An ELISA to detect antipseudomonal IgA antibodies in sera of patients with cystic fibrosis

M M BRETT,* A T M GHONEIM,† J M LITTLEWOOD* Departments of *Paediatrics and †Microbiology, St James's University Hospital, Leeds

SUMMARY An enzyme linked immunosorbent assay (ELISA) to measure free serum IgA antibodies to *Pseudomonas aeruginosa* in patients with cystic fibrosis is described. Results were reproducible and there was no interference from crossreacting antibodies directed against other Gram negative bacteria. Titres were high in patients with *Pseudomonas aeruginosa* infection and differed according to the stage of the infection.

These preliminary results suggest that this assay may be of value in assessing the state of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis.

Pulmonary infection with *Pseudomonas aeruginosa* is an important cause of morbidity and mortality in patients with cystic fibrosis.^{1,2}

In the past there was no specific test to measure the severity and progress of *Pseudomonas aeruginosa* infection in its early stages, which is when antibiotic treatment will probably be most effective. We have previously described an immunoassay (ELISA) that detected serum IgG antibodies to *Pseudomonas aeruginosa*.³ We found that increases in serum IgG titres, indicating the presence of infection, were detected before the isolation of the organism from the respiratory tract in 85-90% patients.^{4,5} This assay seemed to discriminate between infection and harmless, non-pathogenic colonisation among patients with intermittent isolations of *Pseudomonas aeruginosa*.^{4,5} It was also shown to be a specific measure of the severity of infection⁶ but it gave little information about the patient's long term prognosis. In the early stages of *Pseudomonas aeruginosa* lung infection the mucosa of the respiratory tract is the primary site of infection. The role of secretory IgA in combating invading organisms and the association between serum IgA concentrations and the progress of the disease are not well understood. We have therefore developed an immunoassay to detect free serum IgA antibodies against *Pseudomonas aeruginosa* and assessed its value in the early detection of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis.

Patients and methods

Thirty one patients with cystic fibrosis, proved by sweat test, who were attending the paediatric and adult cystic fibrosis clinics at St James's University Hospital, Leeds were investigated.⁷ In 19 of the patients *Pseudomonas aeruginosa* had never been isolated from sputum. Their age ranged from 5 months to 14 years, mean (SE) 6.3 (1.2). *Pseudomonas aeruginosa* had been isolated from sputum on one or more occasions during the previous year in six patients, but they had no recognisable illness attributable to that organism. Their ages ranged from 4-14 years, mean 9.7 (1.4). In six patients *Pseudomonas aeruginosa* had been isolated continuously for at least a year. Their age range was 8-21 years, mean 13.7 (1.8). They had recognisable chest illness attributable to *Pseudomonas aeruginosa*.

Fifteen patients attending the paediatric clinic at this hospital for other conditions acted as controls. Their mean age was 9.2 (1.4) years ranging from 8 months to 15 years. None had any evidence of lung disease or *Pseudomonas aeruginosa* infection.

Serum from patients and controls was stored at -20°C until immediately before use.

Clinical isolates of *Pseudomonas aeruginosa* were identified from sputum samples of patients with cystic fibrosis by the API 20 NE series of biochemical reactions.

Gram negative bacteria other than *Pseudomonas aeruginosa* (*Escherichia coli*, *Enterobacter cloacae*, *Citrobacter diversus*, *Citrobacter amoenitus*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Proteus*

mirabilis), which had been obtained from sputum samples of patients with cystic fibrosis, were used to test the antigenic cross reactivity with *Pseudomonas aeruginosa*. Strains were stored on nutrient agar slopes at 4°C and in nutrient broth at -196°C.

Pseudomonas aeruginosa was serotyped as soon as possible after isolation and identification. The *Pseudomonas aeruginosa* antiserum set produced by Difco (Detroit, Michigan, USA) was used; this is based on the international antigenic typing scheme.⁸

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)
An ELISA was developed to measure IgA antibodies against *Pseudomonas aeruginosa* antigens. Microtitre plates were prepared as previously described.³

A pool of serum samples with high titres was used for a standard curve on each plate. It was diluted between 1/150 and 1/13 000 in phosphate buffered saline, 1% bovine serum albumin. Test serum was diluted 1/200 or 1/1000 in the same diluent. Each sample and standard was incubated in triplicate for 75 minutes at room temperature followed by washing three times with phosphate buffered saline for five minutes. Goat antihuman IgA (α -chain specific) conjugated to horseradish peroxidase (Zymed Laboratories Inc, San Francisco, California, USA) was diluted 1/2000 in phosphate buffered saline, 1% bovine serum albumin, and 100 μ l added to each well and left for two hours at room temperature. The wells were washed three times with phosphate buffered saline for five minutes. The enzyme reaction was as previously described, except that the reaction was permitted to proceed for five minutes.³ Absorption at 492 nm was measured using a Dynatech plate reader. Absorbance was < 0.10 in wells containing no adsorbed antigen, or no test serum, or no labelled antibody. The blank was subtracted from the test absorbance. To standardise the results and eliminate day to day variation this value was converted to a dilution by reference to the standard curve. The figure shows a typical standard curve. The reciprocal of the dilution used for the patient's serum was divided by the reciprocal of the dilution obtained from the standard curve, multiplied by 1000, and expressed as a titre. Serum from each patient was tested against all antigens on the same day. The titre of three samples measured on four occasions gave means (SE) of 1195 (12), 665 (21), and 244 (9.6), respectively.

Gram negative bacteria used in the cross reaction studies were grown overnight as a lawn on cysteine lactose electrolyte deficient (CLED) agar plates at 37°C. Growth from each plate was removed and washed as described for *Pseudomonas aeruginosa*, then all strains were mixed together, each at a concentration of 10^{10} colony forming units/ml, before binding to microtitre plates, fixing, and blocking as described for

Figure ELISA standard curve for serum IgA antibodies to *P aeruginosa* serotype 1.

Pseudomonas aeruginosa. Serum samples (100 μ l) were incubated for 75 minutes at room temperature in these plates before being transferred to plates containing *Pseudomonas aeruginosa*, and the resulting titre compared with that produced by serum incubated only with *Pseudomonas aeruginosa*.

The volume of goat antihuman IgG (α -chain specific, Zymed Laboratories Inc) needed to precipitate serum IgG was determined by an initial titration. Fifty microlitres of serum was diluted 1:10 with phosphate buffered saline, 1% bovine serum albumin. The appropriate volume of goat antihuman IgG was added to 25 μ l diluted serum. Both portions of diluted serum were incubated for one hour at 37°C followed by 18 hours at 4°C. Phosphate buffered saline, 1% bovine serum albumin was then added to give a final volume of 5 ml, followed by centrifugation at 4°C for 30 minutes at 12000 g. The supernatant was removed and tested by ELISA for IgA and IgG antibodies against *Pseudomonas aeruginosa*.

Results

Serum from patients with cystic fibrosis was tested by ELISA against *Pseudomonas aeruginosa* alone, and against *Pseudomonas aeruginosa* after previous reac-

Table 1 Absorbance at 492 nm from serum of patients with cystic fibrosis against *Ps aeruginosa* with and without previous absorption by Gram negative bacteria

Case No	Serum dilution	<i>Ps aeruginosa</i>		Gram negative bacteria only	Previous isolation
		Only	After absorption		
1	1/1000	1.060	1.100	0.218	Chronic <i>Ps aeruginosa</i>
2	1/1000	3.280	3.190	0.347	Chronic <i>Ps aeruginosa</i>
3	1/1000	1.580	1.535	0.065	<i>Ps aeruginosa</i> , <i>E coli</i> , <i>H influenzae</i> , <i>S aureus</i>
4	1/200	0.560	0.630	0.054	<i>Ps aeruginosa</i> , no pathogens
5	1/200	1.060	0.960	0.281	<i>H influenzae</i> , <i>S aureus</i>
6	1/200	0.244	0.237	0.190	<i>H influenzae</i> , <i>S aureus</i>
7	1/200	0.361	0.320	0.233	<i>H influenzae</i> , <i>S aureus</i>
8	1/200	0.302	0.272	0.191	<i>H influenzae</i> , <i>S aureus</i>

tion against Gram negative bacteria. Representative results from eight patients are shown in table 1. The absorbance against *Pseudomonas aeruginosa* was essentially unchanged by previous reaction with Gram negative bacteria in patients from whom *Pseudomonas aeruginosa* was grown continuously (cases 1 and 2). There was no change in absorbance against *Pseudomonas aeruginosa* in one patient from whom *Pseudomonas aeruginosa* was grown intermittently (case 3), and in the other patient the absorbance against *Pseudomonas aeruginosa* was increased by 13% by previous reaction with Gram negative bacteria (case 4). The absorbance against *Pseudomonas aeruginosa* was decreased by 10% by previous reaction with Gram negative bacteria in four patients from whom *Pseudomonas aeruginosa* had never been grown (cases 5–8).

Removal of IgG antibodies from serum by immunoprecipitation had no effect on the IgA titre, so despite the sometimes high concentration of serum IgG antibodies there was no interference with the measurement of IgA antibodies from them.

Serum samples from 15 paediatric patients without cystic fibrosis and with no known *Pseudomonas*

aeruginosa infection were tested for serum IgA antibodies by ELISA (table 2). The sum titre to seven serotypes ranged from less than 105 to 252, with a mean of 139. The 99% confidence interval of the upper limit of normal was 18 to 260. The titres in serum from 19 patients with cystic fibrosis and with no known *Pseudomonas aeruginosa* infection were also low (table 2) and the 99% confidence interval of the upper limit was 24 to 250. There was no correlation between titre and age or sex in either group.

Table 3 shows the titre in serum from six patients from whom *Pseudomonas aeruginosa* had been isolated intermittently. It had been isolated less than 10 times from cases 1–4. Titres greater than control

Table 3 Serum IgA ELISA titre against *Ps aeruginosa* in patients from whom *Ps aeruginosa* was grown intermittently

Case No	<i>Ps aeruginosa</i> serotype							Sum
	1	3	6	9	10	11	NT*	
1	52	15	15	73	58	38	28	279
2	63	103	15	25	114	57	29	406
3	56	15	180	15	174	118	15	573
4	130	15	15	680	115	80	50	1085
5	280	270	68	270	456	400	222	1966
6	816	67	30	630	800	500	645	3490

Serotypes according to the international serotyping scheme,* non-typable strain.

Table 4 Serum IgA titre against *Ps aeruginosa* in patients with cystic fibrosis continually infected with *Ps aeruginosa*

Case No	<i>Ps aeruginosa</i> serotype							Sum
	1	3	6	9	10	11	NT*	
1	170	50	230	420	220	180	90	1360
2	610	300	170	320	710	670	1030	3810
3	320	90	175	360	480	3640	190	5260
4	118	175	350	1590	4350	855	357	7800
5	250	235	450	2940	2080	1610	2500	10100
6	313	1064	222	4000	3130	1890	2500	13100

Serotypes according to the international typing scheme,* non-typable.

Table 2 Serum IgA titre against *Ps aeruginosa* in patients with and without cystic fibrosis with no *Ps aeruginosa* infection

	Patients without cystic fibrosis (n = 15)	Patients with cystic fibrosis (n = 19)
Sum titre to seven serotypes	105–252	105–248
Mean (SE)	136 (13)	139 (10)
Confidence levels of the upper limit 99% CL	18 to 260	24 to 250
Age range	10 months–15 years	5 months–14 years
Mean (SE) (years)	9.2 (1.4)	6.26 (1.2)

Strains used were serotype 1, 3, 6, 9, 10 and 11, and a non-typable strain.

values were present against up to five strains. *Pseudomonas aeruginosa* had been isolated intermittently for six to 12 months from cases 5 and 6. Titres against all serotypes were greater than control values in these two patients.

The titres of six patients from whom *Pseudomonas aeruginosa* had been grown continuously for at least six months are shown in table 4. There was a wide spread of titres to individual strains. Most patients in this group had a higher total titre than patients who grew *Pseudomonas aeruginosa* intermittently, although there was considerable overlap between the two groups.

Discussion

The purpose of this work was to develop a sensitive and specific assay to measure free serum IgA antibodies directed against *Pseudomonas aeruginosa*. Strains representing the six commonest serotypes in our patients and a non-typable strain were used.³ We have previously shown that different strains of the same serotype did not always produce the same IgG titre with one serum sample, which is presumably due to qualitative or quantitative differences in the exposure of lipopolysaccharide or protein antigens. We used the same strains in the assay reported here and in the assay we previously developed to measure serum IgG antibodies.³

The contribution of cross reacting antibodies (those primarily directed against other Gram negative bacteria) to this assay was negligible in patients with *Pseudomonas aeruginosa* infection and not more than 10% in patients with no infection. We also found no cross reaction in the similar assay we reported which measured free serum IgG antibodies to *Pseudomonas aeruginosa*.³ Other workers have reported no significant cross reaction in immunoassays using whole cells or extracellular products.^{9,10,11} The antibody titre resulting from this method of immunoassay is affected not only by antibody concentration, but also by antibody affinity.¹² Short serum incubation times and high serum dilutions will reduce the detection of low affinity antibodies. These will include both cross reacting antibodies and those produced in response to general environmental exposure, whereas antibodies produced in response to a specific infection will be of high affinity.^{12,13}

The serum IgA titre was low in both patients with cystic fibrosis and those who had neither the disease nor a history of *Pseudomonas aeruginosa* infection. The 99% confidence intervals of the upper limit for the two groups of patients were 18 to 260, and 24 to 250, respectively. Patients with cystic fibrosis who had intermittent isolations of *Pseudomonas aeruginosa* or continuous infection by that organism all had titres

above these values. There was a wide spread of titres in both groups of patients and considerable overlap between the groups.

The initial humoral response to bacterial challenge of mucosal surfaces is the production of secretory IgA by plasma cells in the pulmonary mucosa.^{14,15} Secretory IgA agglutinates bacteria and so prevents adhesion to surface epithelial cells, as well as reducing the bacterial growth rate. It also neutralises bacterial exotoxins but is not efficient at bacterial opsonisation.^{14,15,16} If this response is inadequate, IgG antibodies are produced in the local lymph nodes, and to a lesser and variable extent locally, and pass by transduction into the respiratory fluids or into the serum.^{14,16} In the canine respiratory tract the most important immunoglobulin in the upper respiratory tract is secretory IgA, but there is a gradual change so that in bronchial washings the comparative concentrations of IgG and secretory IgA are reversed and approach the ratio found in serum.¹⁴ Most of the IgA released from plasma cells is a dimer that is converted to secretory IgA before release into the respiratory fluids, but about 10% is released as a monomer that then passes into the serum rather than the respiratory fluids.¹⁴

Because secretory IgA is the initial humoral response to infection in the lungs, it is possible that at the onset of infection an increase in specific serum IgA antibodies may occur before an increase in serum IgG antibodies. This could be useful in the management of patients: eradication of bacteria is more likely after early antibiotic treatment of pulmonary infection, given the problem of poor antibiotic access from serum to the intrapulmonary cavity, which is compounded by the tissue damage that occurs during infection. Alternatively, a defective IgA response may be present in some patients, which contributes to pulmonary infection.

The ratio of IgA and IgG concentrations may provide an indication of the depth of pulmonary infection, and also an insight into the reasons for the observed differences in prognosis of different patients with *Pseudomonas aeruginosa* infection.

In conclusion, the assay we have described is a specific measure of free serum IgA antibodies directed against *Pseudomonas aeruginosa* cell surface antigens. The contribution of cross reacting antibodies to this assay is negligible. Antibody titres in patients and in controls with no infection were low, and titres were raised in patients who had *Pseudomonas aeruginosa* infection. The use of this assay in assessing the patients' prognosis and in providing additional information about the state of infection is currently being investigated.

We thank Glaxo Laboratories and the Cystic Fibrosis Research Trust, UK, for financial support.

References

- 1 Pier GB. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. *J Infect Dis* 1985;151:575-80.
- 2 Mearns MB, Hunt GH, Rushworth R. Bacterial flora of respiratory tract in patients with cystic fibrosis, 1950-1971. *Arch Dis Child* 1972;47:902-7.
- 3 Brett MM, Ghoneim ATM, Littlewood JM, Losowsky MS. Development of enzyme linked immunosorbent assay (ELISA) to detect antibodies to *Ps aeruginosa* cell surface antigens in sera of cystic fibrosis patients. *J Clin Pathol* 1986;39:1124-9.
- 4 Brett MM, Ghoneim ATM, Littlewood JM. Serum IgG antibodies in patients with cystic fibrosis with early *Pseudomonas aeruginosa* infection. *Arch Dis Child* 1987;62:357-61.
- 5 Brett MM, Ghoneim ATM, Littlewood JM. Prediction and Diagnosis of early *Pseudomonas aeruginosa* infection in cystic fibrosis in a follow up study. *J Clin Microbiol* 1988 (in press).
- 6 Brett MM, Ghoneim ATM, Littlewood JM. Serum antibodies to *Pseudomonas aeruginosa* in cystic fibrosis. *Arch Dis Child* 1986;61:114-20.
- 7 Gibson LE, Cooke RE. A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine iontophoresis. *Pediatrics* 1959;23:545-9.
- 8 Liu PV, Matsumoto H, Kusama H, Bergen T. Survey of heat-stable major somatic antigens of *Pseudomonas aeruginosa*. *International Journal of Systematic Bacteriology* 1983;33:256-64.
- 9 Cost KM, West CS, Brinson D, Polk Jr HC. Measurement of human antibodies against *Escherichia coli* and *Pseudomonas aeruginosa* using formalin treated whole cells in an ELISA technique. *J Immunoassay* 1985;6:23-43.
- 10 Ericsson Hollsing A, Granstrom M, Vasil ML, Wretling B, Strandvik B. Prospective study of serum antibodies to *Pseudomonas aeruginosa* exoproteins in cystic fibrosis. *J Clin Microbiol* 1987;25:1868-74.
- 11 Kohler RB, Wheat LJ, White A. Rapid diagnosis of *Pseudomonas aeruginosa* urinary tract infections by radioimmunoassay. *J Clin Microbiol* 1979;9:253-8.
- 12 Butler JE, Feldbush TL, McGivern PL, Stewart N. The Enzyme-linked immunosorbent assay (ELISA): a measure of antibody concentration or affinity? *Immunochemistry* 1978;15:131-6.
- 13 Sell S. *Immunology, immunopathology and immunity*. 3rd ed. Hagerstown: Harper and Row, 1980.
- 14 Benfer Kaltreider H. Expression of immune mechanisms in the lung. *Am Rev Respir Dis* 1976;113:347-79.
- 15 Quie PD. Lung defence against infection. *J Paediatr* 1986;108:813-6.
- 16 Moss RB. Immunology of cystic fibrosis: immunity, immunodeficiency and hypersensitivity. In: Lloyd-Still JD, ed. *Textbook of cystic fibrosis*. Bristol: John Wright, 1983:109-51.

Requests for reprints to: Dr M M Brett, Department of Paediatrics and Clinical Health, St James's University Hospital, Leeds LS9 7TF, England.