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Detection of Antibodies to *Pseudomonas aeruginosa* Alginate Extracellular Polysaccharide in Animals and Cystic Fibrosis Patients by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay was developed to detect antibodies to sodium alginate exopolysaccharide purified from three strains of *Pseudomonas aeruginosa* and commercial alginate from seaweed. Good attachment of alginate occurred with polystyrene microtiter plates at pH 7.0 with 0.04 M sodium phosphate buffer. With the enzyme-linked immunosorbent assay procedure, antibodies to alginate (not previously shown to be immunogenic) could be shown in humans and after immunization of mice and rabbits. Antibody to one of the alginates cross-reacted with two other *P. aeruginosa* alginates and commercial seaweed alginate. In animal immunization antibody titers were maximal after a single intravenous injection with an optimal dose of *P. aeruginosa* 3064 alginate. Healthy controls not known to have a previous *P. aeruginosa* infection had low, but detectable, antibody titers to 3064 and commercial alginate. Three cystic fibrosis patients not colonized with *P. aeruginosa* had similar antibody levels. Twenty-eight cystic fibrosis patients colonized with *P. aeruginosa* formed a clearly separate group with antibody titers higher than that of the control and noncolonized cystic fibrosis patients. Antibody titers to 3064 or commercial alginate did not increase during acute *P. aeruginosa* bronchopneumonitis in 16 cystic fibrosis patients or after repeated episodes in 4 patients.

A large percentage of patients with cystic fibrosis (CF) ultimately undergo pulmonary colonization and infection with *Pseudomonas aeruginosa*. After recurrent episodes with pulmonary infection, patients frequently succumb to a *P. aeruginosa* bronchopneumonitis superimposed upon the effects of progressive pulmonary destruction. A high percentage of isolates of *P. aeruginosa* from CF patients exhibit a "mucoid" character, at least on initial isolation (3, 7, 9, 26). The major component of the mucoid material has been identified as the polysaccharide alginic acid (8, 14). The production of alginic acid is only uncommonly detected in association with *P. aeruginosa* isolates from other types of infections.

The role of alginate in the pathogenesis of *P. aeruginosa* pulmonary infections in CF is not understood. It has been demonstrated that the production of this material is energetically very unfavorable for the organism (19). Thus, it seems probable that the substance has an important role to play in maintenance of the organism within a bronchopulmonary environment. It seems likely that the alginate extracellular polysaccharide acts as an antiphagocytic factor (24). It is clear that the polysaccharide capsule of *Streptococcus pneumoniae*, for example, is a potent antiphagocytic factor. Although these findings and the pneumococcal analogy suggest an antiphagocytic role for alginate, Baltimore and Mitchell found that "slime" did not inhibit opsonization of two nonmucoid strains (1). As well, there does not seem to be a difference in the efficacy of clearance of mucoid and nonmucoid strains from the lungs of guinea pigs (4).

Several workers have demonstrated that antibodies are produced to various components of *P. aeruginosa*, both in serum and in pulmonary secretions (2, 7, 9, 11, 18, 23). To date, there has not been a report of the demonstration of anti-alginate antibody in serum, although some effort has been made to detect it (9). One factor that has interfered with the search for anti-alginate antibody has been the lack of a reliable and sensitive procedure for the detection of such antibody.

In this paper we report that development of an enzyme-linked immunosorbent assay (ELISA) with alginate as the antigen, the demonstration

of low levels of anti-alginate antibody in the sera of several patients with CF, and the demonstration that alginate is immunogenic.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains 3064, 1468, and 1591 were clinical isolates from patients with CF.

Purification and characterization of *P. aeruginosa* alginate. *P. aeruginosa* strains were grown on Mueller-Hinton agar (GIBCO Canada, Burlington, Ontario) containing deoxycholate (5 g/liter), sodium thiosulfate (5.4 g/liter), and glucose (20 g/liter). After growth for 7 days, strains generally had a very marked mucoid character. Five to ten milliliters of phosphate-buffered saline was added, and each plate was rotated. Plates were tipped, and the fluid was removed and combined from 5 to 10 plates. The viscous solution was digested with RNase A type 1-A (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.), and DNase I (150 μg/ml, Sigma) for 2 h at 37°C. At that time protease (1.5 mg/ml, purified type XIV; Sigma) was added, and the incubation was continued at 37°C overnight with stirring every 20 min for the first 2 h. After digestion steps the preparation was centrifuged at 10,000 × *g* for 15 min, and the supernatant was recovered (crude alginate).

Alginate from the supernatant was precipitated by adding 4 volumes of 80% ethanol. Alginate was removed by winding it about a glass rod and was redissolved in the original volume of distilled water. Calcium chloride (final concentration, 0.1%) was added to the alginate preparation adjusted to 1 mg of crude alginate per ml. This was centrifuged at 150,000 × *g* for 120 min, and the supernatant was dialyzed against water for 8 h. The supernatant was concentrated to approximately fivefold so that the concentration of crude alginate was 5 mg/ml. Sodium chloride was added to obtain a final concentration of 9 g/liter. Precipitation with 80% ethanol was again carried out, and the precipitate was collected by centrifugation at 30,000 × *g* for 30 min. The precipitate was lyophilized and dissolved at 5 mg/ml in water with 9 g of NaCl per liter added. These ethanol precipitation steps were repeated until no protein or 3-deoxy-D-manno-octulosonic acid could be detected. The final preparation of alginate was suspended at 5 mg/ml (purified alginate).

Assays were performed for 3-deoxy-D-manno-octulosonic acid (10), endotoxin-like activity by the *Limulus* lysate assay (Sigma), DNA (5), RNA (22), acetyl content of alginate (17), protein (15), and uronic acids (25) on preparations containing 5 mg of alginate per ml. Purified preparations were also scanned at 260 nm for nucleic acids, and 100 μg was applied to gel electrophoresis (6) and stained for protein with 1% Coomassie blue. No absorbance or protein was detected by these methods.

ELISA reagents and procedure. Polystyrene plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with purified or commercial alginate at a final concentration of 1.2 or 0.24 μg of alginate per well. Two hundred microliters of the alginate preparation (6 or 1.2 μg/ml) dissolved in 0.04 M sodium phosphate buffer (pH 7.0) was applied to each well of the plate and left at 4°C for a minimum of 15 h. Thereafter, each well was washed three times with the same buffer containing 0.05% Tween 20 (Sigma). Plates were stored at −20°C or used immediately. Alternative coating procedures that were found to be much less successful were identical, except that the suspending buffer was 0.1 or 0.04 M Na_2CO_3 (pH 9.8). To test for anti-alginate antibodies, 0.2 ml of variously diluted serum was added per well and allowed to incubate for 2 h at 25°C. Each well was washed three times with phosphate-buffered saline containing 0.05% Tween 20. Thereafter, 0.2 ml of either rabbit, human, or mouse anti-immunoglobulin G (IgG) conjugated with horseradish peroxidase (Miles Laboratories, Rexdale, Ontario) was added per well and incubated at room temperature for 2 h. The plates were washed with phosphate-buffered saline–Tween 20. The substrate utilized was 0.012% H_2O_2 in citrate phosphate buffer (pH 5.0) containing 0.4 mg of *o*-phenylenediamine dihydrochloride per ml. Reaction mixtures were incubated at 37°C in the dark for 30 min. Control wells were treated identically, except that phosphate-buffered saline replaced the serum (serum control), the conjugated anti-IgG (conjugate control), or the substrate (substrate control). Endpoints were wells with an optical density reading twice that of a serum control. All assay results were examined at 450 nm in a Microelisa Auto Reader (Dynatech).

Cross-reactivity against 14 pneumococcal polysaccharides was determined using the following modification of the above procedure. The preparation used as a source of pneumococcal polysaccharide was the vaccine Pneumovax (Merck Pharmaceuticals, Montreal) diluted in 0.04 M phosphate buffer (pH 7.0) to contain from 15 to 1,500 ng of each of the 14 polysaccharides per ml. Cross-reactivity with the group polysaccharide of *Streptococcus pyogenes* was determined by extracting the group polysaccharide as previously described (12). The extracted material was diluted in 0.04 M phosphate buffer (pH 7.0) to give a final concentration of 5 μg/ml. Each well was coated with 1 μg of the streptococcal preparation. Antisera to provide positive control reactions for pneumococcal and streptococcal polysaccharides, respectively, were Omni sera (Statens Serum Institut, Denmark) used at a dilution of 1:50 and group A streptococcal antiserum (Burroughs-Wellcome Co., Research Triangle Park, N.C.) used at a dilution of 1:50.

P. aeruginosa serotyping antiserum was obtained from Nishimen (Tokyo, Japan) and used at a dilution of 1:16. Commercial seaweed alginate was obtained from Sigma.

Sera. The immunogenicity of alginate and the preparation of positive control sera were determined using New Zealand rabbits of approximately 2 kg and BALB/c mice. Rabbits were immunized by several immunization schedules with either purified or crude *P. aeruginosa* alginate. The immunization schedule used was a first injection of 500 μg of purified alginate followed by a second injection of the same amount in 3 weeks and a third injection in another 10 days. No increase in titer occurred with the second or third injection.

Five mice each were given an initial dose of 25, 60, 125, or 250 μg of purified alginate from strain *P. aeruginosa* 3604 by the intravenous route. Initial antibody titers were determined in 3 weeks, and a second similar injection was given. Further antibody titers were determined at 4 weeks and again at 6 weeks. The immunogenicity of the alginate of strains 1468 and 1591 was compared by using the same immunization

protocol with a 60-μg dose of alginate and by determining antibodies at the times noted for strain 3064.

Human serum was obtained at the time of admission and discharge from 16 CF patients admitted because of acute *P. aeruginosa* bronchopneumonitis and from 4 patients admitted one or two additional times for the same situation. In addition, 28 single serum samples were examined from 28 CF patients colonized with *P. aeruginosa*. All patients had been colonized for a minimum of 2 years. Control serum was obtained from 16 apparently normal volunteers. Three serum samples from two CF patients not colonized with *P. aeruginosa* were also examined.

RESULTS

Purification and characterization of alginic acids from mucoid strains of *P. aeruginosa*. The alginic acid obtained from *P. aeruginosa* strains 3064, 1591, and 1468 have the composition specified in Table 1. Highly concentrated preparations did not contain any detectable protein, RNA, DNA, or 3-deoxy-D-manno-octulosonic acid. Thus, the alginic acid preparation, in terms of the preceding possible contaminating materials, was at least 99.99% pure by weight. Purified preparations contained 7 to 32 ng of endotoxin-like activity per mg of alginate. Crude alginate had greater than 10 μg of entotoxin-like activity per mg of alginate. Alginic acid preparations did not react in the ELISA assay with antisera for each of the *P. aeruginosa* serotypes described by Homma et al. (9a). The serotype antigens for this system are considered for the most part to be lipopolysaccharide. Antiserum raised to the purified alginate preparations did not agglutinate the type-specific strains used for the Homma serotyping system, nor did it agglutinate nonmucoid derivatives of *P. aeruginosa* strains 3064, 1591, and 1468.

ELISA procedure to detect antibodies to *P. aeruginosa* alginic acid. Preliminary studies demonstrated that attachment of the *P. aeruginosa* alginate to polystyrene trays was inconsistent and frequently not detectable at pH 9. The use of a low-ionic-strength phosphate buffer at pH 7.0 allowed consistent reproducible attachment of sodium alginate as determined by endpoint titrations of a standard rabbit antiserum. Of 100 determinations, endpoints were identical in 65 cases and within 1 twofold dilution in 98, and 2 had a twofold difference.

The optimal concentration of alginate antigen was determined by a checker board titration with various concentrations of purified alginate and various dilutions of rabbit, mouse, or human antiserum to alginate. The concentration selected was associated with a linear response between optical densities and a range of serial twofold dilutions of serum. The linear relationship between serum dilution and optical density for the method is shown in Fig. 1 for human and rabbit sera. The use of higher concentrations of alginate to coat polystyrene plates did not produce any significant increase in the capability of the ELISA method to detect lower concentrations of anti-alginate.

TABLE 1. Properties of *P. aeruginosa* alginate from strains 3064, 1468, and 1591

Strain	% Mannuronic	% Guluronic	% Acetylation
3064	66.4	33.6	4.1
1468	34.2	65.8	8
1591	67	33	12

Polylysine used in association with alginate to coat the polystyrene trays did not increase the sensitivity of the ELISA procedure. Polylysine alginate-coated plates were associated with two- or fourfold lower titers of antibody than the direct attachment method.

Cross-reactivity of anti-alginate antibody against other polysaccharides was examined by coating polystyrene trays with a preparation containing 14 pneumococcal polysaccharides or a partially purified preparation of the group specific antigen extracted from a strain *S. pyogenes*. These polysaccharides were attached to the polystyrene under the same conditions as used for attachment of alginate. No cross-reactivity could be demonstrated with wells containing concentrations as high as 1 μg of the streptococcal preparation and 1.5 μg of each of the 14 polysaccharides. Although we did not examine the potential sensitivity of the system, we were able to detect positive ELISA reactions (optical density readings 2 times or greater than that of control wells) in wells containing 14 ng of each of the 14 pneumococcal polysaccharides with commercial omnisera.

Absorption of a preparation of human antiserum with a titer of 1:4,000 with either purified 3064 alginate or seaweed alginate at 4°C for 24 h reduced the titer to less than 1:4 for 3064 or seaweed alginate.

Immune response to alginate in animals and humans. A variety of schedules were used in preliminary studies to determine the optimal immunizing schedule for alginate. Preliminary studies carried out in mice demonstrated that the use of either Freund complete adjuvant or the soluble alginate by the intraperitoneal route of immunization or a combination of subcutaneous Freund adjuvant and intraperitoneal immunization with two intravenous immunizations did not produce significantly greater antibody titers than the immunization schedule described above. It was worthy of note that antibody titers were maximal 3 weeks after the first injection when a 60-μg dose of purified alginate was used as the initial immunizing dose. Table 2 shows antibody

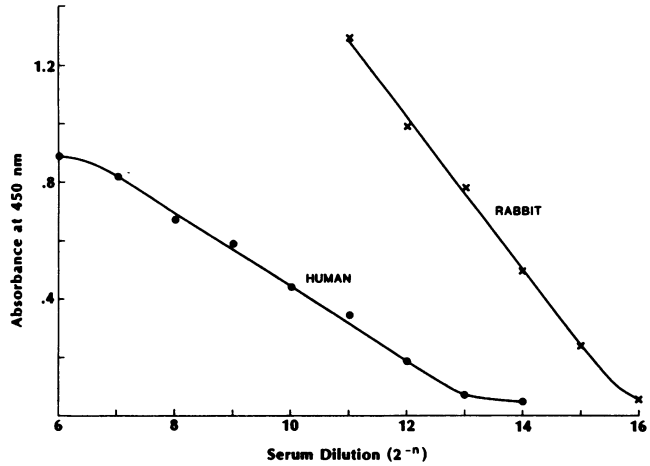

FIG. 1. Relationship of absorbance at 450 nm and serial twofold dilutions of human and rabbit sera in the ELISA procedure with *P. aeruginosa* 3064 alginate as the coating antigen.

titers obtained by immunizing groups of mice with different amounts of alginate. A 60-μg immunizing dose produced a maximal antibody titer at 3 weeks with no subsequent increase in titer. All other doses produced the same titer or within one twofold dilution of it by the end of the second injection. A third injection did not produce any further increase in antibody titers for any of the doses used. Injection of alginate with poly-L-lysine did not enhance the immunogenicity of alginate.

Although the immunization schedule was not so thoroughly explored in rabbits, two observations were made. Antibody titers were maximal after the first immunizing dose, and the use of crude preparations of alginate did not enhance the antibody titer relative to purified alginate.

Figure 2 shows the results of antibody determination done on 28 CF patients colonized with *P. aeruginosa*, 3 CF patients not colonized with *P. aeruginosa*, and 16 control subjects aged 12 to 28 (eight women, eight men) with *P. aeruginosa* 3064 alginate or commerical seaweed alginic acid. None of the controls had experienced a known infection with *P. aeruginosa*, and none had a known disease.

Control subjects had detectable antibody levels, but clearly formed a distinct group from CF patients (Fig. 2). Antibody apparently cross-reacted with 3064 and commercial alginate. Antibody titers were generally about twofold lower with commercial alginate as the antigen, although there was absolute correlation between titers to the two antigens.

Table 3 shows the results of serial antibody titers to 3064 alginate and, in some cases, commercial alginate. Antibody titers to 3064 alginate from paired sera taken at the time of admission for *P. aeruginosa* bronchopneumonitis and 2 or 3 weeks thereafter are given for 16 CF patients. No consistent elevation of antibody could be detected in any of these patients. Four patients were followed for additional time periods, including three patients readmitted for recurrent episodes of *P. aeruginosa* bronchopneumonitis. Additional titer changes were not significant.

An attempt was also made to apply the ELISA procedure to detect IgA antibody in sputum

TABLE 2. Antibody titers in mouse sera to purified *P. aeruginosa* 3064 alginate after intravenous immunization with differing doses of alginate

Dose (μg)	ELISA antibody titer[a] on the following day:		
	21	28	42
250	1:1,600 (4), 1:3,200 (1)	1:3,200 (5)	1:3,200 (5)
125	1:400 (1), 1:1,600 (4)	1:3,200 (4), 1:1,600 (1)	1:3,200 (5)
60	1:3,200 (5)	1:3,200 (5)	1:3,200 (5)
25	1:800 (5)	1:1,600 (5)	1:1,600 (5)

[a] Antibody titers were determined in duplicate on five animals in each group. The numbers within parentheses are the numbers of animals in each group with that antibody titer. Alginate injections were given at 0 time and 21 days after serum was obtained for the antibody titer. A third injection, when used, was given at 35 days. Antibody titers were identical at 42 days to the values above for a two-injection test.

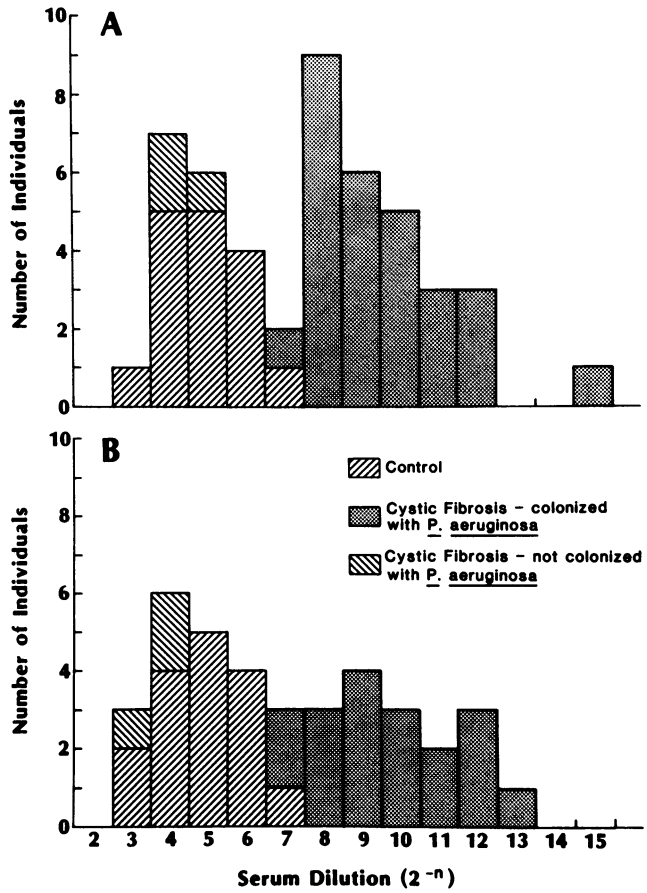

FIG. 2. Distribution of antibody titers determined by the ELISA procedure with serial twofold serum dilutions of healthy controls, CF patients not colonized with *P. aeruginosa*, and CF patients colonized with *P. aeruginosa* with either (A) *P. aeruginosa* 3064 or (B) commercial alginate as the coating antigen.

preparations from CF patients. In several sputum samples examined from three patients, controls lacking alginate were consistently positive. If conjugated anti-IgA antibody was deleted, controls were negative. It seems likely that IgA complexes were attaching directly to the polystyrene and interfered with the detection of anti-alginate IgA. Attempts to block the IgA absorption with bovine serum albumin treatment after the attachment of alginate to polystyrene were unsuccessful.

Antisera raised to alginate from strains 3064, 1468, or 1591 each reacted with the homologous as well as the three heterologous antigens (Table 4). The highest titer was obtained with 3064 alginate, whatever the source of alginate for immunization. Table 5 shows the antibody titers from the two patients infected with strains 1468 and 1591. Serum from the patient with strain 3064 was not available. Results were similar to those obtained in mice demonstrating highest titers to 3064 alginate, but cross-reacting with the three other alginates.

DISCUSSION

The ELISA procedure described herein is the first specific assay for alginate, the major component of the exopolysaccharide capsule-like material of *P. aeruginosa*. The antigen used in the assay is free of all major contaminants, except for minute quantities of endotoxin-like activity. The antigen is reactive with anti-alginate sera, but it is unreactive with anti-lipopolysaccharide sera specific for the Homma *P. aeruginosa* serotypes. Antiserum to *P. aeruginosa* 3064 alginate cross-reacts with seaweed alginate, but not with 15 other bacterial polysaccharides. With these criteria, the test has a very high degree of specificity for alginate.

These studies demonstrate that *P. aeruginosa* alginate is immunogenic in animals in that IgG

TABLE 3. Serial anti-3064 and anti-commercial alginate antibodies in sera from CF patients during acute *P. aeruginosa* bronchopneumonia

Patient	Titer (2^{-n}) after the following week:							
	Anti-3064 alginate				Anti-commercial alginate			
	0	2	3	Other[a]	0	2	3	Other
1	9		9[b]		13		12[b]	
2	12	12[b]		12(13), 12(15),[b] 12(22), 12(24)[b]				12(24)
3	11		10[b]		7		7[b]	
4	8	8[b]		8(9), 8(11),[b] 9(43), 9(45)[b]	9	8[b]		9(9), 8(11),[b] 9(43), 8(45)[b]
5	10	10[b]			5	5[b]		
6	11	11[b]			12	12[b]		
7	15		15[b]		9		10[b]	
8	6	6[b]		8(36), 7(38)[b]	10	10[b]		9(36), 10(38)[b]
9	9			8(5), 8(28), 8(30),[b] 8(38), 8(40)[b]	8			7(5), 8(28), 8(30),[b] 7(38), 8(40)[b]
10	9	9[b]						
11	10	10[b]						
12	8	9[b]						
13	10	10[b]			Not done			
14	9	9[b]						
15	8	8[b]						
16	12	11						

[a] The numbers within parentheses refer to the week serum sample was taken after the initial serum sample.
[b] The serum sample was 2 or 3 weeks after admission for acute *P. aeruginosa* bronchopneumonitis.

antibody is readily detected after immunization. Antibody levels were maximal in most cases after only a single experience with the antigen. In humans there was no significant antibody increase in convalescent sera of 16 CF patients with acute *P. aeruginosa* pneumonia. All patients were previously colonized with *P. aeruginosa*. Thus, anti-alginate antibody does not appear useful diagnostically or for differentiation of infection and colonization. Previous studies have not shown whether antibody was detectable to *P. aeruginosa* alginate in CF patients (7, 9). Our results clearly show that alginate antibody is formed in CF patients to a much greater extent than in controls or in the three CF patients not colonized with *P. aeruginosa*.

Serum anti-alginate is present in *P. aeruginosa* infected CF patients. Why does it not result in clearance of *P. aeruginosa* from the lungs of these patients? Several reasons could account for persistence of strains. The antibody may not reach the lung or it may be "mopped up" by a massive excess of *P. aeruginosa* alginate (13). The antibody may not be an effective opsonin or may not contribute to bactericidal activity. Speert et al. have recently shown that alginate inactivates the classical complement pathway resulting in poor opsonic activity and slowed killing of *S. aureus* (D. P. Speert, Y. Kim, and Y. Grandy, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., Miami Beach, Fla. abstr. no. 349, 1981). Thus, alginate may neutralize the effect of anti-alginate by the above activities. We are currently testing the protective effect of anti-alginate in mice challenged intraperitoneally and intratracheally with mucoid *P. aeruginosa*.

Cross-reactivity of the four alginates indicates that specificity is at least partly directed toward the guluronic-mannuronic component of alginate, as seaweed alginate, unlike the *P. aerugin-*

TABLE 4. Antibody titers in mouse sera to homologous and heterologous *P. aeruginosa* alginate determined by the ELISA procedures

Alginate source	Antibody titers with antigen[a] in ELISA			
	3064	1468	1591	Seaweed alginate
3064	1:16,384	1:640	1:640	1:1,280
1468	1:256	1:256	1:128	1:64
1591	1:1,024	1:128	1:512	1:128
Pooled mouse serum	1:32	1:4	1:4	1:8

[a] Antigen was added at 1.2 μg per well.

TABLE 5. Antibody titers determined by the ELISA to homologous and heterologous antigen in two CF patients

Patient	Strain isolated[a]	Antibody titers to alginate used in ELISA			
		3064	1468	1591	Seaweed alginate
1	1468	1:1,024	1:512	1:256	1:512
2	1591	1:256	1:64	1:128	1:128

[a] These isolates were present at greater than 100 times the quantity of other bacteria present at the time of a recent admission. Quantitation was determined by numerical counts of bacteria in sputum.

osa alginates, is not acetylated. The cross-reactivity is a very useful finding in that a single alginate can be used to screen for anti-alginate antibody from a variety of strains. It seems probable that all alginates will cross-react with a single anti-alginate, although this cannot be absolutely confirmed with the current results.

The alginate of strain 3064 is unusual in the group tested in that it produced higher antibody levels in mice. It also had greater reactivity than either of two infecting strains with the sera of two patients infected with strains other than *P. aeruginosa* 3064. This apparently greater immunogenicity could make it valuable as a potential vaccine antigen. However, as noted above, it is unclear whether anti-alginate is protective. Such antibody could even be harmful if it forms part of immune complexes that exist in CF sera (2, 16, 18, 20) or participates in an Arthus reaction (27). Animal protection studies may help answer this enigma. Previous studies on humans have not shown any beneficial affect of immunization with a *P. aeruginosa* vaccine (21). However, studies have not used alginate as the antigen nor have patients been immunized before colonization with *P. aeruginosa*. We do know that mice tolerate doses of 200 μg of alginate without any evidence of acute toxicity. They also tolerate repeated large injections even with significant antibody levels. These preliminary results are promising enough to encourage further assessment of alginate as a vaccine.

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