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Circulating soluble immune complexes containing pseudomonas antigens in cystic fibrosis

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SUMMARY In order to investigate whether circulating immune complexes containing *Pseudomonas aeruginosa* antigens mediate pulmonary damage in cystic fibrosis, we studied lung function, serum immune complex levels, and immunoglobulin concentrations in relationship to chronic pseudomonas colonisation in 69 affected children. Sixteen of the children with cystic fibrosis had increased levels of immune complexes which contained pseudomonas antigens. There was no significant relationship between lung function corrected for the effect of chronic pseudomonas colonisation and the presence of such complexes or increased levels of complexes detected by Clq binding or raised serum immunoglobulin concentrations. Our results suggest that these abnormalities in cystic fibrosis are secondary effects of chronic infection and they do not provide evidence for immune complex mediated lung damage in this disease.

Recurrent pulmonary infections account for most of the morbidity and mortality[1] in cystic fibrosis (CF). Chronic colonisation with *Pseudomonas aeruginosa* (PA) is particularly likely to occur and is strongly associated with poor lung function.[2 3] High titres of precipitating antibodies to PA correlate with poor prognosis,[2 4] and circulating immune complexes (IC) demonstrated by a variety of methods[5–8] correlate both with PA infection[7] and poor lung function.[9 10] Based on this evidence, it has been suggested that circulating IC may cause localised type III hypersensitivity reactions in the lungs and so produce secondary lung damage in CF.[5 7 10]

To investigate this hypothesis of lung damage mediated by circulating IC we have measured them in the sera of 69 CF patients and analysed them for the presence of PA antigens using an immune complex splitting assay. We then studied the relationship between such complexes, lung function, and chronic PA colonisation by analysis of variance to examine whether the association of circulating IC with poor lung function is causative.

Patients and methods

Sixty-nine CF children who were bled for a previous study and who have been reported[3] were studied. Their ages ranged from 2 to 15 (mean 9) years. Lung function data from that earlier study included forced expiratory flow at 25% vital capacity (FEF^{25}), measured from a maximal expiratory flow volume curve, and residual volume to total lung capacity ratio (RV/TLC), measured by whole body plethysmography. These were the most sensitive measures of airflow obstruction and hyperinflation in the earlier analyses.[3]

Cultures of sputum or cough swabs were obtained every 2 months from these patients and if three consecutive cultures during a period of at least 3 months were positive for PA, the patient was defined as having persistent colonisation with PA.

Blood samples from these patients were separated at room temperature within 2 hours of collection and the sera were stored in aliquots at −70°C.

Immunoglobulin concentrations. Serum concentrations of IgG, IgA, and IgM were measured by radial immunodiffusion[11] and results expressed as IU/ml.

Circulating immune complexes. Two methods were used—namely, a radiolabelled ^{125}I-Clq binding assay (Clq BA)[12] and polyethylene glycol (PEG) precipitation.[13] Clq BA measures precipitation of ^{125}I-Clq by IC in the presence of 3% polyethylene glycol and detects IgM IC and certain subclasses of IgG in IC. Results were expressed as a percentage of the radioactive counts precipitable by 10% trichloroacetic acid.

IgG and IgM IC were also measured independently in the PEG precipitation assay.[13] Serum samples

(250 µl) were mixed with 50 µl 12% PEG in EDTA buffer pH 7·4 and, after overnight incubation at 4°C, precipitated IC were assayed for immunoglobulin content in high sensitivity agar diffusion plates.[11] Each result was expressed as a percentage of the immunoglobulin concentration in the original serum.

Immune complex dissociation assay. A modification of the complex splitting assay described by Paganelli *et al.*[14] was used. The IgG fraction of a high titre rabbit antiserum to PA strains 'PAO' was prepared by absorption with sepharose-staphylococcal protein A (Sigma Chemical Co., St Louis, USA) and after one wash in Tris-HCl buffer (pH 7·6) the IgG was dissociated from the sepharose by 0·2 mol/l glycine-HCl buffer (pH 2·6). The resulting preparation was neutralised with solid Tris and then tested by immunoelectrophoresis against PAO cell wall extract, *Aspergillus fumigatus* serodiagnostic antigen (Bencard Allergy Service, Brentford, Middlesex), and normal human serum. It reacted only with the PAO extract. Positive control PA antigen containing IC in 2- and 4-fold antigen excess were prepared after appropriate titration of the IgG preparation (1 to 10 µg/ml) wtih PAO antigen (163 mg/ml) using ^{125}I labelled PAO as a tracer.[14]

IC were obtained from test and control sera by precipitation in 2% PEG (as before) and the IC from 100 µl serum were resuspended in the same volume of 0·2 mol/l glycine-HCl buffer (pH 2·6). This solution (50 µl) was incubated in each of two polystyrene autoanalyser cups for 18 hours at 22°C. The cups were then washed 5 times with PBS containing 0·1% human albumin and then IgG anti-PA antibody radiolabelled with ^{125}I was added and incubated for 18 hours at 4°C. After four further washes with the PBS-albumin buffer, the autoanalyser cups were counted for one minute and counts per minute corrected for background were calculated. Preformed PA antigen containing IC were suspended in 50% normal human serum and used as positive controls; sera from 9 children with no history of PA infection who had been investigated for coeliac disease were the negative controls.

Statistical methods. Results were analysed using the statistical package for the social sciences[15] at University of London Computer Centre by Student's *t* test, Pearson's least squares regressions (r), and analysis of variance by the multiple regression method.

Results

Pseudomonas antigens in complexes. More complexed PA antigen was detected in the sera of the 69 CF patients than in the 9 patients investigated for coeliac disease (Figure) ($t=4·16$, $P<0·001$). Sixteen CF patients had levels greater than 2 SDs above the mean of the controls but the amount of complexed antigen did not differ significantly between the CF patients chronically colonised with PA and those who were not ($t=1·62$, $P>0·05$), although it was significantly higher in patients with acute exacerbations of pulmonary infection than in those who were well when studied ($t=2·51$, $P<0·02$).

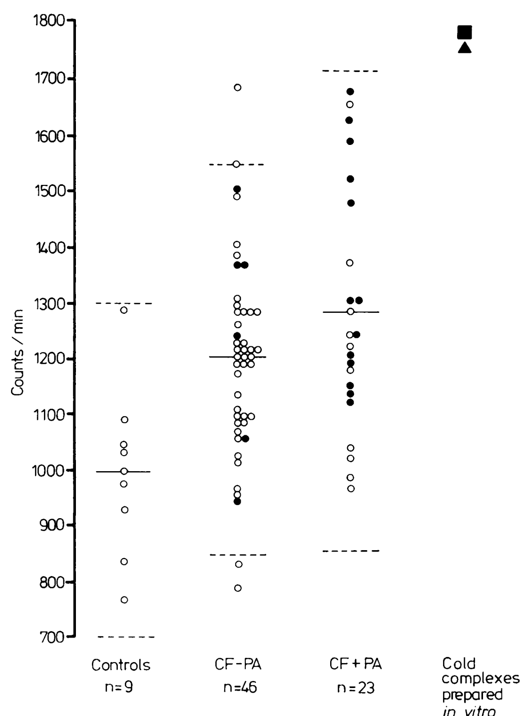

Figure *Levels of complexed* P. aeruginosa *antigen in 23 CF children with chronic* P. aeruginosa *colonisation of the lungs (CF+PA), 46 CF children without such colonisation (CF−PA), and in 9 age-matched controls. CF children with acute exacerbations of pulmonary infection (●) had significantly higher levels than patients who were well (○); ($t=2·51$, $P<0·02$).*

Circulating immune complexes. The mean value of Clq BA in the 69 CF patients was 4·7% which is above our normal upper limit[16] (Table 1) and 36 patients had Clq BA values outside this range. Clq BA was significantly increased in patients chronically colonised with PA (Table 2). No CF patient exceeded the normal range for IgG IC by PEG precipitation; however 2 patients had increased IgM IC (greater than 12·8% of serum IgM). It

Table 1 *Immune complex levels in 69 patients with cystic fibrosis compared with controls*

	Cystic fibrosis patients	*Controls*
Clq binding assay (%), mean (SE)	4·7 (0·2)	2·1 (0·2)
Range	2·4–11·8	≤4·0*
IgG immune complexes (%), mean (SE)	1·3 (0·1)	2·3 (0·1)
Range	0·0–2·8	1·0–3·5
IgM immune complexes (%), mean (SE)	5·9 (0·3)	5·7 (0·4)
Range	1·6–17·8	0·0–12·8

* Determined as the upper limit of normal for our laboratory,[16] measured in 84 uninfected children admitted for cardiac surgery. Clq binding assay expressed as percentage of radioactive counts precipitated by 10% trichloroacetic acid. IgM and IgG immune complexes expressed as percentage of the immunoglobulin concentration in original serum.

Table 2 *Immune complexes, immunoglobulin concentrations, and lung function in 25 CF patients chronically colonised with* P. aeruginosa *and in 44 CF patients without such infection*

	Cystic fibrosis patients	
	Colonised with P. aeruginosa	*Free of* P. aeruginosa *colonisation*
Clq binding assay (%) mean (SD)	5·62 (2·20)***	4·11 (0·96)
IgG immune complexes (%) mean (SD)	1·42 (0·60)	1·21 (0·46)
IgM immune complexes (%) mean (SD)	6·74 (3·25)	5·36 (1·75)
IgG (IU/ml) mean (SD)	204 (60)***	139 (52)
IgA (IU/ml) mean (SD)	141 (64)**	95 (67)
IgM (IU/ml) mean (SD)	170 (93)**	116 (42)
FEF25 (% predicted) mean (SD)	25 (9)***	67 (27)
RV/TLC (%) mean (SD)	55 (13)***	37 (9)

***$P<0·005$, **$P<0·01$.
Clq binding assay, IgG immune complexes, IgM immune complexes results expressed as in Table 1.
Serum IgG, IgA, IgM concentrations expressed as IU/ml.
FEF25 forced expiratory flow at 25% of vital capacity, RV/TLC= ratio of residual lung volume to total lung capacity.

appeared, therefore, that Clq BA was more sensitive to the abnormalities in these patients than the PEG precipitation method.

Immunoglobulin levels. The mean level of serum IgG was 163 IU/ml (SE 7·6) and the mean serum IgM 136 IU/ml (SE 8·4); both exceeded the means of age-matched British schoolchildren.[17] Seven CF children had reduced serum IgA concentrations for age and 2 had levels that were hardly detectable. Many patients had hypergammaglobulinaemia as described in our earlier study[18] and all measured classes of immunoglobulin were significantly increased in CF children with chronic PA colonisation (Table 2).

Lung function. The mean FEF25 in the 54 of 69 CF children for whom data were available was 49% of that predicted for height, and the mean RV/TLC in 49 cases was 45%. There was a pronounced difference in both values according to whether or not the patients were chronically colonised with PA (Table 2).

Correlation analysis. Many of the observations were significantly intercorrelated (Table 3). As previously reported[9 10] there was a significant negative correlation between immunoglobulin levels and Clq BA and lung function. This was also observed for IgM IC but not for IgG IC or PA antigen containing IC. However, with such multiple intercorrelation it was possible that there was a single causative factor with many secondary effects and it seemed likely that PA colonisation was the primary abnormality. We therefore reanalysed the data by analysis of variance so that FEF25 and RV/TLC were adjusted for the influence of chronic PA colonisation. When this was done there was no residual negative correlation between Clq BA, IgG, and IgM, and lung

Table 3 *Correlation coefficients (r) in 69 children with cystic fibrosis*

	Clq binding assay	*IgG immune complexes*	*IgM immune complexes*	*IgG*	*IgA*	*IgM*	*FEF25*	*RV/TLC*
IgC immune complexes	0·36*** (69)							
IgM immune complexes	0·28** (69)	0·56*** (69)						
IgG	0·45*** (69)	0·11NS (69)	−0·04NS (69)					
IgA	0·12NS (69)	0·05NS (69)	0·03NS (69)	0·53*** (69)				
IgM	0·32** (69)	0·15NS (69)	0·04NS (69)	0·59*** (69)	0·39*** (69)			
FEF25	−0·31* (54)	0·17NS (54)	−0·12NS (54)	−0·50*** (54)	0·37** (54)	−0·29* (54)		
RV/TLC	0·32* (49)	0·21NS (49)	0·29* (49)	0·31* (49)	0·34** (49)	0·26* (49)	−0·59*** (47)	

The number of valid cases for each correlation is shown in brackets.
***$P<0·005$, **$P<0·01$, *$P<0·05$.

Table 4 *Variance ratios (F) in analysis of variance on lung function in cystic fibrosis*

Dependent variables	*No of cases*	*Pseudomonas colonisation*	*Clq binding assay*	*IgG*	*IgA*	*IgM*	*Age*
FEF^{25}	44	31·4***	0·2	2·7	0·5	0·5	3·8
RV/TLC	59	16·9***	0·8	0·4	4·6*	0·16	NI

***$P<0{\cdot}001$, *$P<0{\cdot}05$.
NI = not included, as not significant in earlier study.[3]

function (Table 4) although IgA still correlated inversely with RV/TLC (but less so than before).

Discussion

Abnormal levels of circulating IC have been reported before in patients with CF[5–10] and it has been proposed that secondary lung damage may be the result of localised immune complex deposition.[5 7 10] IC have also been demonstrated in the sputum of CF patients[19] and there is a single report of granular deposition of immunoglobulins and complement components in the trachea and lungs of a CF patient.[20]

Pulmonary colonisation with PA is highly prevalent in CF so it was possible that the IC contained PA antigens; indeed haemagglutinating antibodies to PA lipopolysaccharide have been demonstrated in such IC.[8] We have demonstrated the presence of PA antigens within the IC isolated from CF sera by PEG precipitation but there was no significant correlation between these PA antigens containing IC and either poor lung function or overt PA lung colonisation, whereas PA IC were significantly increased in patients with acute exacerbations of pulmonary infection. It was surprising that levels of PA containing IC were not significantly increased in patients chronically colonised with PA. This may reflect either under-diagnosis of PA by standard microbiological methods and our definition of 'chronic colonisation' or intermittent PA infection, perhaps in other sites such as paranasal sinuses. The first of these possibilities is supported by the observation that the 2 patients with the highest levels of PA antigen containing IC in the uncolonised group became persistently colonised soon afterwards. None the less, we have established that the circulating IC in CF may contain PA antigens and that persistent antigenaemia can occur in patients with negative sputum cultures.

These results of IC investigations in CF are compatible with those of earlier studies.[5–10] Our further analysis suggests however, that although circulating IC and impaired pulmonary function are correlated in CF, this correlation is not significant when the effect of PA is allowed for by analysis of variance. The relationship of lung disease to hypergammaglobulinanaemia was similar to that with circulating IC and, therefore, no independent direct relationship was established between either of these immunological phenomena and pulmonary disease. Conversely, we were unable to confirm the reported association of good lung function and subnormal immunoglobulin concentrations[21] when the effect of infection was taken into account.

PA colonisation is the main factor associated with poor lung function.[2 3] Such pulmonary damage could be the effect of exotoxins, proteases, or any of the immunological mediators of inflammation.[22] It is also possible that pulmonary damage could result from *in situ* formation of IC, but our data suggest that circulating IC are not important. However, serum levels of IC in our CF children seemed to be lower than those seen in series which included adults[5 6 8–10] for whom the situation may be different. There was a significant correlation of serum IgA with RV/TLC even when the latter was adjusted for the influence of PA; we interpret this as the effect of other infections. The findings of this study therefore strengthen the view that PA colonisation is of critical importance in CF.[2 3]

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