse the condition of the patient. Complete clinical evaluation at the time of the study was obtained.

According to the Shwachman score the patients were divided into three groups: group 1 comprised 14 patients with a mean age of 18.1 years and scores of less than 55 (moderate or severe disease); group 2 comprised 18 patients with a mean age of 13.9 years and scores of 55–70 (mild disease); and group 3 comprised 25 patients with a mean age of 11.8 years and scores of 70 or more who were well at the time of examination. At the time of the study *Ps aeruginosa* was cultured from the sputum of 49 patients. *Streptococcus pneumoniae* and *Staphylococcus aureus* were each cultured from one patient; these two patients were in group 3. In six patients in group 3 no bacterial growth was detected.

Lung function tests including forced expiratory volume in one second (FEV₁), total gas volume, and residual volume, were performed by whole body plethysmography.

Serum samples were also obtained from 14 healthy subjects who acted as controls; the samples were stored at –20°C until used.

Serum concentrations of IgG, IgA, and IgM were measured by laser nephelometry (Behringwerk). Serum concentrations of IgE were measured by a sandwich enzyme linked immunosorbent assay (ELISA) using a monoclonal antihuman IgE (Diagnostics Pasteur); this showed an excellent correlation ($r=0.97$) with the PRIST technique (Pharmacia). For normal values of IgG, IgA, IgM, and IgE, respectively, according to age, we used the standards of Stiehm and Fudenburg⁸ and Kjellman.⁹

Circulating immune complexes were precipitated out of serum with polyethylene glycol 6000 (Merck) as previously described.¹⁰ Briefly, serum samples were diluted 1/2 with phosphate buffered saline, and were precipitated and washed with 2.5% or 1.5% (IgM) polyethylene glycol. These precipitates were then dissolved in 0.1 M Tris buffered saline containing 1% bovine serum albumin (Sigma) and 0.1% Tween 20 (Merck).

The IgG, IgA, and IgM content of the circulating immune complexes were measured by quantitative ELISA of the polyethylene glycol precipitate as follows: polystyrene microtitre plates (Costar) were coated with 10 µg/ml of class specific antihuman immunoglobulin diluted in carbonate buffer (0.1 M, pH 9.6). Goat antihuman IgG and IgA (Tago), and rabbit antihuman IgM (Dako), were incubated overnight at 4°C, free binding sites were blocked with 150 µl of a 1% bovine serum albumin in carbonate buffer, incubated for 1½ hours at 37°C, and washed five times in physiological saline containing 0.1% Tween 20 in an automated microplate washer (Titertek, Flow Laboratories). One hundred microlitres of polyethylene glycol precipitate diluted with 0.1% Tween 20 was then added to the wells in

duplicate. The immunoglobulin assays were calibrated with a standard protein preparation (Behring) diluted with 0.1% Tween 20. The microtitre plates were then incubated for 2½ hours at 37°C and again washed with physiological saline containing 0.1% Tween 20; the bound immunoglobulins were detected with 100 µl of horseradish peroxidase conjugates. For IgG, IgA, and IgM containing circulating immune complexes, the F(ab')₂ fragments of horseradish peroxidase antihuman IgG, IgA, or IgM, (Tago) were used. After incubation for two hours at 37°C the plates were washed again and the wells filled with 100 µl of 0.4 mg/ml orthophenylene diamine (Sigma) and 0.014% hydrogen peroxide in citrate phosphate buffer (0.1 M, pH 5.0). The colour reaction was stopped after five minutes with 50 µl 2 M sulphuric acid. Optical densities were measured with a microplate photometer (Titertek, Flow Laboratories) at 492 nm with a reference absorbance at 690 nm. The analysis of the calibration curve and the calculation of unknown factors were performed with a four parametric logistic model as previously described.¹¹

Titres of IgG, IgA, and IgM antibodies to *Ps aeruginosa* in serum and circulating immune complexes were quantified with ELISAs. A reference strain of *Ps aeruginosa* from the American Type Culture Collection was submitted to three cycles of freeze thawing, then 20 minutes of sonication, before being diluted 1/100 in carbonate buffer and sonicated until no visible aggregates remained. A total of 100 µl of this antigen solution was coated overnight at 4°C on polystyrene microtitre plates. Free binding sites were then blocked with 150 µl of 1% bovine serum albumin in carbonate buffer and incubated at 37°C for 1½ hours. After five washings with physiological saline containing 0.1% Tween 20, 100 µl of serum, 2.5% polyethylene glycol, or 1.5% polyethylene glycol precipitates diluted with 0.1% Tween 20, were added. A pool of serum samples with strongly positive titres of IgG, IgA, and IgM antibodies to *Ps aeruginosa* was used as a standard. One hundred arbitrary units were attributed to the lowest dilution of standard used, 1/80 for IgA, and 1/40 for IgM antibodies, respectively. Ten units were attributed to the 1/2400 dilution of standard pool for IgG antibody to *Ps aeruginosa*. The remainder of the ELISA (colour development and analysis of the results) was performed as reported for the measurement of class specific circulating immune complexes.

In order to correct for non-specific precipitation of free antibodies to *Ps aeruginosa*, a reference group of 12 patients whose serum samples did not contain IgG, IgA, and IgM circulating immune complexes was constituted. These reference samples

were used to determine 'cut off' concentrations (95th percentile) for antibodies to *Ps aeruginosa* in the polyethylene glycol precipitate.

IgE antibody to *Ps aeruginosa* was measured with a capture ELISA. Microtitre plates were coated with 100 μ l of a 10 μ g/ml solution of monoclonal antihuman IgE (Diagnostics Pasteur) in carbonate buffer. After incubation overnight at 4°C, 150 μ l of 1% bovine serum albumin in carbonate buffer was added to each well and incubated for 1½ hours at 37°C. After five washings with physiological saline containing 0.1% Tween 20, 100 μ l of serum samples diluted 1/5 with 0.1% Tween 20 were added to each well. Plates were then incubated for three hours at 37°C, washed, and 100 μ l of antigens to *Ps aeruginosa* treated with avidin-biotin were added to each well. *Ps aeruginosa* lysate was treated with N-hydroxy-succinimide biotin (Sigma) by the method of Subba Rao *et al.*¹² After incubation for a further two hours at 37°C the microtitre plates were washed, and 100 μ l of a 1/1000 diluted streptavidin peroxidase conjugate (Amersham) were added for 45 minutes at 37°C. Colour development was done as for the other ELISAs. These semiquantitative results were expressed as optical densities (arbitrary units) and calibrated with optical densities obtained from control serum samples.

Results of immunoglobulin, circulating immune complexes, and *Ps aeruginosa* specific antibody assays were transformed logarithmically because of the skewness of the data, and are shown as geometric mean (SD). Significance of correlations among the logarithmically transformed values were calculated by the Pearson correlation coefficient. For comparison between groups, non-parametric one way analysis of variance (Kruskal-Wallis) was used.

Results

Mean serum IgG, IgA, and IgM concentrations were inversely correlated with the Shwachman score (table 1). The differences among the groups were only significant for IgG and IgA. When compared with age matched control subjects most patients with raised concentrations of immunoglobulin were found to be in the group with the most severe clinical disease. Serum IgE concentrations differed significantly among the different groups, with the highest concentrations being in patients in group 2.

Concentrations of circulating immune complexes containing IgG and IgA were significantly increased compared with the control population (table 2), and were highest in the patients in group 1. Concentrations of IgM containing circulating immune complexes were not associated with clinical state, though they were raised in 16 (28%) of the patients. Concentrations of the different circulating immune complexes also correlated significantly with the different serum immunoglobulin concentrations (results not shown).

Antibodies to *Ps aeruginosa* of the IgG and IgA classes were found in most patients (table 3). There was a significant increase with evolving clinical state, with a tenfold difference in IgA antibodies specific to *Ps aeruginosa* between the patients with the worst and the best clinical score. Titres of IgM and IgE antibodies specific to *Ps aeruginosa* were raised in 17 (30%) of the patients but did not correlate with clinical state. There was a moderately significant correlation ($p < 0.05$) among IgG, IgA, and IgM antibodies specific for *Ps aeruginosa* and serum concentrations of IgG, IgA, and IgM ($r = 0.44, 0.31, \text{ and } 0.46$, respectively).

Class specific antibodies to *Ps aeruginosa* could be

Table 1 Mean (SD) serum concentrations of immunoglobulins in patients with cystic fibrosis and number of patients (%) with raised concentrations

Immunoglobulin	Shwachman score			p Value
	<55 (n=14)	55-70 (n=18)	≥70 (n=25)	
IgG:				
Mean (SD) concentration (g/l)	22.08 (16.52-29.51)	18.08 (14.08-23.21)	12.61 (8.54-18.63)	<0.001
No (%) of patients with raised concentration	10 (71)	13 (72)	7 (28)	
IgA:				
Mean (SD) concentration (g/l)	4.11 (2.57-6.58)	2.44 (1.44-4.15)	1.37 (0.73-2.57)	<0.001
No (%) of patients with raised concentration	10 (71)	4 (22)	2 (8)	
IgM:				
Mean (SD) concentration (g/l)	1.88 (1.14-3.11)	1.76 (1.07-2.90)	1.18 (0.40-3.44)	0.262
No (%) of patients with raised concentration	10 (71)	14 (78)	19 (76)	
IgE:				
Mean (SD) concentration (kU/l)	85 (15-478)	219 (49-982)	54 (14-211)	0.032
No (%) of patients with raised concentration	4 (31)	11 (61)	5 (22)	

detected in the polyethylene glycol precipitate in some patients. Significantly higher titres of IgG and IgA antibodies to *Ps aeruginosa* were found in the groups with the worse clinical states. In most of the samples containing circulating immune complexes, we found raised titres of antibodies to *Ps aeruginosa* (tables 4 and 5).

Ps aeruginosa was not isolated from the sputum of eight patients. These were all in group 3 (Shwachman score 70 or more) and their mean age was 7.7 years (range 4.2–15.6), which is less than the mean

age of the whole group. Three of these patients had raised titres of IgA antibodies to *Ps aeruginosa* and one of them had raised titres of IgE antibodies to *Ps aeruginosa*. The subject who had *S pneumoniae* in his sputum also had raised serum concentrations of IgM circulating immune complexes, and raised titres of IgM to *Ps aeruginosa* and IgM antibodies to *Ps aeruginosa* were found in the circulating immune complexes. None of the other subjects who did not have *Ps aeruginosa* in their sputum had raised circulating immune complexes.

Table 2 Mean (SD) concentrations of class specific circulating immune complexes expressed as arbitrary units and number (%) of patients with raised concentrations

Class specific circulating immune complexes containing:	Control population	Shwachman score			p Value
		<55 (n=14)	55-70 (n=18)	≥70 (n=25)	
IgG:					
Mean (SD) concentration	7.5 (5.8-9.9)	76.7 (33.1-177.7)	55.1 (22.2-137.0)	26.3 (12.8-54.1)	0.030
No (%) of patients with raised concentration		10 (75)	12 (67)	3 (10)	
IgA:					
Mean (SD) concentration	3.2 (1.7-6.2)	8.41 (2.4-29.4)	4.8 (1.7-13.7)	1.8 (0.5-5.9)	<0.001
No (%) of patients with raised concentration		7 (50)	4 (22)	2 (8)	
IgM:					
Mean (SD) concentration	5.0 (2.7-9.0)	10.8 (4.8-24.3)	11.4 (5.5-23.6)	8.2 (4.1-16.8)	0.302
No (%) of patients with raised concentration		5 (36)	7 (39)	4 (16)	

Table 3 Mean (SD) titres of antibodies specific for *Ps aeruginosa* expressed as arbitrary units and number (%) of patients with raised titres

Titres of antibodies specific for <i>Ps aeruginosa</i>	Control population	Shwachman score			p Value
		<55 (n=14)	55-70 (n=18)	≥70 (n=25)	
IgG:					
Mean (SD) titre	130 (98-174)	1588 (523-4817)	428 (137-1339)	176 (43-713)	<0.001
No (%) of patients with raised titres		13 (93)	13 (72)	12 (48)	
IgA:					
Mean (SD) titre	8 (4-16)	757 (230-2490)	143 (35-584)	61 (12-299)	<0.001
No (%) of patients with raised titres		14 (100)	16 (89)	18 (72)	
IgM:					
Mean (SD) titre	88 (45-174)	148 (63-351)	137 (59-317)	124 (62-250)	0.697
No (%) of patients with raised titres		5 (36)	5 (28)	7 (28)	
IgG:					
Mean (SD) titre	11 (5-25)	40 (14-117)	48 (12-202)	54 (23-213)	0.638
No (%) of patients with raised titres		3 (21)	7 (39)	7 (28)	

Table 4 Mean (SD) titres of antibodies specific for *Ps aeruginosa* in the polyethylene glycol precipitate expressed as arbitrary units

Titres of antibodies specific for <i>Ps aeruginosa</i>	Shwachman score			p Value
	<55 (n=14)	55-70 (n=18)	≥70 (n=25)	
IgG	4.9 (0.9-25.5)	2.1 (0.4-10.1)	1.1 (0.3-3.5)	0.014
IgA	2.2 (0.7-7.2)	0.9 (0.4-2.0)	0.5 (0.2-1.2)	<0.001
IgM	3.3 (0.8-14.2)	4.9 (1.7-14.2)	2.6 (0.4-16.6)	0.590

Table 5 Number of patients with circulating immune complexes and raised titres of antibody to *Ps aeruginosa* in the polyethylene glycol precipitate

Antibody specific for <i>Ps aeruginosa</i>	Shwachman score			Total No
	<55	55-70	≥70	
IgG	4/6	1/4	0/1	5/11
IgA	5/7	0/4	1/2	6/13
IgM	3/5	3/7	2/4	8/16

Table 6 Significance of correlations among respiratory function tests and other measurements

Measurement	FEV ₁		Total gas volume		Residual volume	
	Correlation coefficient	p Value	Correlation coefficient	p Value	Correlation coefficient	p Value
Shwachman score	0.70	<0.001	-0.57	<0.001	-0.70	<0.001
Concentration of:						
IgG	-0.52	<0.001	0.34	<0.008	0.41	0.002
IgA	-0.57	<0.001	0.30	0.018	0.39	0.003
IgM	-0.24	0.044	0.05	0.36	0.07	0.315
Circulating immune complexes containing:						
IgG	-0.51	0.007	0.37	0.042	0.41	0.026
IgA	-0.51	<0.001	0.20	0.08	0.29	0.021
IgM	-0.34	0.008	0.09	0.278	0.11	0.217
Antibodies specific to <i>Ps aeruginosa</i> :						
IgG	-0.47	<0.001	0.34	0.007	0.36	0.006
IgA	-0.39	0.003	0.33	0.011	0.37	0.004
IgM	-0.15	0.142	0.23	0.058	0.24	0.046
Antibodies to <i>Ps aeruginosa</i> in circulating immune complexes:						
IgG	-0.52	<0.001	0.21	0.078	0.24	0.048
IgA	-0.60	0.001	0.30	0.020	0.34	0.008
IgM	-0.27	0.031	0.10	0.257	0.14	0.176

FEV₁ and residual volume correlated well with the Shwachman score (table 6). Significant correlations were also found between FEV₁, total gas volume, and residual volume on the one hand, and concentrations of immunoglobulins and circulating immune complexes, and titres of antibodies to *Ps aeruginosa*, on the other.

Discussion

In this group of patients with cystic fibrosis colonisation of the respiratory tract with *Ps aeruginosa* was detected in 49 (86%). Only in eight patients in group 3 could *Ps aeruginosa* not be detected in the sputum. Most centres treating patients with cystic fibrosis have reported similar isolation rates, particularly in patients with advanced disease.¹³

There have been several reports of the association between serum immunoglobulin concentrations and clinical state in patients with cystic fibrosis.^{14 15} Most reports have focused on IgG, and studies concerning total IgM or IgA or concerning antibodies specific to *Ps aeruginosa* are scarce. There

have also been various reports about the presence and importance of circulating immune complexes, but class specific circulating immune complexes have seldom been studied in patients with cystic fibrosis.

As in previous studies, significant differences in total immunoglobulin concentrations among the three clinical groups were found, except for IgM; the IgM concentrations were equally raised in the three groups. In a study by Turner *et al* an association was also detected between high concentrations of IgG and IgA (but not IgM) and recurrent infection.¹⁴ On the other hand, low concentrations of immunoglobulins have been reported to be associated with milder lung disease and slower progression of pulmonary impairment.¹⁵ None of our patients had hypogammaglobulinaemia. Many other studies have shown variable rises in immunoglobulin concentrations in serum samples from patients with cystic fibrosis.¹⁶ Twenty one (37%) of our subjects had raised concentrations of IgE. This confirms the finding of Wallwork and McFarlane who found raised concentrations of IgE in 32% of

their patients.¹⁶ Several other workers have reported raised total IgE and raised titres of allergen specific IgE antibodies in association with positive skin prick tests in patients with cystic fibrosis.^{14 17} Turner *et al* reported a clear association between IgE concentrations and the number of recent respiratory tract infections.¹⁴ Furthermore, it has been shown that recurrent infection and inflammation of the bronchial tree lowers the threshold of irritant receptors leading to bronchial hyper-reactivity.¹⁸

Though raised concentrations of circulating immune complexes have been described,¹ the presence of class specific circulating immune complexes has not been well studied in patients with cystic fibrosis. In our study, concentrations of IgG and IgA containing circulating immune complexes were highest in patients with advanced disease, whereas concentrations of IgM containing circulating immune complexes were not associated with worsening of the clinical state. Whether circulating immune complexes containing IgG or IgA are actively concerned in the progression of the lung disease needs to be elucidated. That circulating immune complexes containing IgG and IgA could be partly responsible for the progression of the disease and not solely reflecting the duration of colonisation with *Ps aeruginosa* is suggested by the finding of immune complex deposition in the respiratory tracts of patients with cystic fibrosis,¹ and by the development of cutaneous vasculitis in some patients with cystic fibrosis. Another indication of the pathogenic role of these immune complexes comes from the *in vitro* experiments that showed a release of mediators from inflammatory cells by circulating immune complexes containing IgG or IgA in patients with cystic fibrosis.¹⁹ Recent clinical studies also suggest that circulating immune complexes are an independent risk factor for death from cystic fibrosis, and that they are also associated with poor prognosis. Other workers, however, have suggested that the concentration of circulating immune complexes cannot be used as markers for infectious exacerbations, but have to be considered as indicators of the chronic nature of the disease.

Antibodies specific to *Ps aeruginosa* were found in most patients, with significant differences in IgG and IgA antibodies specific to *Ps aeruginosa* among the three groups. The highest titres were found in patients with the worst clinical state. These data confirm the results of other studies that showed that high titres of IgG antibody specific to *Ps aeruginosa* were associated with poor clinical state, and that it is a sensitive and specific marker for the severity and progress of infection with *Ps aeruginosa* in patients with cystic fibrosis.²⁰ Titres of IgA antibodies

specific to *Ps aeruginosa* have not been widely studied and it is therefore interesting that they were the most often raised, even in patients who were clinically well—18 out of 25 (72%) of the patients with less severe disease had raised titres of IgA antibodies specific for *Ps aeruginosa*—or in patients without *Ps aeruginosa* in their sputum samples (three of eight). Because titres of IgA antibodies to *Ps aeruginosa* were significantly lower in serum samples from control subjects than in those from patients with good or excellent clinical state, and because these titres increase with the severity of the disease, it might be that IgA antibodies to *Ps aeruginosa* are specific and sensitive detectors of infection by *Ps aeruginosa* in young children with cystic fibrosis. Furthermore they could indicate previous infection with *Ps aeruginosa* in patients with cystic fibrosis who were not currently growing *Ps aeruginosa* in their sputum.

The highest titres of IgA antibodies to *Ps aeruginosa* in circulating immune complexes were found in the patients with the most progressive disease, there being significant differences among the three clinical groups. Antigens and antibodies to *Ps aeruginosa* have been detected in circulating immune complexes by a number of workers, and have been associated with colonisation by *Ps aeruginosa* and advanced disease. Moss and Hsu showed that patients with cystic fibrosis who had increased concentrations of circulating immune complexes, these complexes contained IgG antibody to *Ps aeruginosa*.⁵ Other authors showed that circulating immune complexes contained antibody to *Ps aeruginosa* lipopolysaccharide and had endotoxin activity.⁴

In conclusion it seems that IgA is a sensitive marker of colonisation by *Ps aeruginosa* in patients with cystic fibrosis. Concentrations of total IgA, specific circulating immune complexes containing IgA, and IgA antibody titres to *Ps aeruginosa* are closely associated with the clinical progression of cystic fibrosis as judged by the Shwachman score and lung function tests. The use of IgA antibody to *Ps aeruginosa* as an early marker of colonisation by *Ps aeruginosa* or as an indicator of previous infection with *Ps aeruginosa* in patients with cystic fibrosis needs further study but could be important, particularly in young children from whom it is difficult to collect sputum for culture.

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