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Synthesis and Characterization of a *Pseudomonas aeruginosa* Alginate-Toxin A Conjugate Vaccine

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Alginate from *Pseudomonas aeruginosa* 3064 was depolymerized by controlled heating in dilute acid. The resulting depolymerized alginate (M_r < 60,000) was covalently coupled to toxin A with adipic acid dihydrazide as a spacer molecule and carbodiimide as a linker. The resulting conjugate was composed of toxin A and depolymerized alginate at a ratio of 4:1 and possessed an M_r of 260,000. The conjugate was nontoxic and nonpyrogenic. While native alginate (M_r > 640,000) given in a range of doses was poorly immunogenic in mice and rabbits, the conjugate induced high levels of antibody which bound to native alginate. Rabbits, but not mice, also produced an antitoxin immunoglobulin antibody response. Alginate derived from three other strains of *P. aeruginosa* competed with the homologous 3064 alginate for binding to anticonjugate antibody. This indicates that the conjugate elicits an antibody response able to recognize heterologous alginates. The serum from rabbits immunized with the conjugate was effective at promoting the uptake and killing of mucoid strains of *P. aeruginosa* by human polymorphonuclear leukocytes. In contrast, immunization with native alginate did not engender an opsonic antibody response. Rabbit anticonjugate antibody also neutralized the cytotoxic potential of toxin A.

Severe pulmonary infections due to *Pseudomonas aeruginosa* are a frequent occurrence among individuals with cystic fibrosis (CF) (14, 33). A substantial proportion of CF patients will eventually succumb to chronic progressive lung disease caused primarily by *P. aeruginosa*. Strains of *P. aeruginosa* isolated from the lungs of CF patients often display a mucoid phenotype due to the copious production of alginate, an acetylated polysaccharide composed of randomly repeating blocks of manuronic and guluronic acids (10, 11, 13).

Alginate is believed to be a key factor in the pathogenesis of *P. aeruginosa* pulmonary infections in CF patients. Alginate interferes with the clearance of *P. aeruginosa* because of its antiphagocytic properties (2, 29). It also acts as an adhesin for mucoid strains of *P. aeruginosa* (18, 28). Furthermore, the considerable amounts of alginate produced during an acute pulmonary exacerbation greatly hamper normal pulmonary functions by blocking airways.

Immunization with purified alginate has been shown to afford strain-specific protection against experimental *P. aeruginosa* pulmonary infections in rats (34). Antialginate antibody can promote the uptake and killing of mucoid *P. aeruginosa* by human polymorphonuclear leukocytes (1). In addition, elevated levels of opsonic antialginate antibodies were found to correlate with freedom from *P. aeruginosa* infection in a subgroup of older CF patients (24). Therefore, immunization of CF patients with an alginate-based vaccine may provide a clinical benefit either by preventing lung colonization or by facilitating the clearance of *P. aeruginosa*.

Alginates isolated from various strains of *P. aeruginosa* express conserved and strain-specific epitopes (4, 21, 23). While alginate is immunogenic in several species of animals, it is only moderately immunogenic in humans (12a; unpublished observations). In an attempt to enhance its immunogenicity, alginate in depolymerized form (D-ALG) was covalently coupled to *P. aeruginosa* toxin A to yield a conjugate vaccine. The resulting conjugate was nontoxic, nonpyrogenic, and evoked significantly better antialginate antibody responses than native alginate did.

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MATERIALS AND METHODS

Purification of alginate and toxin A. Alginate was purified from the culture supernatant of strain 3064 (mucoid CF isolate; provided by D. E. Woods, University of Calgary, Calgary, Alberta, Canada) by slight modification of previously described techniques (4, 21). Briefly, the purification scheme involved repeated ethanol fractionation; $CaCl_2$ precipitation; treatment with DNase, RNase, and pronase; extraction with hot phenol; and ultracentrifugation. Purified alginates from *P. aeruginosa* 6680, 8813, and 8839 were provided by S. S. Pedersen, Copenhagen, Denmark. The characteristics of these alginates have been described elsewhere (21).

Toxin A was purified as previously described from the culture supernatant of *P. aeruginosa* PA103 (8). Purity was greater than 97%, as determined by high-pressure liquid chromatography.

Analytical methods. The percent protein was determined by the method of Lowry et al. (17) with bovine serum albumin as a standard. The percent total carbohydrate was determined with the tryptophane reaction (31). The percent nucleic acids was determined by measuring the A_{260} of a 1-mg/ml solution of alginate. For conversion purposes, 50 μg of nucleic acid per ml equaled an A_{260} of 1. Lipopolysaccharide (LPS) content was measured by a *Limulus* lysate assay (Coatest; KabiVitrum AB, Mölndal, Sweden) using *Escherichia coli* O111 LPS as a standard. Uronic acid content was measured with the carbazole reaction (3). O-acetyl group content was quantitated by the method of Kabat and Mayer (15) with acetylcholine as a standard. The level of pyrogenicity was determined by intravenous administration of graded doses of antigen to 2- to 2.5-kg New Zealand White rabbits. Body temperature was recorded at 0.5-h intervals

for 4 h postadministration. A pyrogenic response was defined as a ≥0.3°C rise in temperature at any time point.

High-pressure liquid chromatography. A Zorbax GF-250 (resolution limits of 16,000 to 670,000 M_r; DuPont Co., Wilmington, Del.) coupled to a Uvicon 735LC monitoring system (Kontron AG, Schlieren, Switzerland) and a Spectra-Physics SP4290 integrator (Spectra-Physics, San Diego, Calif.) was used. Dextrans with molecular masses of 10, 40, 70, and 500 kDa were used as standards.

ADP ribose-transferase activity. ADP ribose-transferase activity of toxin A and the D-ALG–toxin A conjugate was determined with wheat germ elongation factor 2 as a substrate (9).

Conjugate synthesis. Alginate was depolymerized by heating a 4-mg/ml solution in 1% (vol/vol) acetic acid at 121°C for 30 min. After cooling, the solution was extensively dialyzed against pyrogen-free water and lyophilized. D-ALG ($M_r \leq$ 60,000) was covalently coupled to toxin A as follows. To 25 mg of toxin A and 25 mg of carbodiimide (EDEC; Fluka AG, Buchs, Switzerland) was added 50 mg of adipic acid dihydrazide (ADH; Fluka AG) in phosphate-buffered saline (PBS), pH 7.4. The solution was gently stirred for 2 h at 22°C, and the pH was maintained at 4.8 to 4.9 by the addition of 0.3 N HCl. The resulting toxin A-ADH solution was filter sterilized to remove insoluble material. Approximately 13 mol of ADH were incorporated into each mole of toxin A, as determined by a reaction with 2,4,6-trinitrobenzenesulfonic acid. ADH was used to generate a standard curve.

D-ALG (100 mg/ml in distilled water) was added to a fivefold excess (wt/wt) of EDEC and 50 mg of toxin A-ADH. The pH was maintained at 4.8 to 4.9 by the addition of 0.3 N HCl for 3 h at room temperature with stirring. The solution was concentrated to about 10 ml with an Amicon YM-30 membrane filter (Amicon Corp., Danvers, Mass.). The concentrate was chromatographed over a Sephadex G-75 column (3 by 95 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in sterile distilled water. The conjugate was eluted in the void volume fractions which were pooled, concentrated with an Amicon YM-30 membrane filter (Amicon Corp.), and filter sterilized.

Preparation of vaccines. Native alginate and the D-ALG–toxin A conjugate were filter sterilized. The antigen content was adjusted by dilution in sterile PBS. For adsorption to $Al(OH)_3$, an equal amount of sterile $Al(OH)_3$ (0.8% wt/vol) was mixed with the antigen preparation (twice the desired final concentration), incubated for at least 8 h at room temperature, and aseptically bottled.

Immunogenicity studies. Mice (NMRI strain, 18 to 20 g) in groups of five and rabbits (New Zealand White, 2 to 2.5 kg) in groups of three to six were immunized intramuscularly on days 0 and 14 with various doses of antigen. Rabbits received either 10 or 50 μg of native alginate, 50 or 100 μg of D-ALG, or 50 μg of D-ALG as the conjugate (equal to 250 μg of conjugate). Mice received 0.1, 1, or 10 μg of native alginate or 10 μg of D-ALG as the conjugate (equal to 50 μg of conjugate). Serum samples were obtained on days 0, 14, and 28 for analysis.

ELISA. Each well of a Dynatech Immulon microtiter plate (Dynatech AG, Buchs, Switzerland) was coated with 100 μl of either a 10-μg/ml solution of alginate in PBS, pH 7.4, or a 10-μg/ml solution of toxin A in 0.1 M $NaCO_3$, pH 9.6, and incubated overnight at 25°C. Prior to use, the plates were washed three times in PBS, pH 7.4, containing 0.05% (wt/vol) Tween 20 (PBS-T). Serial dilutions of serum samples in PBS-T (100 μl per well) were added to the plates, which were then incubated at 25°C for 3 h. The plates were washed three times in PBS-T, 100 μl of a 1:2,500 dilution (in PBS-T) of peroxidase-labeled goat anti-mouse or goat anti-rabbit immunoglobulin G (IgG) (Nordic Immunology, Tilburg, The Netherlands) was added to each well, and the plates incubated at 25°C for 2 h. After the wells were washed three times in PBS-T, 100 μl of 2,2′-azino-di(3-ethyl)-benzylthiozoline (Böhringer Mannheim, Mannheim, Federal Republic of Germany; 10 mg/ml in 0.1 M $NaPO_4$, pH 4.0) was added to each well and the color was allowed to develop at 25°C for 30 min. The A_{405} was read with a Dynatech MR5000 enzyme-linked immunosorbent assay (ELISA) reader. The A_{405} was converted into ELISA units by multiplying the reciprocal of the serum dilution which yielded a value that fell onto the linear portion of the titration curve (A_{405}, 0.2 to 1.0) by the actual A_{405} value.

A competitive ELISA to determine the degree of cross-reaction between alginate purified from various strains of *P. aeruginosa* and strain 3064 was performed as follows. The serum from one rabbit hyperimmunized with alginate purified from strain 3064 (in complete Freund adjuvant) was diluted in PBS-T to yield an A_{405} of approximately 0.8, which falls within the linear portion of the dilution curve. Alginate from *P. aeruginosa* 3064, 6680, 8813, and 8839, plus 3064 D-ALG, LPS purified from strain 3064, and toxin A, were diluted in PBS-T and added to the anti-3064 serum to give the following final concentrations: 1, 5, 25, and 125 μg/ml. The antigen-antiserum solutions were incubated for 30 min at 37°C. To washed plates coated with 3064 alginate, 100 μl of anti-3064 antiserum alone or with various concentrations of competing antigens was added to duplicate wells and incubated for 3 h at 25°C. The ELISA was performed as described above. The inhibition dose (ID_{50}) was defined as the concentration of antigen that reduced the A_{405} by 50% of the value obtained with antiserum alone.

Opsonophagocytic assay. A mucoid variant of *P. aeruginosa* 3064 or 6680 was grown to mid-log phase on Trypticase soy broth (BBL, Cockeysville, Md.) containing glucose, at 37°C with gentle shaking. The bacteria were washed in cold, PBS, pH 7.4, and suspended in Eagle medium containing 0.1% (wt/vol) gelatin at a final concentration of approximately 10^7 bacteria per ml. The bacteria were incubated with serially diluted test sera or Eagle medium alone for 30 min at 37°C and resuspended in Eagle medium. An equal volume of human polymorphonuclear leukocytes (2.3×10^7/ml) containing normal rabbit serum (2%, vol/vol) as a complement source (which contained no antialginate antibody, as determined by ELISA) was added. An aliquot of this suspension was immediately taken, serially diluted, and plated onto agar plates for base-line bacterial counts. The remaining samples were incubated at 37°C for 120 min with continuous gentle mixing. Bacterial counts were again determined by plate counts. The percentage of the initial bacterial inoculum killed was calculated by the following formula:

$$\frac{\text{bacterial counts (all reagents except test serum)} - \text{bacterial counts (all reagents including test serum)}}{\text{bacterial counts (all reagents except test serum)}} \times 100$$

The titer was defined as the reciprocal of the highest dilution of serum resulting in a ≥75% reduction in the initial bacterial counts.

Toxin A neutralization assay. The ability of various sera to neutralize the cytotoxicity of toxin A was determined in a HEp-2 cell assay as previously described (9).

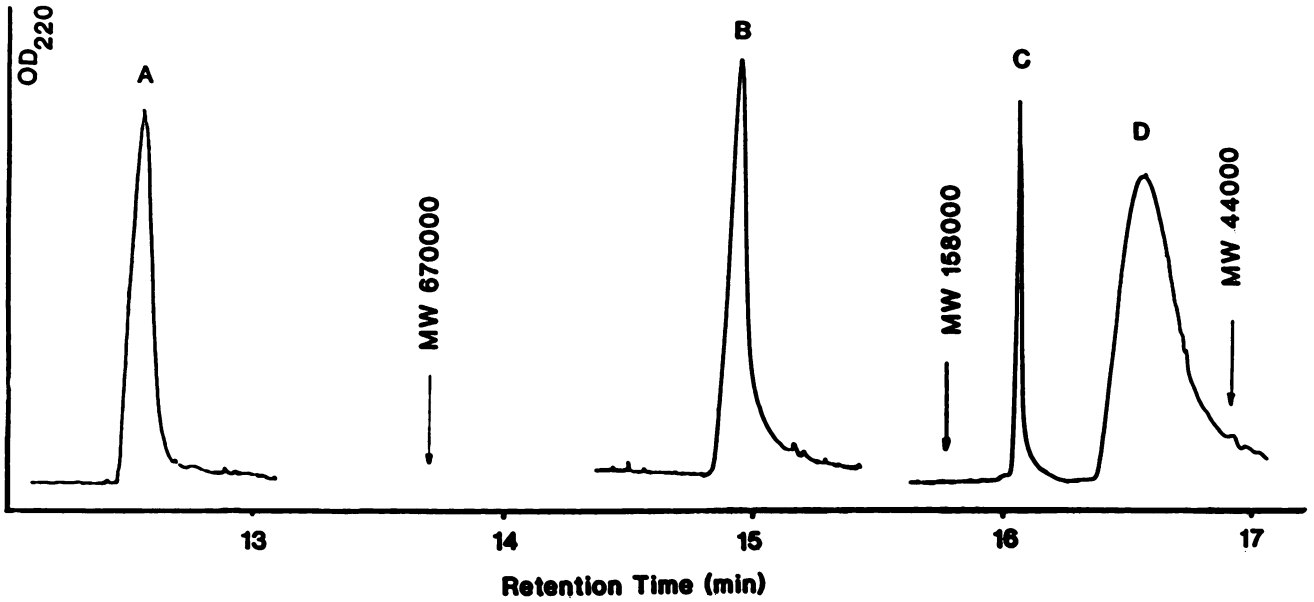

FIG. 1. High-pressure liquid chromatography of native alginate (peak A), D-ALG–toxin A conjugate (peak B), toxin A (peak C), and D-ALG (peak D). Retention times of molecular weight (MW) markers are indicated. OD_{220}, Optical density at 220 nm.

RESULTS

Initial attempts to couple native alginate ($M_r > 640,000$) to toxin A gave poor yields of conjugate. This was most likely due to the large molecular weight of native alginate ($M_r > 2 \times 10^6$) compared with that of toxin A ($M_r = 6 \times 10^4$) and to the viscosity of the reaction mixture. We therefore sought to reduce the molecular weight of native alginate as a means of facilitating conjugate formation. Controlled heating of alginate for a short period of time in dilute acetic acid resulted in a substantial reduction in its molecular weight ($M_r < 60,000$; Fig. 1). Such D-ALG still reacted with a rabbit antiserum produced against native alginate (as determined by ELISA), as evidenced by the ability of such antiserum to react with D-ALG-coated plates.

D-ALG could be coupled to toxin A in the presence of EDEC with ADH as a spacer molecule with good yields (Fig. 1). Various characteristics of native alginate, D-ALG, and the D-ALG–toxin A conjugate are shown in Table 1. Native alginate and D-ALG were composed primarily of uronic acid-containing carbohydrate. D-ALG contained roughly 33% of the O-acetyl groups of native alginate. The conjugate was composed of 81% toxin A and 19% D-ALG by weight. The conjugate possessed a molecular weight of 260,000, substantially greater than the weight of either unconjugated D-ALG or toxin A-ADH. All three preparations were nonpyrogenic when administered to rabbits at a dose of 10 μg/kg of body weight. The conjugate was nontoxic when 50 μg of conjugate (equal to 40 μg of toxin A protein) was administered intraperitoneally to mice and was devoid of the ADP ribose-transferase activity characteristic of toxin A.

The ability of native alginate, D-ALG, and the conjugate vaccine to produce an antialginate IgG antibody response in mice and rabbits is shown in Table 2. Native alginate (50 μg per dose for rabbits and 10 μg per dose for mice) administered intramuscularly did not engender a significant increase in antibody levels in either rabbits or mice. Lesser amounts of alginate (0.1 and 1 μg per dose for mice and 10 μg per dose for rabbits) were also poorly immunogenic (data not shown). D-ALG (50 μg per dose) was also nonimmunogenic in rabbits. In contrast, immunization with the D-ALG–toxin A conjugate elicited a strong antialginate IgG antibody response. A single dose elicited a substantial (6- to 10-fold) rise in antibody levels in both mice and rabbits. A booster dose resulted in a further rise in antibody levels, which were modest in rabbits but pronounced in mice.

The ability of anticonjugate antibody to recognize alginate purified from different mucoid strains of *P. aeruginosa* as determined in a competitive binding ELISA is shown in Table 3. Alginate from strains 3064 (homologous), 6680, and 8813 inhibited the binding of anticonjugate antibody to alginate purified from strain 3064 at a final concentration of <1 μg/ml (ID_{50}, <1 μg/ml). The ID_{50} values for alginate purified from strain 8839 and D-ALG were 1.25 and 1.4 μg/ml, respectively. LPS of the same serotype as that expressed by strain 3064 and toxin A did not compete for antibody binding ($ID_{50} > 125$ μg/ml).

TABLE 1. Characteristics of alginate, D-ALG, and the D-ALG–toxin A conjugate[a]

Prepn	Composition[b] (%)		μM O-acetyl[c] group/mg of carbohydrate	M_r[d]
	Protein[e]	Carbohydrate[f]		
Alginate (3064)	<1.0	>95	14.8	2×10^6
D-ALG	<1.0	>95	5.2	6×10^4
D-ALG–toxin A conjugate	81	19	8.7	2.6×10^5

[a] The smallest quantity of antigen needed to evoke a ≥0.3°C rise in body temperature was >10 μg/kg for each preparation.

[b] Nucleic acids composed <1.0% of each preparation, as determined with the A_{260}; LPS composed <0.1% of each preparation, as determined by *Limulus* lysate assay.

[c] Determined by the method of Kabat and Mayer (15) with acetylcholine as a standard.

[d] Determined by high-pressure liquid chromatography.

[e] Determined by the method of Lowry et al. (17) with bovine serum albumin as a standard.

[f] Determined with the tryptophane reaction (31).

TABLE 2. Ability of native alginate and D-ALG–toxin A conjugate to elicit an antialginate immune response in mice and rabbits

Animal[a]	Immunogen	Geometric mean [IgG ELISA units (range)]		
		Preimmune	Day 14	Day 28
Rabbits	Native alginate	1.6 (1.4–2.0)	2.0 (1.5–2.5)	2.4 (2.0–2.7)
	D-ALG	3.2 (2.8–3.6)	ND[b]	3.9 (3.5–4.4)
	D-ALG–toxin A conjugate	2.3 (1.9–3.0)	27.0 (9.0–96.0)	108.0 (39.0–197.0)
Mice	Native alginate	≤0.2[c] (≤0.2)	0.3 (0.2–0.5)	0.3 (0.2–0.7)
	D-ALG–toxin A conjugate	≤0.2 (<0.2)	1.3 (0.6–2.6)	73.4 (20.0–143.0)

[a] Rabbits (groups of three for native alginate or D-ALG and groups of six for the D-ALG–toxin A conjugate) were immunized with 50 μg of native alginate or D-ALG or 50 μg of D-ALG conjugated to toxin A (equal to 250 μg of conjugate) adsorbed to alum on days 0 and 14. Mice (groups of 5) were immunized with 10 μg of native alginate or 10 μg of D-ALG conjugated to toxin A (equal to 50 μg of conjugate) on days 0 and 14.
[b] ND, Not done.
[c] Lower limit of detection was 0.2 ELISA units.

The ability of antibody induced by native alginate or the conjugate vaccine to promote the uptake and killing of mucoid *P. aeruginosa* 3064 or 6680 by human polymorphonuclear leukocytes is shown in Table 4. None of the sera from the six rabbits which received native alginate enhanced phagocytic killing (titer, <2). In contrast, immunization with the conjugate vaccine evoked a substantial opsonic antibody response against both strains. The mean titer postimmunization was higher for the homologous strain (3064). However, the titer rises were comparable.

The abilities of sera from rabbits or mice immunized with either native alginate or the conjugate vaccine to neutralize toxin A are shown in Table 5. As would be expected, immunization with alginate did not induce either an antitoxin A-binding (IgG ELISA) or a neutralizing-antibody response. Although the conjugate did not elicit an antitoxin A response in mice, high levels of both toxin A binding and neutralizing antibodies were elicited in rabbits.

DISCUSSION

Pulmonary infections due to *P. aeruginosa* continue to be a leading cause of morbidity and mortality among CF patients. The introduction of more-effective antipseudomonal antibiotics, particularly the DNA gyrase inhibitors (quinolones), has markedly improved the quality of life and prolonged the average age of CF patients. As with other classes of antibiotics, resistance to these new-generation agents will undoubtedly become a problem. Strains of *P. aeruginosa* resistant to quinolones have already been isolated from CF patients during therapy (5), a recurrent problem with *P. aeruginosa*.

Initial colonization of the respiratory tract by *P. aeruginosa* is believed to occur with strains expressing smooth LPS (22). With time, there is a phenotypic shift so that the majority of isolates express a mucoid phenotype and rough LPS. There is an increasing body of evidence to indicate that the early-colonizing strains also produce a small amount of alginate (20, 22). Thus, sera of CF patients harboring only nonmucoid *P. aeruginosa* strains produced a significantly greater amount of antialginate IgG and IgA prior to the emergence of mucoid variants (20). On the basis of the above-described findings, the induction of either anti-LPS or antialginate antibodies prior to colonization could, in theory, offer a degree of protection.

Attempts to bolster resistance to *P. aeruginosa* in CF patients by immunization with LPS-based vaccines have yielded disappointing results. In several studies, no clinical benefit could be attributed to vaccination (16, 25). Immunization of colonized patients, who already possessed elevated levels of anti-LPS antibodies, did not improve their clinical status even though a vigorous immune response was mounted (25). Immunization of a small number of noncolonized patients also appeared to be of limited benefit (16). However, interpretation of the latter study is complicated by a lack of detail concerning the magnitude and duration of the immune response and the seroepidemiology of the infecting strains. Recently Winnie et al. (32) have shown that administration of intravenous immune globulins (known to contain antibodies to various serotypes of *P. aeruginosa* LPS and to toxin A [26]) early in the course of a *P. aeruginosa* pulmonary exacerbation resulted in a more rapid clinical improvement, suggesting a role for at least adjunctive antibody therapy.

The demonstration that antialginate antibody can promote the clearance of mucoid *P. aeruginosa* via opsonophagocytic killing (1), coupled with the finding that elevated levels of

TABLE 3. Ability of D-ALG–toxin A conjugate to elicit an antibody response recognizing heterologous alginate

Prepn	ID_{50}[a] (μg/ml)
Alginate from strain:	
3064	<1
6680	<1
8813	<1
8839	1.25
D-ALG	1.40
LPS	>125
Toxin A	>125

[a] The quantity of competing antigen required to inhibit 50% binding of anti-D-ALG–toxin A conjugate to homologous 3064 alginate.

TABLE 4. Opsonic antialginate antibody engendered by immunization with alginate of the D-ALG–toxin A conjugate

Immunogen[a]	Test strain	Mean opsonic titer[b] (range)	
		Preimmunization	Postimmunization
Alginate	3064	<2	<2
	6680	<2	<2
D-ALG–toxin A	3064	16 (10–20)	112 (80–320)
	6680	4 (<2–20)	40 (20–80)

[a] Rabbits (groups of five or six) were immunized with 50 μg of alginate (strain 3064) or D-ALG–toxin A conjugate (total, 250 mg) on days 0 and 14.
[b] The reciprocal of the highest dilution of serum resulting in ≥75% bacterial killing in the presence of complement and human polymorphonuclear leukocytes.

TABLE 5. Toxin A-neutralizing antibody engendered by immunization with D-ALG–toxin A conjugate

Immunogen[a]	Animal	Geometric mean[b]			
		ELISA units		μg of toxin A neutralized/ml of serum[c]	
		Pre-immune	Post-immune	Pre-immune	Post-immune
Alginate	Mice	<1	<1	<0.3	<0.3
	Rabbits	<10	<10	<0.3	<0.3
D-ALG–toxin A conjugate	Mice	1	3	<0.3	<0.3
	Rabbits	11	260	<0.3	3.1

[a] Mice and rabbits were immunized with 10 and 50 μg of alginate antigen, respectively, on days 0 and 14.
[b] Preimmune, day 0, postimmune, day 28.
[c] The lower limit of detection was 0.3 μg of toxin A neutralized per ml of serum.

opsonic antialginate antibody appear to confer protection against infection in a subpopulation of CF patients (24), has stimulated considerable interest in the development of an alginate-based vaccine. Unfortunately, native alginate was found to be poorly immunogenic in healthy adult volunteers (12a; unpublished observations). In an attempt to circumvent this problem, we coupled serologically reactive low-molecular-weight alginate to toxin A using techniques previously shown to yield a vaccine suitable for human use (7). Toxin A was selected as a carrier protein because of prior findings that antitoxin A antibodies may exert a protective effect against *P. aeruginosa* infections (6, 27).

The D-ALG–toxin A conjugate vaccine described here was far more effective at eliciting both alginate-binding IgG antibodies and opsonic antibodies than was native alginate in two animal species. There was evidence that the conjugate behaved as a T-dependent antigen, as indicated by increased antialginate IgG antibody levels following a second immunization. It was somewhat surprising that alginate purified from strain 3064 was poorly immunogenic in both mice and rabbits. We selected alginate produced by this strain on the basis of prior studies showing that immunization with such alginate engendered in laboratory animals an antibody response which bound to alginates purified from other strains of *P. aeruginosa* (4). However, in previous studies in which alginate was found to be immunogenic in animals, high doses were administered by either the intravenous or intraperitoneal route (4, 12) or alginate was combined with potent adjuvants. The native alginate used in these studies possessed a high molecular weight ($>2 \times 10^6$) and was acetylated. Therefore, structural alterations alone cannot be responsible for its poor immunogenicity.

Of prime importance was the fact that the conjugate vaccine induced antibodies with the desired functional attributes. Antibody directed against the alginate moiety was cross-reactive with heterologous alginates, as determined by a competitive binding ELISA. Anticonjugate antibody was also able to promote the uptake and killing of two mucoid strains of *P. aeruginosa* by human polymorphonuclear leukocytes. In addition, immunization with the conjugate induced toxin A-neutralizing antibodies in rabbits. Therefore, the conjugation method employed preserved critical epitopes on both the toxin A and alginate vaccine components.

The conjugate described herein possesses those traits desired in an alginate-based vaccine, e.g., being nontoxic, nonpyrogenic, and able to elicit functional antibodies against alginate and toxin A. However, the evaluation of any anti-*P. aeruginosa* vaccine in CF patients raises several issues. Foremost among these is the possibility that immune complexes formed among alginate, LPS, toxin A, and their respective antibodies are an important contributing factor to the deteriorating clinical picture observed during a pulmonary exacerbation caused by *P. aeruginosa* (19, 30). A major concern is that vaccination would result in an irreversible priming of the immune system, which would potentiate immune complex formation and disease progression. Arguing against this is the fact that most normal children and children with CF have low levels of naturally acquired antibody to both *P. aeruginosa* alginate and LPS (19, 20, 24). However, it would appear that he majority of such antialginate antibody is not opsonic and, therefore, is nonprotective (19, 24).

We have recently immunized a small number (~20) of young noncolonized CF patients with a multivalent *P. aeruginosa* O-polysaccharide-toxin A conjugate vaccine. After up to 1 year of surveillance, immunization had no discernible adverse effect on these patients' clinical statuses in spite of eliciting long-lasting high titers of antibodies against LPS and toxin (28a). Therefore, induction of anti-*P. aeruginosa* antibody in and of itself does not appear to have a deleterious effect. Given the facts that (i) once pulmonary colonization occurs, it is impossible to eradicate *P. aeruginosa* from the lungs, (ii) colonization eventually progresses to outright disease, and (iii) exacerbations are recurrent and difficult to manage, a vaccine which would even delay the sequence of events would be of great benefit.

The conjugate vaccine described herein is currently being evaluated for safety and immunogenicity in healthy adult volunteers.

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