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Purification, Characterization, and Immunological Cross-Reactivity of Alginates Produced by Mucoid *Pseudomonas aeruginosa* from Patients with Cystic Fibrosis

SVEND STENVANG PEDERSEN,* FRANK ESPERSEN, NIELS HØIBY, AND GEOFFREY H. SHAND

Statens Seruminstitut, Department of Clinical Microbiology at Rigshospitalet, Juliane Mariesvej 28.2, DK-2100 Copenhagen Ø, Denmark

Received 14 November 1988/Accepted 6 January 1989

Alginates from nine mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis were purified by repeated ethanol precipitation, nuclease digestion, anion-exchange chromatography, dialysis, and lyophilization. Uronic acid constituted 72% of the dry weight when mannuronolactone was used as the internal standard in the carbazole-borate assay for uronic acids. The average degree of acetylation was 16%, and the ratio of mannuronic acid to guluronic acid was 4.7. No homopolymeric blocks of guluronic acid were found when analyzed by nuclear magnetic resonance spectroscopy. Contaminating proteins were denatured by heating, and during purification the content of protein relative to alginate fell from 566 to 0.9%. The content of lipopolysaccharide was 0.012%. No immunological or biological activity was attributable to the protein or lipopolysaccharide content as estimated by immunoblotting, enzyme-linked immunosorbent assay (ELISA), and a neutrophil chemotaxis assay. Rabbits were hyperimmunized with *P. aeruginosa* alginates and alginate from the seaweed *Laminaria hyperborea*, and an ELISA that detected alginate-specific antibodies was developed. Antibodies to *P. aeruginosa* alginate were detected by ELISA in 1:4,000 dilutions of serum from patients with cystic fibrosis with chronic *P. aeruginosa* lung infection. The serological cross-reactions between serum from the nine patients with cystic fibrosis and the corresponding *P. aeruginosa* alginates were investigated and showed considerable heterogeneity. This finding indicates that *P. aeruginosa* alginate from more than one *P. aeruginosa* strain should be used in serological tests. There was no serological cross-reactivity between *P. aeruginosa* and *Laminaria hyperborea* alginate in either rabbits or patients with cystic fibrosis.

The life expectancy of patients with the inherited disease cystic fibrosis (CF) is determined mainly by the clinical course of progressive respiratory insufficiency caused by chronic *Pseudomonas aeruginosa* lung infection. The prognosis is significantly poorer in patients with CF infected with a characteristic mucoid phenotype of *P. aeruginosa* than in those infected with nonmucoid strains (29). Evidence is accumulating that the pathogenesis of lung damage in CF involves a persistent infection associated with an exuberant antibody response leading to local formation of immune complexes, activation of polymorphonuclear leukocytes, and release of tissue-damaging proteolytic enzymes (28, 42). The interaction of the mucoid substance from *P. aeruginosa* with host defenses may contribute to this process by its antiphagocytic (53), antigenic (3, 5, 46, 57), and polyclonal B-cell activation (11, 45) properties.

In 1964, Linker and Jones reported that the mucoid substance produced by *P. aeruginosa* was a polysaccharide consisting of mannuronic acid and guluronic acid (36). The mucoid exopolysaccharide is chemically similar to the alginate from brown seaweed except that *P. aeruginosa* alginate is O acetylated (9, 15, 36). In previous studies of alginate from *P. aeruginosa*, a limited number of strains have been used as the source of alginate, and the extraction and purification procedures have differed widely resulting in varying degrees of purity of the final preparations (3, 5, 46, 55, 57).

In the present study, we have purified alginate from nine *P. aeruginosa* strains isolated from patients with CF. During the isolation we compared purification procedures used by other investigators. We have evaluated the immunogenicity of *P. aeruginosa* alginates in rabbits and humans by means of an enzyme-linked immunosorbent assay (ELISA) and have investigated the immunological cross-reactivity among the different *P. aeruginosa* alginates and alginate isolated from seaweed (SW alginate).

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* was isolated from patients with CF as previously described (26). Mucoid colonies were selected and subjected to multiple passages (approximately 30) on 5% horse blood agar; those that were stably mucoid were stored at room temperature in the dark in sealed meat extract agar. Subsequently, nine stably mucoid isolates were used for separation and purification of alginate.

Antigens. A sonicated soluble extract of *P. aeruginosa* (St-Ag) was prepared as previously described (29). SW alginate extracted from the seaweed *Laminaria hyperborea* was a gift from Grindsted Products A/S, Brabrand, Denmark. Protanal LF 10/60, another SW alginate, was obtained from Protan A/S, Drammen, Norway.

Purification of *P. aeruginosa* alginate. Mucoid strains were grown on a solid agar medium with peptone (1%), glucose (2%), and NaCl (0.5%), pH 7.5 (Truche agar) for 72 h at 35°C, which gave a confluent layer of mucoid substance. The mucoid layer was harvested with 10 ml of phosphate-buffered saline (PBS), pH 7.2. Pilot experiments showed that harvesting with PBS or with 0.8 M EDTA (46) was comparable with regard to the viscosity of the final solution and ease of separation by centrifugation. The collected mucoid layers from each strain were pooled and stirred vigorously on a magnetic stirrer for 1 h to obtain a uniformly consistent

* Corresponding author.

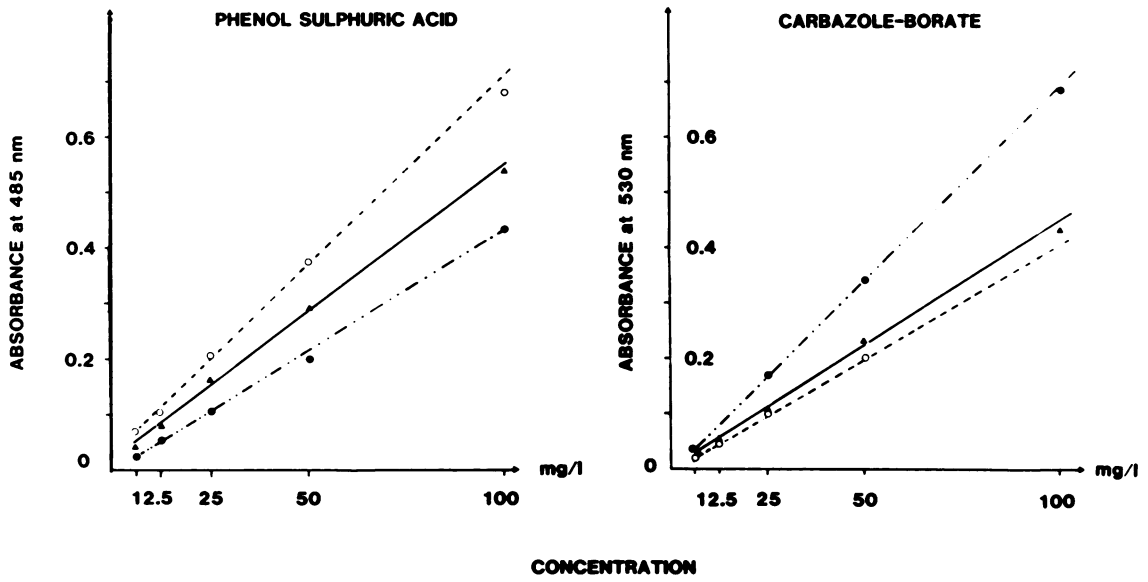

FIG. 1. Comparison of the phenol-sulfuric acid assay for determination of total carbohydrate content with the carbazole-borate assay for uronic acid on three uronic acid standards. Symbols: ●, D-mannuronolactone; ▲, SW alginate; ○, Protanal SW alginate.

solution. The viscous solution was centrifuged for 4 h at 17,700 × *g* at 4°C to remove whole bacteria. The clear supernatant was heated for 30 min at 80°C to kill viable bacteria and to denature proteins and centrifuged at 17,700 × *g* for 30 min. The pellet containing denatured proteins was discarded. In some experiments, the supernatant was autoclaved instead of being heated at atmospheric pressure. The supernatant was precipitated by the addition of cold absolute ethanol to a final concentration of 80% (vol/vol). The ethanol precipitation procedure was decided upon after prior testing comparing precipitations with concentration gradients from 0 to 80% (vol/vol) and from 96 to 80% (vol/vol).

Ethanol precipitation (0 to 80% [vol/vol]) was also compared with precipitation with propan-2-ol (0 to 75% [vol/vol]). After 1 to 2 h at 4°C, the precipitated alginate was collected with a bent-glass rod, squeezed dry, and washed twice in 80% (vol/vol) ethanol and once in 96% (vol/vol) ethanol. The precipitate was dissolved in sterile 0.9% saline, and the ethanol precipitation and washing procedure was repeated once. The resulting precipitate was dissolved in PBS supplemented with 10 mM $MgCl_2$ and 1 mM $CaCl_2$. Enzyme digestion to remove contaminating RNA and DNA was carried out with type 1A RNase A (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and type IV DNase I (100 μg/ml; Sigma) for 4 h at 37°C. Enzymes were inactivated and denatured by heating for 30 min at 80°C. After centrifugation, the ethanol precipitation procedure was repeated once. Ion-exchange chromatography was performed by the method described by Pier et al. (47) with a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) with an ammonium carbonate gradient, after comparing it with the method described by Sherbrock-Cox et al. (55) with a Dowex anion-exchange resin (Sigma) with a stepwise elution with sodium chloride. The precipitate was dissolved in 0.025 M ammonium carbonate before loading onto the anion-exchange column. The loading was 1 ml of alginate solution per ml of anion exchanger. A gradient of 0.025 to 1.0 M ammonium carbonate was run with a peristaltic pump to a volume 10 times the bed volume. The eluate was continuously monitored for A_{280}. Fractions (5 ml) were collected and assayed for content of uronic acid by the carbazole-borate method (see below). Positive fractions were pooled and dialyzed three times for 24 h against demineralized sterile water, and the dialysate was lyophilized.

For the final preparations, the column matrix was used only once and then discarded, as initial experiments showed that alginatelike material remained on the matrix despite elution with 2 M ammonium carbonate, resulting in impaired flow. The acetyl groups may be removed from alginate by mild alkaline hydrolysis. The influence of the elution procedure on acetylation was tested by incubating alginate with the slightly alkaline elution buffer (pH 8.6) at room temperature for 20 h with 1 M ammonium carbonate. The incubated sample and a control were dialyzed, lyophilized, and assayed for acetyl content.

Biochemical analyses. Total carbohydrate was determined colorimetrically by the phenol-sulfuric acid assay (14) with a concentration of 3% phenol. A_{485} was measured in a D.B. spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). An internal standard of D-mannuronolactone (Sigma) and SW alginate was included in each run. Total uronic acid content was measured by the modified carbazole-borate assay (33) with D-mannuronolactone and SW alginate as internal standards, and A_{530} was measured. The assay was also performed after drying of the lyophilized sample at 120°C for 4 h followed by P_2O_5 exsiccation in vacuum for 2 days. The assays for total carbohydrate and uronic acids were compared with standards of mannuronolactone, SW alginate, and Protanal. Parallel estimations were performed for total carbohydrate and uronic acid. If mannuronolactone was used as the internal standard, the alginate content of the two seaweed samples was underestimated by 33 to 42%. If, on the other hand, mannuronolactone was used as the standard in the phenol-sulfuric acid assay, the total carbohydrate content in the alginates was overestimated by 32 to 75% (Fig. 1). Contamination with protein was quantitated by the Folin assay (38). Bovine serum albumin, dissolved either in saline or 1 mg of SW alginate per ml, was used as the internal standard.

Lipopolysaccharide (LPS) was determined by a quantita-

tive *Limulus* amoebocyte assay (1) and is expressed in equivalents of the endotoxic activity of an *Escherichia coli* LPS standard. The thiobarbituric acid assay (TBA) was used for gross determination of LPS content with ketodeoxyoctusolonic acid (Sigma) as the standard, and A_{550} was read (31). This method also estimates unsaturated uronic acid residues (50). The degree of acetylation of alginate was measured by the hydroxylamine assay (40) with beta-D-glucosepenta-acetate (Sigma) as the standard, and A_{520} was measured.

All tests were performed in duplicate, and no colorimetric assay was accepted if the coefficient of correlation of the internal standard was less than 0.975. The ratio of mannuronic to guluronic acid was determined by gas chromatography (61), and the composition of the uronate residues was determined on two samples by nuclear magnetic resonance (NMR) spectroscopy (20).

Electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis for characterization of alginate was carried out by a modification of the tube gel method (6) adapted for vertical slab gels (16). The gel had a polyacrylamide concentration of 6% with 2% bisacrylamide. Alginate samples were also run in a 5% stacking and 14% separating gel. Samples were mixed with an equal volume of glycerol and 0.01% bromophenol blue and electrophoresed at 220 V until the marker dye had run 10 cm. Gels were stained overnight with 0.08% (wt/vol) alcian blue in 7% (vol/vol) acetic acid and destained in 7% (vol/vol) acetic acid.

Contamination in the alginate samples was investigated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 5% polyacrylamide in the stacking gel and 14% in the separating gel (17) and staining with Coomassie brilliant blue for protein (54) and a modified silver stain for LPS (59). Experiments with Coomassie brilliant blue showed that it also stained alginate. All color was retained in a dialysis bag when a mixture of alginate and Coomassie brilliant blue was dialyzed against demineralized water. In a control dialysis bag without alginate, the color diffused into the dialysis fluid. Likewise, the color was retained after prolonged dialysis of a suspension of calcium SW alginate beads containing Coomassie brilliant blue.

Immunoblotting was by a modification of the method of Towbin (58) as previously described in detail (54).

Patient serum. Alginate-producing strains of *P. aeruginosa* were isolated from nine patients with CF (mean age, 18 years; range, 13 to 34 years) who had had chronic *P. aeruginosa* infection for a mean duration of 8 years (range, 8 to 16 years). The mean number of *P. aeruginosa*-precipitating antibodies in patient serum determined by crossed immunoelectrophoresis was 21 (range, 11 to 42; values in normal controls, 0 to 1 [29]). Serum from each patient was obtained at the same time that the mucoid strain was isolated and was stored at −20°C until analysis. Control serum was pooled from 10 infants with CF (CF-P/Y; mean age, 6.5 months) and from 15 older patients with CF (CF-P/O; mean age, 17 years; range, 12 to 29 years) with no history of *P. aeruginosa* infection.

Immunization of rabbits. Groups of outbred albino rabbits (New Zealand White type Ssc:CPH; Statens Seruminstitut, Copenhagen, Denmark) were hyperimmunized with SW alginate, a mixture of three highly immunogenic purified *P. aeruginosa* alginates (see below), and *P. aeruginosa* St-Ag. The vaccine was given to three rabbits in each group and was mixed with an equal quantity of Freund incomplete adjuvant. Antigen (0.5 mg) was injected subcutaneously five times with 2-week intervals followed by monthly boosters (22). Serum was obtained before immunization, 1 week after the initial immunization schedule was finished, and then at monthly intervals. Serum was stored at −20°C until use. The immunoglobulin fraction (immunoglobulin G and A) was purified with ammonium sulfate precipitation and ion-exchange chromatography (22). Crossed immunoelectrophoresis has shown (24) that serum (St-Ab) from rabbits immunized with St-Ag reacts with 64 different antigens, mainly protein but also LPS, of *P. aeruginosa*. Rabbit antibodies to *P. aeruginosa* LPS (serotype O:3, O:4, and O:9) were a gift from the Department of Hospital Infection, Statens Seruminstitut, Denmark.

ELISA. A series of experiments were performed to select the type of plate, the blocking procedure, and the washing procedure which consistently gave the lowest nonspecific reading of background optical density (OD) of patient serum. The following 96-well plates were tested: Immunoplate type I, Microwell, and Microwell Module (Nunc, Roskilde, Denmark). Coating was carried out with SW or *P. aeruginosa* alginate dissolved in PBS, pH 7.4, as prior experiments showed no difference when it was compared with a bicarbonate buffer at pH 9.6. The optimal concentration of coating antigen was determined by varying the concentration of alginate. Binding was achieved by 1 h of incubation at 35°C on a shaking table followed by overnight incubation at 4°C. Binding to the plates was tested by treating coated plates with native avidin followed by incubation with peroxidase-conjugated biotin (Dakopatts, Glostrup, Denmark). Absorbance following application of purified antibodies from hyperimmune rabbits was measured to confirm binding of alginate to the plates. The blocking procedure was determined following experiments with bovine serum albumin plus Triton X-100, Tween 20, gelatin, heat-inactivated fetal calf serum, or newborn calf serum. The blocking procedure was performed on a shaking table at 35°C for 1 h. Washing was performed with PBS plus 0.1% Tween 20 and 0.5 M NaCl by leaving the buffer in the wells for 3 min.

Rabbit or patient serum was diluted in the blocking buffer and titrated in twofold dilutions. After incubation of serum for 1 h at room temperature, plates were washed three times. Bound antibodies were allowed to react with peroxidase-conjugated second antibody for 1 h at 35°C. For assays of rabbit serum, swine anti-rabbit immunoglobulin (Dakopatts) diluted 1:1,000 in PBS plus 0.1% Tween 20 was used. Peroxidase-conjugated goat anti-human immunoglobulin G (Tago, Burlingame, Calif.) diluted 1:2,000 was used for assays of human sera. After being washed six times, 0.1 ml of 0.1 M sodium citrate solution (pH 5.0) containing 2.2 mM 1,2 phenylendiamide-dihydrochloride and 6.5 mM H_2O_2 was added, and incubation was carried out in the dark for 30 or 60 min. The reaction was stopped by the addition of 0.1 ml of H_2SO_4 (1 M), and the OD was read on an automatic plate reader (Bio-Rad, Richmond, Calif.) at A_{492}.

The specificity of ELISA was tested by absorbing immune serum for 1 h at 35°C with increasing concentrations of either *P. aeruginosa* or SW alginate before it was applied to the plates. The contribution to the absorbance of a reaction of anti-LPS antibodies with contaminating LPS was tested by applying specific O-group rabbit antisera to plates coated with *P. aeruginosa* alginate which were purified from strains of those particular serotypes.

Cross-reactivity of *P. aeruginosa* and SW alginate. Serum from three patients with CF with chronic *P. aeruginosa* lung infection was tested for reactivity against SW alginate immobilized to an ELISA tray. The mixture of highly immunogenic *P. aeruginosa* alginates (see below) used for immu-

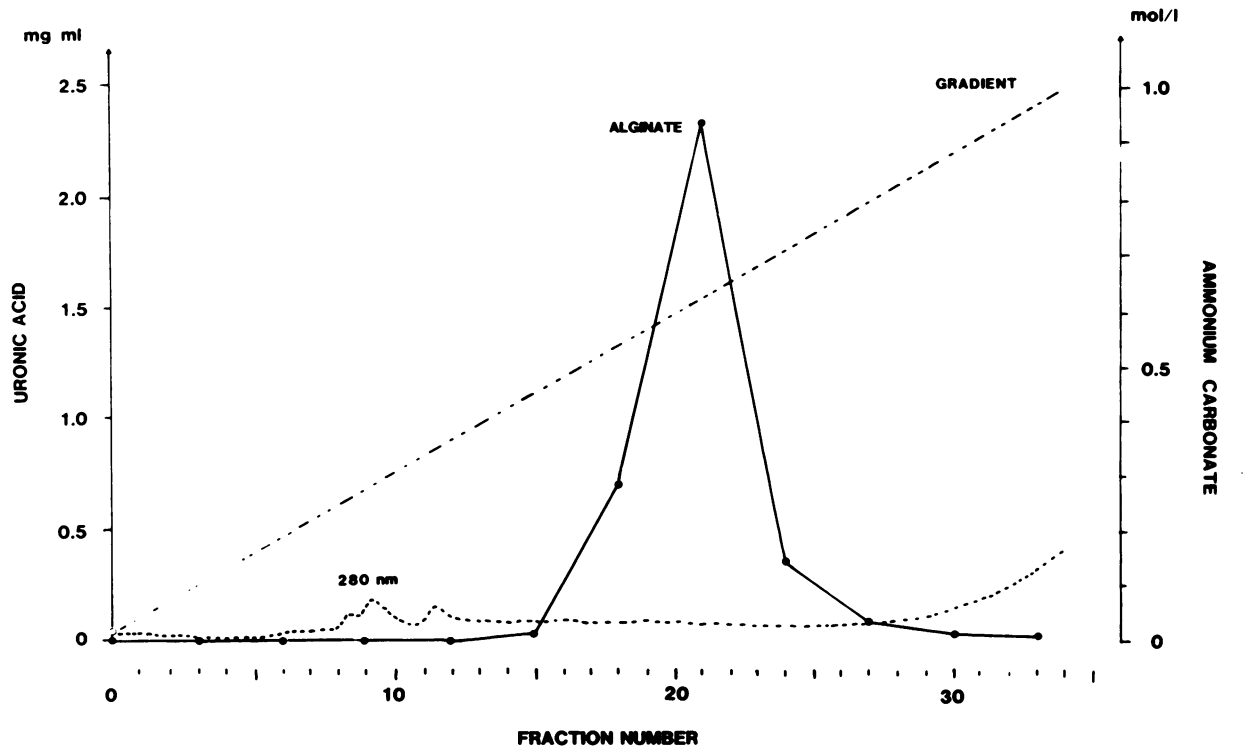

FIG. 2. Elution profile of a *P. aeruginosa* alginate by DEAE-Sephacel ion-exchange chromatography with a linear gradient of ammonium carbonate as elution buffer. The alginate was partly purified by repeated ethanol precipitation before it was applied to the column. Protein content was monitored continuously by A_{280}. Purified alginate was obtained from fractions 17 to 24, which were pooled.

nization of rabbits, absorption experiments, and ELISA was purified from mucoid *P. aeruginosa* harbored by these patients at the same time the serum samples were collected. Serum from groups of three rabbits, immunized with either SW alginate or *P. aeruginosa* alginate, was tested for reaction with *P. aeruginosa* and SW alginate by ELISA. The serum samples were either unabsorbed or absorbed with SW or *P. aeruginosa* alginate prior to ELISA. Absorption was carried out in glass tubes for 1 h at 35°C followed by overnight incubation at 4°C.

RESULTS

Purification of alginate. The bacteria consistently produced copious amounts of mucoid substance on the medium used. *P. aeruginosa* alginate was cleared of whole bacteria following centrifugation without any obvious facilitation with EDTA (46). The supernatant of the harvested material contained approximately five times more protein than uronic acid (Table 1). No difference was observed in the content of uronic acid, acetyl, protein, and LPS following the different precipitations with ethanol and propan-2-ol. Ethanol was eventually chosen because of lower cost. Heat treatment at 80°C ensured sterility of the preparation. Autoclaving markedly reduced the viscosity, indicating depolymerization of alginate, and also decreased the immunogenicity (see below). After precipitation, the *P. aeruginosa* alginate contained between 0.07 to 0.6% RNA. Nucleic acids were removed with nucleases which eliminated A_{254}. *P. aeruginosa* alginate eluted between 0.45 to 0.55 to 0.7 to 0.8 M ammonium carbonate from the columns (Fig. 2). The alkaline buffer did not cause deacetylation of the alginates. The results obtained with the Sephacel column were, in our hands, more reproducible than those obtained with the Dowex anion-exchange method described by Sherbrock-Cox et al. (55) because the alginates eluted at different molarities. The yield was higher from the Sephacel column, and the use of ammonium carbonate buffer was furthermore advantageous because it vaporized during lyophilization.

The procedures employed (Table 1) resulted in a substantial reduction of protein relative to uronic acid.

Purity of *P. aeruginosa* alginate. Table 2 shows the results of the biochemical analyses performed on the nine *P. aeruginosa* alginate preparations. With SW alginate as the standard, all of the dry weight could be accounted for as uronic acid, whereas 72% was accounted for with mannuronolactone as standard. Less than 1% of the alginate weight was protein, and approximately 0.01% had endotoxinlike activity. The degree of acetylation ranged from 0 to 28%. Apart from ketodeoxyoctusolonic acid, TBA measures unsaturated hexuronic moieties, and no increase was seen during purification. Some reaction was, however, seen in TBA following autoclaving (data not shown).

NMR spectroscopy performed on two samples confirmed that no other uronic acid acids were present except mannu-

TABLE 1. Removal of protein from the mucoid substance of *P. aeruginosa* during purification

Treatment	mg of protein/ml (± SD)	% Protein of alginate (± SD)
Harvest supernatant	13.1 ± 6.2	566 ± 311
1st ethanol precipitation	0.8 ± 0.3	21 ± 7
2nd ethanol precipitation	0.5 ± 0.3	11 ± 8
Ion-exchange chromatography	0.05 ± 0.02	0.9 ± 0.5

TABLE 2. Biochemical analysis of nine alginates isolated from *P. aeruginosa*

Biochemical composition	Mean ± SD (range)
Uronic acid[a] with:	
Mannuronolactone standard	72 ± 11 (53–86)
SW alginate standard	102 ± 18 (78–138)
Acetyl[b]	16 ± 8 (0–28)
Protein[b]	0.9 ± 0.5 (0.38–2.2)
LPS[b]	0.0123 ± 0.01 (0.0005–0.03)
RNA	ND[c]
Ratio of mannuronic: guluronic acid	4.7 ± 2.6 (2.5–9.4)

[a] In percent dry weight.
[b] In percent uronic acid content.
[c] ND, Not detectable.

ronosyl (M) and guluronosyl (G). The NMR scan showed that the composition of uronic acid was either MM or MG, whereas GG blocks were absent. All samples contained more M than G, as the M:G ratio exceeded 1 in all samples. In some samples, nearly 90% of the material was M (Table 2).

With polyacrylamide gel electrophoresis, the alginate showed in the upper quarter of the vertical slab gel (Fig. 3). During purification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a progressive reduction of protein bands. In the final preparation, only a high-molecular-weight band stained. We conclude that this band was alginate, as its position was closely similar to that of the only band on a duplicate gel stained with alcian blue. Coomassie brilliant blue staining of alginate was confirmed in experiments where sodium and calcium alginate were mixed with Coomassie brilliant blue and dialyzed. Running the *P. aeruginosa* St-Ag together with alginate showed that alginate did not retard the migration of proteins. The lower detection level for protein with the soluble sonic extract was 80 ng of protein (54). The gel was loaded with 60 μg of alginate, and accordingly the protein content was less than 0.13%. Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis did not show the ladder pattern which is characteristic for LPS (17).

Immunoblotting of the alginate with St-Ab showed no immunoreactivity.

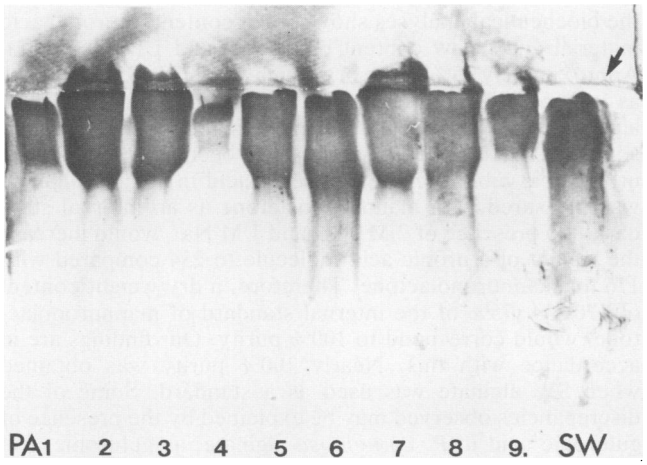

FIG. 3. Polyacrylamide gel electrophoresis of purified *P. aeruginosa* alginates 1 to 9 and SW alginate stained with alcian blue. For details see Materials and Methods. The arrow in the upper right corner indicates the boundary between stacking and running gels.

TABLE 3. Cross-reactivity between *P. aeruginosa* alginate and homologous and heterologous patient serum in ELISA

Serum	*P. aeruginosa* alginate reactivity[a] in serum:										SW alginate reactivity
	1	2	3	4	5	6	7	8	9	Mean	
1	**4**	3	2	2	1	4	2	4	3	2.8	2
2	4	**4**	4	4	1	4	4	4	4	3.7	2
3	3	1	**1**	0	1	2	0	4	4	1.8	2
4	4	4	4	**4**	1	4	4	4	4	3.9	3
5	4	4	4	4	**1**	4	4	4	4	3.9	2
6	4	1	0	0	0	**2**	0	3	1	1.2	2
7	4	2	2	1	2	4	**1**	4	4	2.7	3
8	4	2	1	0	0	2	0	**4**	2	1.7	2
9	4	2	2	1	1	4	1	4	**4**	2.6	3
Mean	3.9	2.6	2.2	2.1	0.9	3.3	1.8	3.9	3.3		2.3
CF-P/Y	0	0	0	0	0	0	0	0	0	0	0
CF-P/O	2	0	0	0	0	1	0	2	1	0.7	0

[a] 4, Very strong reaction (OD > 1.5); 3, strong reaction (1.0 < OD < 1.5); 2, moderate reaction (0.5 < OD < 1.0); 1, weak reaction (0.2 < OD < 0.5); 0, no reaction (OD < 0.2). Homologous reactions are shown in boldface type.

Development of ELISA. Alginate attached equally well to the three types of plates tested, but because of consistently lower nonspecific background absorbance, Microwell polystyrene plates were used in all subsequent experiments. Binding occurred in a dose-dependent manner, and a linear correlation (correlation coefficient $r = 0.995$) was seen between the concentration of the coating alginate antigen and absorbance when tested with avidin-biotin. The dose dependency was confirmed with hyperimmune rabbit antibodies to SW alginate. At high concentrations of alginate, the absorbance leveled off, indicating saturation of binding sites (data not shown). The antibody reaction with untreated and heat-treated (80°C) SW alginate gave similar absorbances, whereas autoclaving reduced absorbance by approximately 50% (data not shown). Sonication of the alginate antigen did not enhance its immunoreactivity.

Several substances were tested for blocking residual binding sites after being coated with antigen. PBS plus 0.1% Tween 20 gave the best blocking effect on uncoated plates treated with CF serum, while on coated plates it did not mask the specific antigen-antibody response as the other blocking buffers did. Titration experiments showed ELISA to be optimal if serum from the patients was diluted 1:4,000 in the blocking buffer. It was possible to reduce the absorbance by more than 90% if hyperimmune rabbit serum was absorbed with SW alginate prior to application on the ELISA plate. The reduction in OD was dose dependent (data not shown). No antigen-antibody reaction was observed when *P. aeruginosa* alginate-coated plates were allowed to react with O group-specific rabbit hyperimmune serum.

Immunogenicity of alginate. The results of duplicate ELISAs of the reactions between *P. aeruginosa* alginate and sera from patients with CF are shown in Table 3. Five of nine (no. 1, 2, 4, 8, and 9) patients with CF with chronic *P. aeruginosa* lung infection had serum antibodies in a 1:4,000 dilution which reacted strongly with the homologous *P. aeruginosa* alginate preparation. The reaction to the homologous serum was weaker but still detectable for the remaining four *P. aeruginosa* alginates.

Four *P. aeruginosa* alginates (no. 1, 6, 8, and 9) had very strong reactions with the majority of heterologous patient sera. Numbers 1, 6, and 8 were pooled and used for

TABLE 4. Serological cross-reactivity between alginates from *P. aeruginosa* and SW alginate tested by ELISA[a]

ELISA plates coated with:	Response (%)(range) of rabbit anti-serum against:				Response (%)(range) of CF serum absorbed with:	
	SW alginate absorbed with:		*P. aeruginosa* alginate absorbed with:			
	SW alginate	*P. aeruginosa* alginate	SW alginate	*P. aeruginosa* alginate	SW alginate	*P. aeruginosa* alginate
SW alginate	3 (1–4)	92 (88–95)	4 (2–6)	13 (10–15)	19 (14–23)	100 (100–100)
P. aeruginosa alginate	2 (0–4)	2 (1–4)	95 (93–97)	2 (0–4)	100 (99–100)	15 (5–23)

[a] The response is in percent unabsorbed response of the homologous serum, and data are the means (range) of triplicate experiments with sera from three individuals in each group. The response of unabsorbed heterologous serum is ≤5% of the response of homologous serum.

immunization of rabbits. Together these three antigens reacted strongly with all nine serum samples tested. One *P. aeruginosa* alginate (no. 5) had only a weak reaction with homologous and heterologous serum, although the serum of patient 5 had a very strong reaction to all other *P. aeruginosa* alginates. A different pattern is exemplified by *P. aeruginosa* alginate 6, which had strong cross-reactions with heterologous serum samples, whereas the serum from patient 6 reacted strongly only with two *P. aeruginosa* alginates (no. 1 and 8). All *P. aeruginosa*-infected patients were able to mount an antibody response against one or more *P. aeruginosa* alginates, and apart from *P. aeruginosa* alginate 5, all alginates reacted with one or more serum samples. There was no apparent correlation between M:G ratio or the degree of acetylation and the immunogenicity of the *P. aeruginosa* alginates. All chronically infected patients with CF had antibodies which reacted with SW alginate, but the response was always less than the response to the most reactive *P. aeruginosa* alginate. The serum pool from young patients with CF (CF-P/Y) without *P. aeruginosa* had no antibodies against either *P. aeruginosa* or SW alginate, whereas older noncolonized patients (CF-P/O) had low to moderate reactions against *P. aeruginosa* alginate 1, 6, and 8 and no reaction against the other antigens.

Preimmunization rabbit serum did not contain antibodies against either SW or *P. aeruginosa* alginate. The rabbits responded to immunization with SW alginate, but the development of antibody response was slow, as two of three rabbits had an antibody response just above background absorbance after the first booster. However, after further monthly boosters for 6 months, all had high titers of antibodies. All rabbits immunized with *P. aeruginosa* alginate showed a strong antibody response after the first booster.

Immunological cross-reactivity between SW and *P. aeruginosa* alginate. There was no serological cross-reaction between SW and *P. aeruginosa* alginate in either rabbits or patients with CF (Table 4). Serum from rabbits immunized with SW alginate did not react at all with any of the nine purified *P. aeruginosa* alginates, and serum from rabbits immunized with *P. aeruginosa* alginate did not react to SW alginate. Serum from patients with CF infected with *P. aeruginosa* reacted with SW alginate, the response being 30% of the response against *P. aeruginosa* alginate (Table 4). This response was specific for SW alginate, because it could be absorbed with SW alginate and not *P. aeruginosa* alginate. The patient antibody response to *P. aeruginosa* alginate was specific, as no absorption occurred with SW alginate (Table 4).

DISCUSSION

The mucoid exopolysaccharide from *P. aeruginosa* is a linear heteropolysaccharide of mannuronic and guluronic acid linked by 1 to 4 glycosidic linkages (9). Apart from the presence of acetyl groups, it resembles the alginic acid which can be isolated from a variety of brown algae (43). Only a few bacteria have been reported to produce alginate. These include *Azotobacter vinelandii* (18); *P. aeruginosa* (36); *P. mendocina*, *P. fluorescens*, and *P. putida* (19); and *P. syringae* pv. *glycinea* (16). Of these, only *P. aeruginosa* is considered pathogenic to humans. Mucoid *P. aeruginosa* is isolated mainly from patients with chronic illnesses (23, 39) and is characteristically associated with the lung disease of patients with CF (13, 23), where it is associated with a poorer prognosis than is infection with nonmucoid strains (29). Ninety percent of patients with CF acquire this ultimately fatal lung infection with *P. aeruginosa* before the age of 16 years (44). Colonization usually occurs initially with classic nonmucoid strains, but after a variable length of time, mucoid phenotypes predominate. The occurrence of mucoid *P. aeruginosa* is associated with an increased antibody response against this bacterium, both in patients with CF (27) and in patients without CF (25). The role of the mucoid exopolysaccharide in the persistence and pathogenesis of CF lung infection has therefore been a subject of considerable interest (Editorial, Lancet **ii**:1372–1373, 1986).

The purification of the mucoid substance produced by *P. aeruginosa* should ensure removal or inactivation, or both, of other immunogenic or biologically active substances such as proteins, toxins, and LPS. At the same time, the purification must be done without any significant change in the structure and properties of the alginate.

The procedure used in this study was a modification of methods described by other investigators (46, 47, 55), and the biochemical analyses show a high content of uronic acid material and a low content of protein and LPS (Table 2). Approximately 75% of the dry weight could be accounted for as uronic acid with mannuronolactone as a standard. The alginate was very hydrophilic, and despite extensive drying, some water molecules may still have been attached to each uronic acid molecule. When uronic acid in sodium alginate was measured with mannuronolactone as an internal standard, the presence of 2 M H_2O and 1 M Na^+ would increase the weight of a uronic acid molecule to 234 compared with 176 for mannuronolactone. Therefore, a dry weight content of 176/234 (75% of the internal standard of mannuronolactone) would correspond to 100% purity. Our findings are in accordance with this. Nearly 100% purity was obtained when SW alginate was used as a standard. Some of the discrepancies observed may be explained by the presence of guluronic acid in *P. aeruginosa* alginate, as guluronic acid reacts differently in the carbazole-borate assay from mannuronic acid (33).

Alginate of higher purity was found by Bryan et al. (5). However, their finding of more than 99.99% purity was

obtained by subtracting the amount of contaminants from a theoretical value of 100%. Using gas chromatography, Pier et al. (46) found >99% uronic acid in three preparations but did not account for 100% of the dry weight in their results. Our results are similar to those of others (15, 37) who found the alginate content to be 50 to 73 and 59 to 80%, respectively.

The assays for total carbohydrate and uronic acid did not correlate well (Fig. 1). Sherbrock-Cox et al. (55) found that virtually all of the carbohydrate in the 0.8 M fraction in their study was uronic acid, but they did not give the concentration of phenol used in the phenol-sulfuric acid assay. This may be important, as measurement of uronic acids requires a fairly low concentration of phenol (14). The presence of other sugars in our final preparation cannot be ruled out, although NMR spectroscopy of two alginate samples indicated that no other uronic acids were present. By NMR scan, no GG blocks were seen, which confirms that a major difference between SW and *P. aeruginosa* alginate is the absence of GG blocks in the latter (49, 55, 56). Therefore, the M:G ratio will never be less than one (51), as also shown in Table 2. Another major difference between algal and bacterial alginate is the presence of O-acetyl groups on the latter. The presence of acetyl may prevent subsequent extracellular epimerization to L-guluronosyl (12, 56). The physical properties of alginate are to a large degree determined by the divalent cationic chelating properties of guluronic acid, and a regulation of the degree of acetylation provides the bacteria with a means of controlling the chemical composition and physical properties of the surrounding capsule of exopolysaccharide (56). The degree of acetylation in the final preparation (Table 2) is in accordance with the findings of other investigators (15, 49).

Ketodeoxyoctusolonic acid is a characteristic constituent of LPS linking the O-polysaccharide chain to core lipid A (60) and is estimated to account for 3% of the weight of *P. aeruginosa* LPS (48). TBA (31) has been widely used for estimating LPS content (5, 46). In our hands, the lower limit for detection of LPS by measuring the ketodeoxyoctusolonic acid content by TBA is 0.6% in a highly viscous (5 mg of alginate per ml) sample, as the lowest standard giving a readable absorption is 1 μg of ketodeoxyoctusolonic acid per ml. However, 5 mg of alginate per ml usually yields some turbidity, and the use of a lower concentration of alginate is therefore associated with an elevation of the detection limit. Furthermore, an error may be introduced as TBA also measures unsaturated uronic acid molecules (50). TBA should therefore not be used for determining the content of LPS from alginate samples. Determination of endoxinlike activity by the *Limulus* amoebocyte lysate assay is very sensitive and can measure the activity of picogram quantities of LPS (1). However, the *Limulus* assay is not specific for LPS. Other polysaccharides may react with coagulogen in the coagulation reaction with the *Limulus* assay, albeit much more weakly than LPS (2), and it is not known whether alginate reacts in this way. Very low levels of LPS (100 pg/mg of alginate) were obtained by Learn et al. (35) using repeated precipitation with propan-2-ol. We tested whether precipitation with this less polar solvent could reduce the LPS content as compared with ethanol and found the LPS concentration in the microgram range to be the same with the ethanol and propan-2-ol methods. The difference may be due to differences in methodology. However, with ion-exchange chromatography, it was possible to reduce LPS more than 1,000-fold. The purification procedure did not alter the size of *P. aeruginosa* alginate since polyacrylamide gel electrophoresis did not show any sign of breakdown, and TBA did not increase during purification. The purity of the alginate was furthermore confirmed by polyacrylamide gels stained with Coomassie blue and silver, where no bands of proteins and LPS were found. The band seen with Coomassie brilliant blue was alginate, as experiments showed that alginate binds Coomassie brilliant blue very firmly.

The *P. aeruginosa* alginates did not react with St-Ab in immunoblotting and with anti-LPS in ELISA. In a neutrophil chemotaxis assay *P. aeruginosa* alginate did not function as a chemoattractant, which indicates that LPS, which is a very powerful chemoattractant in fresh human serum (41), is virtually absent (unpublished observations).

We therefore conclude that our preparations are undegraded *P. aeruginosa* alginate of high purity and that they are devoid of immunological and biological activity caused by proteins and LPS.

Chronic *P. aeruginosa*-infected patients with CF had antibodies against *P. aeruginosa* alginate. All nine bacterial alginates were immunogenic when allowed to react with the homologous patient serum (Table 3). All but one reacted very strongly in a dilution of 1:4,000. No reaction was seen with a pool of serum from young noncolonized patients with CF. Older noncolonized patients with CF had detectable levels of antibodies but still lower levels than those of the colonized patients. This observation is in accordance with that of Pier et al. (47), who found alginate antibodies in older noncolonized patients with CF. They have suggested a role for these opsonophagocytic killing antibodies in resistance to *P. aeruginosa* infection. Another possibility may be that these patients had been exposed to mucoid *P. aeruginosa* at some stage without the bacteria having been detected in sputum cultures. In previous investigations of the antibody response against *P. aeruginosa* alginate (5, 47), only alginate isolated from one *P. aeruginosa* strain has been used, on the assumption that similar results would be obtained if other strains were used. The heterogeneity in the immune response of different patients against different *P. aeruginosa* alginates observed in this study (Table 3) indicates that the use of only one *P. aeruginosa* alginate for serologic tests is not acceptable.

Four alginates reacted strongly with all the other heterologous sera. One other alginate was only weakly immunogenic with homologous and heterologous sera. This implies that some of the alginates have common immunodeterminants. Rabbits immunized with a mixture of three of the strongly reactive alginates reacted with all the other alginate samples, including the one which had low reactivity with patient serum. One patient serum sample reacted very strongly with all the other alginates except the homologous alginate, but no serum was found that had clearly the strongest reaction only to the homologous alginate. However, the antibodies present in the patient serum reflect the total antigenic stimulus to the patient immune apparatus, and several different mucoid strains of *P. aeruginosa* may be present simultaneously in the lung. The ELISA developed by using *P. aeruginosa* alginate from three different strains will be applied in our laboratory for determination of the immune reaction of patients with CF to the extracellular polysaccharide.

Alginate, whether of algal or bacterial origin, is immunogenic in rabbits, confirming the findings of others (5, 46). However, it took several booster vaccinations before two of the three rabbits immunized with SW alginate developed a good antibody response.

It was fairly simple to achieve binding of alginate to the microwell plates by dissolving the antigen in PBS. It was therefore not necessary to perform any of the coating procedures recommended for attaching polysaccharides to ELISA plates, such as coupling to poly-L-lysine (21) or using capture antibodies (8). The binding could be visualized by using native avidin and biotin. The binding shown in our experiments is probably nonspecific because avidin, a 40-kilodalton glycoprotein, does not resemble the heparin-binding lectin (13 to 16 kilodaltons) from rat lungs, which has a high affinity for alginate (10). Avidin is strongly alkaline and can bind to the negatively charged sulfate groups of heparin in mast cells (7) and possibly to polyanionic alginate.

Serum from SW alginate-vaccinated rabbits did not react with any of the *P. aeruginosa* alginates, and *P. aeruginosa* alginate-immunized rabbits did not recognize any immunodeterminants in SW alginate. The same reaction was also seen with serum from a patient with CF infected with mucoid *P. aeruginosa*. This difference in serological cross-reactivity was confirmed by the absorption experiments (Table 4). The majority of bacterial polysaccharides consist of fairly small, repeating units of from one to six saccharide residues (32), and antibodies are generated against small portions (determinant groups) of the polysaccharide ranging from two to four monosaccharide units (4). One of the major differences between SW and *P. aeruginosa* alginate is the presence of homopolymeric G blocks in SW alginate. If antibodies against SW alginate are directed solely against a combination of monosaccharides in which two to four L-guluronic acid residues are linked together, this combination will not be found in bacterial alginate and may explain the lack of cross-reactivity. Monoclonal antibodies against alginate have been found to have specificity for guluronic acid (30, 34). SW alginates have widespread use as food additives (52), as part of implantable biomaterials, and as mucoprotectives in patients with peptic ulcer. The implication of the above findings is that if humans reacted immunologically to the ingestion of SW alginate and to the GG block combination there would be little, if any, cross-reaction with an ELISA with *P. aeruginosa* alginate as antigen.

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