Antibody response of fibrocystic patients to homologous 0-typable and 0-defective isolates of *Pseudomonas aeruginosa*

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Abstract

Antibody titres to *Pseudomonas aeruginosa* of sera from 60 adult fibrocystic patients were determined in an enzyme linked immunosorbent assay (ELISA) with whole cells of homologous isolates which had been classified according to 0-antigen state by their reactivity with 0-typing antisera. Patients who were continuously colonised with *Ps aeruginosa* gave the highest titres: range 1500-64000 (mean 11000) and 500-48000 (mean 9000) with homologous 0-typable and 0-defective isolates, respectively. Lower titres to both varieties of isolates were obtained with recently colonised patients, and non-colonised patients gave titres with reference laboratory strains marginally above those of healthy controls. Serum titres of patients with sequential isolates were strain dependent and did not correlate with the 0-antigen state of the strain. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of these sera and strains showed antibody binding primarily to high molecular weight 0-repeating units of lipopolysaccharide.

It is concluded that the 0-antigen of the strain of *Ps aeruginosa* used in the ELISA test does not influence the titre obtained with fibrocystic sera, and it is recommended that serum titres should be assessed with a panel of homologous isolates from patients.

The lungs of patients with cystic fibrosis are frequently colonised with or infected by *Pseudomonas aeruginosa* despite the fact that serum antibodies, often in high titre, towards the organism and its products are evident.¹⁻³ Approaches to the choice of test antigens vary considerably among laboratories but there is general agreement that antibody titres to *Ps aeruginosa* antigens rise when the infection progresses to a chronic state. The apparent decrease of titres to somatic antigens after treatment, as described by Brett *et al*,⁴ contrasts, however, with the finding that serum titres to *Pseudomonas* exoproteins are not influenced by exacerbations of pulmonary infection or antibiotic regimens.⁵ This contradiction may be due to the choice of strain or antigen for the determination of antibody

in a particular patient's sera and the length of time the patient has been colonised.

Lipopolysaccharide (LPS) is the dominant structure of the outer membrane of *Ps aeruginosa*, and serotype specificity is determined by the repeating 0-polysaccharide side chains present in smooth strains.⁶ Most *Ps aeruginosa* from the serum samples of patients with cystic fibrosis, however, are rough or defective in 0-specific antigens.⁷ The value of using smooth test strains for the determination of serum antibody in such patients colonised with rough strains is therefore debatable. We set out to examine whether there were differences between the antibody titres of adult fibrocystic patients towards their homologous isolates, taking account of the 0-antigenic state of the strains.

Methods

Serum samples were obtained from 54 adult fibrocystic patients who were colonised or infected with *Ps aeruginosa* in their sputum and six patients who were negative for the organism by culture. A panel of six control sera from healthy subjects was also included. Sputum was liquefied by the addition of 1 mg/ml (w/v) pancreatin (BDH) and incubated at 37°C for two hours and plated on King's A agar⁸ and *Pseudomonas* Isolation Agar (Difco). Non-pigmented cultures were identified as *Ps aeruginosa* by the scheme of King and Phillips.⁹

Ps aeruginosa strains were serotyped by slide agglutination with 17 0-typing sera of the International Antigenic Typing Scheme¹⁰ and two serum samples towards LPS defective variants.¹¹ LPS was prepared from an overnight tryptone soy broth culture (5 ml) of a strain by the proteinase K digestion method.¹² Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% resolving gels.¹³ LPS bands in gels were detected with a modified silver stain¹⁴ with carbonate developer (0.28 M sodium carbonate containing 0.05% (v/v) formaldehyde). Electrotransfer and immunoblotting were performed as described previously.¹⁵

For the ELISA, strains were grown on tryptone soy agar overnight at 37°C and the growth was harvested in carbonate buffer (pH 9.6) and adjusted by dilution to an optical density of 0.06 at 600 nm in the same buffer. Flat-bottomed microtitre plates (Sterilin) were coated overnight at 37°C with 100 µl of

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Table 1 ELISA titres of patients and controls toward 0-typable and 0-defective isolates of *Ps aeruginosa*

Patient group (n)	Mean ELISA titre (range)	
	0-typable isolates	0-defective isolates
All patients (60)	9000 (1000–64000)	7500 (500–48000)
Recently colonised* (16)	3500 (1000–4000)	4500 (1000–7000)
Continuously colonised* (38)	11000 (1500–64000)	9000 (500–48000)
Non-colonised† (6)	1500 (200–6400)	1500 (200–6400)
Controls† (6)	200 (100–400)	200 (100–400)

*Titre with homologous isolates recovered prior to serum date.

†Titre with serotype strain 06 and reference 0-defective strain.¹¹

bacterial suspension and washed three times in phosphate buffered saline (pH 7.4) with 0.05% (v/v) Tween 20 (PBST). Serum samples were diluted serially in PBST and 100 µl volumes were incubated at 37°C for two hours. The plates were washed five times with PBST, and 100 µl of peroxidase conjugated anti-total immunoglobulin (Sigma) was added to each well and incubated for a further two hours at 37°C. After three washes in PBST the reaction was developed with 100 µl of 0-phenylene diamine reagent (Sigma), and stopped after 15 minutes with 12.5% (v/v) sulphuric acid, and the absorbance read at 450 nm. Serum specimens were tested in duplicate and repeated on at least one occasion. Batch variation was controlled by the use of a standard rabbit antiserum (anti-06) and a whole cell suspension of the homologous serotype strain which gave reproducible absorbance values. The titre of a patient's serum was taken as the reciprocal of the dilution of serum which gave 50% of the maximum absorbance obtained with the standard rabbit serum.

Results

Representative colonies from primary plates of sputum specimens were purified and serotyped. Fifty two of the 54 culture positive patients were colonised with serologically unclassified strains—that is, they were either not agglutinated by any of the 0-sera (not typable) or gave multiple reactions with three or more sera (polyagglutinable). All unclassified strains reacted with sera to 0-defective

antigens.¹¹ Heavily mucoid or dry colonies which could not be emulsified in saline were not examined further. Twenty one patients also harboured 0-typable strains and the serotypes found in order of prevalence were 06 (n = 7), 03 (n = 5), 09 (n = 3), 011 (n = 2) 01 (n = 2), 02 and 010 (n = 1 of each). One patient harboured two strains of different serotypes.

Table 1 gives the mean and range of ELISA titres of the 60 fibrocystic sera and the six control sera with, where appropriate, 0-typable and 0-defective homologous isolates, or the serotype strain 06 that was included as a reference control. Each of the control sera gave titres of 400 or less and the maximum titre obtained with a colonised patient's serum was 64000. The mean titres of colonised patients with both groups of isolates were similar but patients who were continuously colonised (more than three years) gave titres generally two fold higher than those recently colonised; a wider range of titres was also found in the former group. Overall, patients had higher titres against 0-typable than 0-defective strains, but this was reversed for patients recently colonised with *Ps aeruginosa*. One of the non-colonised patients gave a titre of 6400 with both strain 06 and a reference 0-defective variant,¹¹ and two others had titres of 1000 with strain 06 but were within normal limits of the 0-defective strain.

It was clear that a cut-off of clinically important titre, which would distinguish between newly colonised and persistently colonised patients, could not be selected. Furthermore, as an average of six isolates was recovered from each of the 54 patients positive for *Ps aeruginosa* it was necessary to examine in detail the antibody titres of selected patients with each of their isolates recovered at the time of the serum sample or before that date. Immunoblotting of LPS extracts separated by SDS-PAGE was also performed to identify differences in antibody binding with LPS constituents.

Case 1 (table 2) had first been colonised about six months before the serum collection date with a serotype 09 non-mucoid strain. When the serum sample was taken two polyagglutinating variants of mucoid and non-mucoid colonial forms were isolated. The highest ELISA titre was recorded for the mucoid *Ps aeruginosa* variety, although the response to the original 09 strain was similar; the second *Ps aeruginosa* variant gave a much lower titre. Type specific antibody, judged by the response to the serotype 09 reference strain, was of a similar titre to that obtained with the homologous 09 strain.

Silver stains of the LPS profiles of isolates from case 1 (fig 1a) showed numerous densely stained bands in the upper part of the gel of the 09 isolates, corresponding to high molecular weight repeating units of LPS. Isolates 2a and 2b contained only low molecular weight LPS core components, evidenced by the lack of bands in the upper gel. The corresponding immunoblot with this patient's serum (fig 1b) showed antibody binding to the ladder bands, but no reaction with LPS core components of any of the isolates was visible.

Table 2 Serum ELISA titres of patients with homologous isolates of *Ps aeruginosa*

Case No	Duration (months)	Sample/ isolate	Mucoid	Serotype	Titre
1	1 6*	1	—	09	2000
		2a	+	Polyagglutinable	4000
		2b	—	Polyagglutinable	500
2	2	a	+	06	1500
		1b	—	06	2000
		1c	—	06	8000
		2a	+	06	6000
		2b	—	06	3000
		3	+	06	16000
	4 5 6	4	+	06	8000
		5a	+	Polyagglutinable	64000
		5b	—	Polyagglutinable	4000
		6a	+	Polyagglutinable	30000
		6b	—	Unclassifiable	4000
		7	—	Unclassifiable	12000
3	9*	7a	—	06	6000
		7b	—	Polyagglutinable	1500
	1	1a	+	Polyagglutinable	6000
		1b	—	Non-typable	4000
		1c	—	Non-typable	4000
	2*	2a	+	Polyagglutinable	8000
		2b	—	Polyagglutinable	10000
		2c	—	Polyagglutinable	8000

*Serum sample taken.

Figure 1a Silver stain of LPS extracts of isolates from case 1 after SDS-PAGE.

Figure 1b Immunoblot with homologous serum diluted 1 in 1000.

Figure 2a Silver stain of LPS extracts of isolates from case 2 after SDS-PAGE.

Figure 2b Immunoblot with homologous serum diluted 1 in 1000.

In contrast, case 2 had had *Ps aeruginosa* for seven years and 13 isolates recovered over eight months before the serum collection dates were available. Table 2 shows that the ELISA titre for the serotype 06 strain ranged from 1500 to 16000 and the titre varied towards the same 0-serotypable phenotype in different samples. For example, the titre with the isolate 1a was about 10-fold lower than that with isolate 3. Variation in titre was also found with 0-defective strains, as shown with isolates 5a (64000) and 5b (4000) from the same specimen.

SDS-PAGE of 10 isolates showed that despite the lack of specificity in agglutination tests only one of the *Ps aeruginosa* strains (5b) was grossly deficient in high and medium molecular weight LPS components (fig 2a). Furthermore, antibody binding to LPS core and lipid A was either extremely weak or non-existent when compared with bands formed by antibody to other LPS components.

Case 3 had also persistently harboured *Ps aeruginosa* for at least seven years and the six isolates recovered over two months were serologically atypical and two of them were mucoid (table 2). The titres ranged from 1500 to 10000 and no association between the phenotype of the isolate and titre was evident. SDS-PAGE showed variation in LPS banding pattern, as found with other patients' isolates, and immunoblotting showed weak antibody binding only to high molecular weight LPS. This pattern of reactivity to different titres with various isolates was repeated in a further six serum samples and similar immunoblot profiles were obtained.

Discussion

Brett *et al* showed that an ELISA test with

whole cells of standard strains of *Ps aeruginosa* could be used to measure serum IgG antibodies to cell surface antigens in paediatric fibrocystic patients and that titres were correlated with the stage and severity of infection.¹⁶ We began this study with the view that because many of the strains of *Ps aeruginosa* from adult patients are defective in the synthesis of O-repeating units which confer serotype specificity, it was probably inappropriate to use strains in the ELISA that would not be representative of those commonly isolated from patients.

We classified isolates from patients according to their serotype and found that those who had been culture positive for several years generally gave higher titres with both O-specific and O-defective strains than those who had been recently colonised. This contrasts with the findings of a recent study which concluded that the specificity of the antibody response with *Pseudomonas* LPS increased with the duration of infection.¹⁷ We were unable to study sequential sera, however, and did not differentiate between IgG and IgM antibody; the latter is apparently similar in concentration in chronic and non-colonised fibrocystic patients.¹⁷ Nevertheless, we can confirm that non-colonised patients seldom had higher titres of antibody against either antigenic variety of *Ps aeruginosa* and were only marginally above those of controls.

Serum titres towards sequential isolates of individual patients showed that the antibody response was highly strain dependent, but the magnitude of titres did not correlate with the O-antigenic state of the isolates. Brett *et al* found that the laboratory strain of the serotype that produced the highest ELISA titre with a patient's serum sample was not always the same serotype as the sputum isolate.¹⁶ They reasoned that this was probably due to several factors, including failure to detect the serotype in the patient's sputum, antibiotic treatment, or the delay of serum IgG formation. Alternatively, antibody may be directed against other antigenic structures such as outer membrane proteins which are antigenically conserved. One might expect, however, that the antibody titre of a single serum sample with a variety of strains would be similar if the antibody reacted with a conserved antigen. Moreover, the conserved outer membrane protein antigens are highly immunogenic and in rabbits produce titres far in excess of those engendered by LPS, and titres are reproducible with different strains.¹⁸

SDS-PAGE showed that minor variations in LPS banding profile resulted in a loss of serotype specificity as some strains that were *Ps aeruginosa* by slide agglutination contained O-repeating units while other *Ps aeruginosa* strains showed a complete loss of ladder bands. Nevertheless, immunoblotting often illuminated O-bands not detected by silver staining; a feature also observed by Cochrane *et al*.¹⁹

Immunoblots of LPS extracts of sequential isolates from three patients confirmed the antigenic activity of LPS in vivo, and the contrast in the intensity of antibody binding to high and medium molecular weight LPS, com-

pared with core components was quite noticeable. Hancock *et al* reported that 74% of chronically infected fibrocystic patients had antibody that bound to core LPS²⁰ and a recent Danish study seems to support this.¹⁷ Our inability to detect clinically important antibody binding to core LPS may be a reflection of our methodology, but we consider this unlikely as positive blots with core LPS with hyper-immune rabbit sera had been obtained in routine tests. In our experience core LPS strains are less immunogenic than those with complete LPS and therefore may give rise to lower antibody reactivity in ELISA and immunoblotting. The response of both of the persistently colonised patients may have been directed mainly towards the more immunogenic portions of the LPS.

Jacobsen *et al* suggested that antibodies to *Ps aeruginosa* core LPS were present in chronic but not acute *Pseudomonas* infection, and 11 of 18 fibrocystic patients had demonstrable serum antibody to core LPS.²¹ Further studies are clearly needed with purified and chemically characterised LPS constituents to identify the importance of antibody responses to various regions of the molecule.

We conclude that an ELISA method with whole bacterial cells is a sensitive measure of serum antibody in cystic fibrosis. The titre of the response of individual patients' serum is strain dependent, however, and does not correlate with the O-antigen composition of the isolate. Our data therefore question the use of standard laboratory strains in the ELISA and we suggest that a variety of isolates recovered from the sputum should be tested individually to determine the range of titres of patients' sera.

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