

Vaccine Potential of *Pseudomonas aeruginosa* O-Polysaccharide-Toxin A Conjugates

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Serologically reactive O-polysaccharide from nine serotypes of *Pseudomonas aeruginosa* were covalently linked to toxin A via reductive amination, with adipic acid dihydrazide serving as a spacer molecule. The conjugates were composed of toxin A/O-polysaccharide ratios ranging from 1.17:1 to 3:1. All possessed an average M_r of $>10^6$, were devoid of ADP ribosyltransferase activity associated with toxin A, and were nontoxic for mice and guinea pigs. The conjugates were stable from toxic reversion when stored at 37°C for 28 days. The conjugation condition used preserved a substantial proportion of critical epitopes on the toxin A molecule as shown by the ability of toxin A-neutralizing monoclonal antibodies to react with the various conjugates. All nine conjugates were capable of evoking an antitoxin A and an antilipopolsaccharide immunoglobulin G (IgG) response in mice and rabbits. Rabbit antitoxin A IgG was capable of neutralizing the cytotoxic effect of toxin A, whereas mice immunized with any of the conjugates were protected against toxin A intoxication. Rabbit anti-conjugate IgG, when passively transferred to mice, was highly effective at preventing fatal *P. aeruginosa* burn wound sepsis.

Nosocomial infections due to *Pseudomonas aeruginosa* present a major problem among numerous populations of compromised patients (4, 6). Treatment of *P. aeruginosa* infections is greatly hampered by innate and acquired antibiotic resistance (15, 22). Therefore, fatality rates of 25% or greater are not uncommon for *P. aeruginosa* bacteremia or pneumonia (3, 10). These findings have served as an impetus to investigate the feasibility of controlling *P. aeruginosa* infections through immunological means (for reviews, see references 2 and 8).

Human immunity appears to be conferred by humoral antibody directed against serospecific determinants expressed by lipopolysaccharide (LPS) or against toxin A (1, 5, 21, 27). It is interesting that antitoxin A antibody and anti-LPS antibody may provide protection through an independent and additive mechanism(s) (21). It therefore appears that optimal protection against *P. aeruginosa* would be obtained by use of a vaccine capable of engendering both anti-LPS and antitoxin A antibodies.

In a prior study (8), we have described a conjugate vaccine produced by covalently coupling serologically reactive O-polysaccharide (O-PS) derived from *P. aeruginosa* immunotype 5 LPS to toxin A. This vaccine was nontoxic and capable of engendering both anti-LPS and antitoxin A immune responses in animals. Existing epidemiological evidence indicates that numerous serotypes of *P. aeruginosa* can cause life-threatening disease in humans (6, 20). Since the monosaccharide composition of the O-PS moiety of *P. aeruginosa* can vary substantially from serotype to serotype (12, 16), we were interested in determining the feasibility of applying the conjugation method described above to the relevant serotypes of *P. aeruginosa* LPS. In the present report, we describe the synthesis and characterization of nine O-PS-toxin A conjugate vaccines using O-PS from those serotypes of *P. aeruginosa* most frequently associated with serious disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PA220 (Habs serotype 6) and W-18 (serotype 10) were provided by B. Wretling, Danderyd Hospital, Danderyd, Sweden. *P. aeruginosa* E-576 (serotype 2/5) and PA53 (serotype 1) were gifts from A. Cross, Walter Reed Army Institute of Research, Washington, D.C. Strains 8505 (serotype 3) and 6511 (serotype 4) were provided by T. L. Pitt, Public Health Laboratory Service, London, England. Strains ATCC 27313 (serotype 11), ATCC 27316 (serotype 7), and ATCC 27318 (serotype 5), were gifts of M. Fisher, Parke, Davis & Co., Detroit, Mich. Cultures were grown on Trypticase soy broth plus glucose (BBL Microbiology Systems, Cockeysville, Md.).

Purification of LPS and O-PS. LPS was extracted from washed whole cells by the phenol-water method of Westphal et al. (25). Crude LPS pellets obtained by ultracentrifugation at $100,000 \times g$ for 3 h were treated with DNase, RNase, and pronase as previously described (9). O-PS was isolated from acid-treated LPS as described elsewhere (8). O-PS isolated in this manner possessed a molecular weight of $<50,000$.

Purification of toxin A. Toxin A was purified from the supernatant of fermentor-grown cultures of *P. aeruginosa* PA103 as previously described (7). The purity of the final preparations was between 92 and 98% as determined by high-pressure liquid chromatography (HPLC; see below). The mean lethal dose for purified toxin A was 0.15 to 0.30 μg (depending on the lot tested) when administered intraperitoneally to female NMRI mice weighing 18 to 20 g each.

Synthesis of O-PS-toxin A conjugate. Oxidized O-PS was coupled to toxin A as described elsewhere (8). Briefly, O-PS was oxidized in the presence of 0.1 M NaIO_4 . Free NaIO_4 was removed by dialysis, and the O-PS was lyophilized. Adipic acid dihydrazide (ADH; Fluka AG, Buchs, Switzerland) was introduced into toxin A with carbodiimide. Toxin A-ADH was coupled to oxidized O-PS by combining equal amounts of reagents (5 mg/ml) in sterile water. After 6 h of incubation at 22°C, NaCNBH_3 was added, and the reaction

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mixture was stored for 5 days at 22°C. After dialysis, the mixture was filtered over a column (gel bed, 5 by 45 cm) of AcA34 (LKB-Produkter, Bromma, Sweden). The material which eluted at the void volume (M_r , >350,000) was pooled and lyophilized.

The carbohydrate content of the conjugates was determined by the tryptophan method (26) with purified O-PS as a standard. The protein content was measured by the method of Lowry et al. (18) with toxin A as a standard.

Enzymatic activity assay for toxin A. The ADP ribosyltransferase activity of toxin A was determined with wheat germ extract as a substrate (13). Native toxin A or conjugates were treated with urea-dithiothreitol before assay. For testing of the conjugates, the equivalent of 100 ng of protein was tested per assay sample. Native toxin A, when tested at 10 ng per assay, yielded values at least fourfold greater than background (all reagents minus toxin). Values less than twofold above background were considered negative.

HPLC. Native toxin A, O-PS, and conjugates were analyzed by HPLC with a Du Pont GF-250 column as previously described (8).

Cytotoxicity assay. The ability of antisera to neutralize the cytotoxic activity of toxin A was determined with a HEp-2 cell assay system described elsewhere (8).

Toxicity assay. The lethal effect of toxin A or conjugates was determined for female NMRI mice weighing 18 to 20 g each. Each mouse received the equivalent of 200 µg of toxin A protein as a conjugate (equal to 1,000 mean lethal doses of native toxin A) intraperitoneally. Groups of six mice were used and observed for 14 days postchallenge.

Immunogenicity studies. New Zealand White rabbits (2 to 2.5 kg each) were immunized intramuscularly on days 0, 14, and 28 with 50 µg of conjugate. Venous blood samples were obtained on days 0, 14, 28, and 42, and the serum was collected and frozen.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) for quantitation of antitoxin A and anti-LPS immunoglobulin G (IgG) antibodies were performed as described elsewhere (8). The titer was defined as the reciprocal of the highest dilution of serum yielding an A_{405} of ≥ 0.4 , a value at least 2 standard deviations above background.

Protection studies. The murine burn model of Stieritz and Holder was used (24). Mice (18 to 20 g; female; NMRI strain) were used in groups of six. For passive-protection studies, IgG was isolated from pooled rabbit serum (three rabbits per pool) after immunization with the conjugate vaccines as described elsewhere (12). Each mouse received 0.2 ml of IgG (unconcentrated) intravenously 24 h before challenge. Mortality was recorded for a minimum of 5 days postchallenge. For active immunization studies, mice (groups of six) were immunized intramuscularly with 1 µg of conjugate in 100 µl of 0.5% Al(OH)₃ or with Al(OH)₃ alone on days 0 and 14. The challenge was performed on day 28.

MAbs. Monoclonal antibodies (MAbs) to toxin A were produced as follows. Mice (BALB/c) were repeatedly immunized with sublethal doses of purified toxin A. Immune spleen cells were fused with the nonproducer myeloma cell line X63-Ag8.653 (14) by previously described techniques (17). Cell culture supernatants from resulting hybridomas were assayed for production of antitoxin A by the dot-immunobinding assay (11) with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). Seven independently derived hybridomas were identified which produced antibody reactive with toxin A and were able to neutralize the cytotoxic potential of toxin A in the HEp-2 cell assay system (see above). Hybridoma lines designated

TABLE 1. Characteristics of O-PS-toxin A conjugates

Conjugate (O-PS serotype)	Composition (% [dry wt])		Enzymatic activity ^a	Toxicity (LD ₅₀ [µg]) ^b	Mol wt ^c
	Protein (toxin A)	Carbohydrate (O-PS)			
1	54	46	ND ^d	>200 ^e	>10 ⁶
2	55	45	ND	>200	>10 ⁶
3	73	27	ND	>200	>10 ⁶
4	66	34	ND	>200	>10 ⁶
5	62	38	ND	>200	>10 ⁶
6	75	25	ND	>200	>10 ⁶
7	70	30	ND	>200	>10 ⁶
10	69	31	ND	>200	>10 ⁶
11	66	34	ND	>200	>10 ⁶
Toxin A	>95	ND	4.2 × 10 ²	0.15–0.3 ^f	66,000

^a In picomoles of ADP ribose transferred per microgram of protein.

^b Determined by intraperitoneal injection in mice. The values shown for the conjugates are expressed as the amount of toxin A protein administered.

^c Determined by HPLC.

^d ND, Not detected. Values were less than two-fold above background.

^e Highest amount given; all animals survived.

^f Range for five lots.

as TA-57, 94, 117, 121, 379, and 417 produced IgG1, and TA-313 produced IgG2a antibody as determined by the dot-immunobinding assay with antisera specific for murine immunoglobulin subclasses (Serotec, Oxon, England).

The reactivity of these MAbs with the various O-PS-toxin A conjugates, native toxin A, and toxin A-ADH was analyzed by the dot-immunobinding assay with horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad) and 4-chloro-1-naphthol to visualize membrane-bound antibody-antigen complexes.

RESULTS

Covalent coupling of O-PS to toxin A via reductive amination, with ADH serving as a spacer molecule, was found to be an effective method of constructing conjugates regardless of the O-PS serotype (Table 1). The composition of the nine conjugates was similar, with a ratio of protein to carbohydrate ranging from 1.17:1 (serotype 1) to 3:1 (serotype 6). All preparations were devoid of enzymatic activity and toxicity. The conjugates possessed an average molecular weight of greater than 10⁶, eluting as a single broad peak in the void volume when analyzed by HPLC (Fig. 1). These findings indicate that considerable intramolecular cross-linking occurs during conjugate formation and that each preparation is most likely composed of several high-molecular-weight species. O-PS-toxin A conjugates could be synthesized in a highly reproducible manner. Six consecutive lots of serotype 10 conjugate gave a protein to carbohydrate ratio between 1.5:1 and 2.5:1. The six lots were of comparable molecular weights (>10⁶) and nontoxic.

The stability of the conjugates to toxic reversion was determined in the following manner. Each preparation was incubated at 37°C for 28 days, at which time they were assayed for toxicity by intraperitoneal injection mice (200 µg of conjugate protein per mouse) and for enzymatic activity. All nine conjugates remained nontoxic after incubation, and there was no evidence of an increase in enzymatic activity. Conjugates were pooled and lyophilized to form a nine-valent vaccine. A 0.5-ml volume containing a total of 225 µg of O-PS (25 µg per serotype) and 372 µg of toxin A was injected intraperitoneally per mouse either immediately after reconstitution or after storage at 37°C for 28 days. No toxicity was noted.

A series of MAbs which recognized critical epitopes expressed by native toxin A was used to determine what effect the conjugation conditions had on the antigenic makeup of the toxin A moiety (Table 2). MAbs TA-117, 379, and 417 reacted with toxin A, toxin A-ADH, and all conjugates, indicating that these epitopes are not significantly altered during conjugate synthesis. MAb TA-313 reacted with all antigens except the serotype 6 conjugate. In contrast, MAbs TA-57 and TA-121 did not react with any conjugate, indicating the destruction of at least two neutralizing epitopes during synthesis. Interestingly, MAb TA-57 appeared to recognize a toxin A determinant destroyed by coupling to ADH.

Next, the ability of these conjugates to evoke an immune response in rabbits was determined (Table 3). All nine preparations were capable of inducing IgG antibody which bound both toxin A and LPS of the appropriate serotype. Antitoxin A IgG was also capable of neutralizing the cytotoxic potential for toxin A for HEp-2 cells. There were considerable differences in the titers of antibodies to toxin A and LPS induced by the various conjugates. In regard to the anti-LPS response, the serotype 2 conjugate was by far the most immunogenic (mean titer, 23,200). Similarly, serotype 10 and 11 conjugates evoked the most vigorous antitoxin A

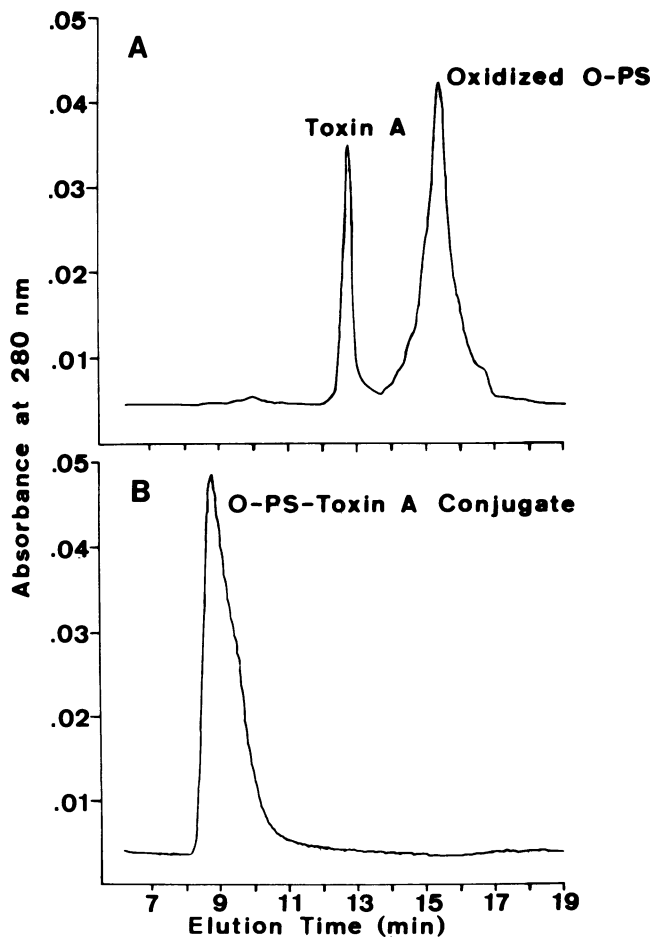


FIG. 1. Analysis of native toxin A, serotype 10 O-PS, and toxin A-O-PS conjugate by HPLC. A Du Pont GF-250 column (gel filtration; resolving range, about 10⁴ to 10⁶ daltons) was used. Panels: A, native toxin A and oxidized O-PS; B, toxin A-O-PS conjugate.

TABLE 2. Reactivity of toxin A-neutralizing MAbs, O-PS-toxin A conjugates, toxin A, and toxin A-ADH^a

Conjugate (O-PS serotype)	Reactivity with the following MAb:						
	TA-57	TA-94	TA-117	TA-121	TA-313	TA-379	TA-417
1	-	+	+	-	+	+	+
2	-	+	+	-	+	+	+
3	-	+	+	-	+	+	+
4	-	+	+	-	+	+	+
5	-	+	+	-	+	+	+
6	-	+	+	-	-	+	+
7	-	+	+	-	+	+	+
10	-	+	+	-	+	+	+
11	-	+	+	-	+	+	+
Toxin A-ADH	-	+	+	+	+	+	+
Toxin A	+	+	+	+	+	+	+

^a Determined by the dot-immunobinding technique. Symbols: +, strong reaction; -, no reaction.

response, with serotype 5 being the weakest immunogen. In most cases, the toxin A-neutralizing capacity correlated well with the antitoxin A ELISA titer, the exceptions being serotypes 5 and 10.

The conjugates were next evaluated for ability to protect mice against toxin A intoxication (Table 4). All of the conjugates evoked an antitoxin A IgG antibody response. Although the amount of each conjugate used to immunize the mice was standardized as to the amount of toxin A protein, the immune response was found to vary considerably. All mice which were immunized with a conjugate were protected against challenge with native toxin A. The level of protection, for the most part, correlated well with the antitoxin A titer in serum.

The protective capacity of anti-conjugate IgG was evaluated in a murine burn sepsis model (Table 5). IgG was isolated from sera of rabbits immunized with each of the nine conjugates and passively transferred to mice 24 h before challenge with homologous *P. aeruginosa*. All immune IgG preparations were found to afford substantial protection when compared with normal rabbit serum (NRS). The absolute degree of protection ranged from 11-fold (serotype 4 conjugate) to >10,000-fold (serotype 2 conjugate). This wide variance in protection achieved with anti-conjugate IgGs can most likely be attributed to the innate virulence of the

TABLE 3. IgG response in rabbits following vaccination with O-PS-toxin A conjugates^a

Conjugate (O-PS serotype)	Mean IgG ELISA titer (range) for ^b :		μg of toxin A neutralized/mg of IgG ^c
	LPS	Toxin A	
1	1,650 (1,550-1,700)	1,717 (1,300-2,250)	1.2
2	23,200 (23,000-23,500)	1,625 (1,500-1,750)	2.9
3	227 (150-335)	1,143 (590-1,900)	1.3
4	470 (220-900)	532 (215-700)	1.1
5	715 (255-960)	273 (195-365)	4
6	6,050 (2,600-9,500)	1,425 (950-1,900)	2.25
7	203 (80-290)	1,163 (910-1,500)	2.5
10	1,500 (1,250-1,750)	12,800 (10,600-15,000)	0.7
11	2,825 (2,750-2,900)	21,000 (15,500-26,500)	14.9

^a Rabbits (groups of three) were immunized intramuscularly with 50 μg of conjugate in 0.5% Al(OH)₃ on days 0, 14, and 28.

^b Values are for sera obtained 14 days after the final immunization (day 42).

^c Toxin A-neutralizing capacity was determined in the HEp-2 cell cytotoxicity assay. Serum samples from rabbits were pooled, and the IgG was isolated and tested for neutralizing capacity.

TABLE 4. Protection against toxin A intoxication following immunization with various conjugate vaccines

Conjugate immunogen (serotype) ^a	Mean antitoxin A IgG ELISA titer (range) ^b	LD ₅₀ (μg of toxin A/mouse)
None	<10	0.25
1	605 (80–950)	1.6
2	49 (33–75)	0.8
3	970 (620–1,500)	>12.8 ^c
4	378 (92–600)	>6.4 ^c
5	858 (740–960)	>6.4 ^c
6	735 (250–1,700)	5.13
7	807 (475–1,300)	>6.4 ^c
10	95 (30–265)	4.29
11	123 (75–250)	0.8

^a Mice (groups of six) each received 10 μg of toxin A protein as a conjugate in 0.1 ml of phosphate-buffered saline intramuscularly on days 0 and 14. Mice were challenged with graded doses of toxin A on day 28.

^b Represents the average of five individual serum samples collected on day 28.

^c Highest dose tested; >50% of the animals survived.

challenge strain used since the 50% lethal dose (LD₅₀) values for groups receiving anti-conjugate IgG were all comparable (>3 × 10⁵).

Prior studies have shown that anti-LPS antibody to *P. aeruginosa* confers protection predominantly in a serotype-specific manner (2, 6, 21, 27). To extend this finding to the current conjugate vaccines, the following experiment was done. Pre- and postimmune anti-serotype 10 IgGs were passively transferred to mice 24 h before challenge with graded doses of serotype 10 or 6 *P. aeruginosa*. Whereas

TABLE 5. Protection against fatal *P. aeruginosa* burn wound sepsis by transfer of rabbit anti-conjugate IgG

IgG administered ^a	LD ₅₀	Fold protection ^b
NRS	10 ³	
Anti-serotype 1 conjugate	10 ⁶	>1,000
NRS	<10 ³	
Anti-serotype 2 conjugate	>10 ⁷	>10,000
NRS	3.2 × 10 ⁴	
Anti-serotype 3 conjugate	>10 ⁶	31
NRS	9 × 10 ⁴	
Anti-serotype 4 conjugate	>10 ⁶	11
NRS	8.6 × 10 ²	
Anti-serotype 5 conjugate	4.3 × 10 ⁵	500
NRS	5.5 × 10 ¹	
Anti-serotype 6 conjugate	2.7 × 10 ⁵	49,000
NRS	<1.4 × 10 ³	
Anti-serotype 7 conjugate	3.2 × 10 ⁵	>228
NRS	10 ⁴	
Anti-serotype 10 conjugate	5.5 × 10 ⁶	550
NRS	3.2 × 10 ³	
Anti-serotype 11 conjugate	1.2 × 10 ⁶	375

^a Each mouse received 0.2 ml of rabbit IgG intravenously about 24 h before challenge with a homologous serotype of *P. aeruginosa*. Groups of six animals were used.

^b Calculated by dividing the LD₅₀ for groups which received anti-conjugate antibody by the LD₅₀ value for animals which received normal rabbit IgG.

TABLE 6. Immunogenicity and protective capacity of O-PS-toxin A conjugates in mice

Immunogen ^a	IgG ELISA titer ^b		Mean LD ₅₀
	toxin A	LPS	
Al(OH) ₃	10	<10	<10 ^{3c}
Serotype 2 conjugate	170	870	>10 ^{6d}
Al(OH) ₃	<10	<10	2 × 10 ¹
Serotype 5 conjugate	800	210	5.6 × 10 ⁴
Al(OH) ₃	<10	<10	<10 ^{1c}
Serotype 6 conjugate	505	440	5.8 × 10 ⁶
Al(OH) ₃	<10	<10	4.5 × 10 ¹
Serotype 10 conjugate	3180	300	9.6 × 10 ⁵

^a Mice were immunized intramuscularly on days 0 and 14 with 1 μg of conjugate in 0.1 ml of 0.5% Al(OH)₃ or with Al(OH)₃ alone. The challenge was on day 28.

^b The titers shown are mean values for three animals.

^c Low dose tested; more than 50% of the animals died.

^d High dose tested; more than 50% of the animals survived.

anti-conjugate IgG increased the LD₅₀ value 100-fold for the homologous serotype challenge strain, there was no difference in LD₅₀ values for mice which received preimmune IgG and those which received postimmune IgG before challenge with the serotype 6 strain.

Next, four conjugates were used to immunize mice to document their protective capacity when used in an active-immunization model (Table 6). All four induced both anti-toxin A and anti-LPS IgG antibody. Mice were protected to a high degree (10³-fold [serotype 2] to 5.0 × 10⁵-fold [serotype 6]) after vaccination.

DISCUSSION

Serospecific anti-LPS antibody has been shown to play a key role in protection against *P. aeruginosa* infection (1, 2, 5, 6, 21). Recent reports have also suggested that antitoxin A antibody can increase the chances for survival after an episode of *P. aeruginosa* sepsis (5, 21). The fact that the survival rate for bacteremic patients was the highest among those with elevated antibody levels to both antigens in serum indicates that a vaccine capable of engendering an immune response to both toxin A and LPS would provide optimal protection against *P. aeruginosa* (21). Prior studies from our laboratory have shown that a monovalent O-PS-toxin A conjugate vaccine fulfilled this requirement (8). In the current report, we have extended these studies to include O-PS antigens from those serotypes of *P. aeruginosa* most frequently associated with bacteremia (6, 20).

Serologically reactive O-PS could be isolated from all nine serotypes of LPS subjected to brief acid hydrolysis. This material could readily be coupled to toxin A by reductive amination with ADH as a spacer molecule. This procedure yielded high-molecular-weight conjugates composed of O-PS and toxin A at ratios of 1.17:1 to 3:1. The enzymatic activity of toxin A was destroyed by the introduction of ADH, thereby rendering these conjugates nontoxic. Of critical importance was the finding that toxic reversion did not occur when the conjugates were stored at physiological temperatures for an extended time. The stability of these conjugates is further evidenced by the fact that the HPLC elution profile of a serotype 10 conjugate stored for 9 months was nearly identical to that seen immediately after its synthesis.

All conjugates were capable of eliciting an antitoxic and a serospecific IgG antibody response, indicating that the con-

jugation method used preserved a substantial proportion of epitopes on both the toxin A and O-PS moiety. In the case of toxin A conjugate component, this was confirmed by analysis with antitoxin A MAbs which recognized neutralizing epitopes. The magnitude of the immune response, especially to the O-PS component, was found to vary considerably among the various conjugates. These differences appeared to be related not to vaccine composition, e.g., the ratio of toxin A to O-PS, but rather to the innate immunogenicity of the O-PS moiety. This, in turn, could be influenced by the stability of critical antigenic epitopes upon exposure to low pH.

Antibody evoked after immunization with the conjugate vaccine displayed the desired functional attributes. Therefore, antitoxin A antibody neutralized both the cytotoxic and in vivo lethal potentials of native toxin A. Furthermore, anti-conjugate antibody was capable of preventing fatal *P. aeruginosa* burn wound sepsis. Since antitoxin A antibody alone appears to confer little protection in this model system (7, 23), we conclude that the anti-LPS antibody is the critical factor and that the conjugates can engender protective anti-LPS antibody.

The current study demonstrates the feasibility of developing a safe and effective polyvalent *P. aeruginosa* O-PS-toxin A conjugate vaccine capable of eliciting antibody directed against toxin A and against those serotypes of *P. aeruginosa* most commonly associated with bacteremic infections (6, 20). Preliminary results indicate that combining the nine serospecific conjugates herein described does not substantially diminish their immunogenicity in animals as compared with when they are administered individually (unpublished data). We have also found a serotype 10 O-PS-toxin A conjugate vaccine to be safe and immunogenic when administered to healthy adults (manuscript submitted for publication). We are now planning to extend these studies to the evaluation of a polyvalent conjugate vaccine.

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