Preparation and Characterization of a Nontoxic Polysaccharide-Protein Conjugate That Induces Active Immunity and Passively Protective Antibody Against Pseudomonas aeruginosa Immunotype 1 in Mice

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Pseudomonas aeruginosa is often a virulent pathogen in hosts whose immune system is compromised by neoplastic disease (30) or major thermal injury (21). Despite therapy with appropriate antibiotics, the mortality rate from P. aeruginosa bacteremia is high in these debilitated patients (14). Numerous studies have indicated that immunoglobulin G (IgG) antibody to lipopolysaccharide (LPS) is protective in experimentally infected animals (6, 22). A heptavalent vaccine containing LPS of the seven immunotypes of P. aeruginosa (13) is effective in inducing antibodies in humans (19) that are protective in experimentally infected animals (12). Attempts to immunize patients at high risk of P. aeruginosa infection with this vaccine, however, have been only moderately successful due in part to the potent endotoxin activity of LPS. Local and systemic adverse reactions to endotoxin, including fever, malaise, and pain at the injection site, can limit vaccine dosage (25, 35).

In this study, we describe the preparation of a nontoxic P. aeruginosa vaccine consisting of purified low-molecular-weight polysaccharide (PS) derived from immunotype 1 LPS covalently coupled to bovine serum albumin (BSA) and characterize the protective activity of hyperimmune mouse serum in experimentally infected burned and normal mice. (This paper was presented in part at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1983.)

MATERIALS AND METHODS

Bacteria and inocula preparation. P. aeruginosa 1369 (Fisher-Devlin-Gnabasik immunotype 1) was obtained from J. A. Bass, Shriners' Hospital for Crippled Children, Galveston, Tex. The strain was kept at −60°C in brain heart infusion broth containing 10% glycerol. Working cultures were maintained on brain heart infusion agar slants at 4°C. On the day of an animal challenge, overnight cultures on brain heart infusion agar slants were subcultured onto fresh agar slants. After 4 to 5 h of incubation at 37°C, cells were harvested by washing the slants with phosphate-buffered saline (pH 7.4). Cells were washed once and adjusted to the desired concentration with saline. Inocula levels were determined by correlating plate counts with a 660-nm spectrophotometric reading of cell suspensions.

Mice. Female Swiss-Webster mice (weight, 18 to 26 g) were obtained from Simonsen (Gilroy, Calif.). For each study, mice were matched by age and weight. They were housed at 10 mice per cage and freely given water and mouse chow.

Extraction and purification of LPS. P. aeruginosa was cultured to late log phase in 10 liters of chemically defined glucose-glutamate salts medium (16) in a 14-liter Virtis fermenter. Cells were killed by addition of formaldehyde to 0.37%, harvested by centrifugation, washed once with distilled water, and lyophilized. Yields of cells were 20 to 30 g (dry weight). LPS was extracted by the hot phenol-water method of Westphal et al. (33). After dialysis to remove phenol, crude LPS was treated with RNase and DNase (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M acetate buffer (pH 5.0) for 24 h. The pH was adjusted to 7.0 with NaOH, and LPS was treated with pronase (Sigma) for 24 h. Low-molecular-weight substances were removed by dialfiltration through a hollow fiber ultra filter (HIX-50; Amicon Corp., Lexington, Mass.).

Preparation of conjugate vaccine. LPS was hydrolyzed in dilute acetic acid (8). Insoluble lipid A was removed after centrifugation at 10°C. The supernatant fluid was adjusted to pH 7.0 with NaOH, and the remaining lipid A was removed by three extractions with 2 volumes of CHCl₃-methanol (2:1; vol/vol). PS in the aqueous phase was concentrated by rotary evaporation under vacuum. PS was fractionated by water elution on a Bio-Gel A-5m column (100 by 2.6 cm),
followed by fractionation with water elution on a Sephadex G-25 column (100 by 2.6 cm). PS in the Sephadex G-25 void volume was lyophilized. Dextran (Pharmacia Fine Chemicals, Piscataway, N.J.) molecular weight standards were used to estimate the molecular weight of eluted PS fractions. PS was oxidized with NaIO₄ to generate aldehyde groups (29). Oxidized PS was purified by water elution on a Sephadex G-25 column (100 by 2.6 cm) and lyophilized. Essentially fatty acid-free BSA (Sigma) was coupled to 1,4-diaminobutane (Aldrich Chemical Co., Milwaukee, Wis.) to increase the number of BSA amino groups (24). Oxidized PS was coupled to aminoethyl-BSA by reductive amination with sodium cyanoborohydride (3). The reaction products were fractionated by water elution on a Sephadex G-100 column (100 by 1.0 cm). Fractions containing both carbohydrate and protein were collected.

**High-pressure liquid chromatography.** The elution profiles of BSA, aminoethyl-BSA, and the aminobutyl-BSA-PS conjugate were examined on an Altex high-pressure liquid chromatograph fitted with a Spherogel TSK-3000 column (7.5 mm by 60 cm) and a UV detector. The eluting buffer was 0.2 M sodium phosphate (pH 6.8).

**Chemical assays.** Assay methods for 2-keto-3-deoxyoctonate (32), protein (20), RNA (4), DNA (1), total carbohydrate (9), reducing sugar (28), and free amino groups (18) have been described previously.

*Limulus amoebocyte lysate.* The activities of LPS, 2 lots of PS, BSA, and 2 lots of BSA-PS conjugate were quantitated with reagents from Associates of Cape Cod. The Limulus assay was sensitive to 0.015 ng of *Escherichia coli* 0113 LPS per ml.

**Active immunization.** Mice (weight, 18 to 20 g) were injected once weekly for four times by the subcutaneous route with 0.1-ml saline solutions of the following substances: 37 μg of aminobutyl-BSA, 5 μg of PS, a mixture of aminobutyl-BSA and PS, or the conjugate containing 5 μg of PS covalently linked to 37 μg of BSA. Sera were obtained before immunization and after injections two through four. Mice were challenged after injection four with 10 times the 50% lethal dose (LD₅₀) of *P. aeruginosa* 1369 by the intraperitoneal route.

**Passive immunization of normal mice.** Mice (weight, 18 to 20 g) were passively immunized intraperitoneally with 0.05 to 0.10 ml of sera. Two hours later, mice were challenged intraperitoneally with 10 to 20 times the LD₅₀ of *P. aeruginosa* 1369.

**Passive immunization of burned mice.** Dorsal fur of mice (weight, 26 g) was clipped from head to tail. A 0.05-ml volume of serum or immunoglobulin was administered intraperitoneally. Two hours later, mice were anesthetized by an intraperitoneal injection of sodium pentobarbitol (60 to 80 mg/kg of body weight). An asbestos cloth with a 5.8-cm² oval hole was placed over the back, and mice were given approximately a 10% full thickness body surface burn for 5 s with a Fischer gas burner. Inoculum suspended in 0.5 ml of saline was injected subcutaneously into the burn site (5). Burned mice were observed for 15 days afterward.

**Serum fractionation.** A volume of 1.9 ml of pooled sera from mice immunized with conjugate vaccine was fractionated by phosphate-buffered saline (pH 7.4) elution on a Sephadex G-200 column (100 by 2.6 cm). Each fraction containing protein was tested by agar gel immunodiffusion against goat anti-mouse IgG, IgA, and IgM (Kirkegaard and Perry Laboratories). Only fractions containing a single immunoglobulin class were used for passive immunization. Immunoglobulin concentration was adjusted to the same concentration as found in serum by ultrafiltration on a Amicon PM10 membrane.

**Enzyme-linked immunosorbent assay.** The enzyme-linked immunosorbent assay (ELISA) was essentially that described by Engvall and Perlmann (11), as adapted to microtiter plates by Voller et al. (31). Wells of polystyrene microtiter plates (Cooke Laboratories, Alexandria, Va.) were coated with 200 μl of carbonate buffer (pH 9.6) containing 10 μg of LPS per ml. Alkaline phosphatase-conjugated rabbit anti-mouse IgG and IgM were obtained from Miles Laboratories, Inc., Elkhart, Ind. The substrate p-nitrophenylphosphate was obtained from Sigma. The yellow color that developed was read at 405 nm on a Dynatech MR580 ELISA spectrophotometer. The titer was considered to be the highest dilution of pooled sera that gave an absorbance at 405 nm of 0.100 after 30 min of incubation at 23°C. All plasma samples were run in duplicate, with reference serum included on each microtiter plate.

**Statistics.** The significance of protection conferred by active and passive immunization was determined by the Fisher exact test.

**RESULTS**

**PS antigen.** Fractionation of immunotype 1 PS on Bio-Gel A-5m yielded well-separated, high-molecular-weight (1.5 × 10⁵ to 5 × 10⁵) and low-molecular-weight (0.2 × 10⁴ to 4 × 10⁴) peaks (Fig. 1). Low-molecular-weight PS was further purified from the void fraction of Sephadex G-25. The yield of immunotype 1 PS after acidic acid hydrolysis of LPS was 15%, with approximately 50% of the total PS in the low-molecular-weight range. To exclude the possibility of contamination of PS with trace amounts of LPS, only the low-molecular-weight fraction (<4 × 10⁴) was used for conjugate vaccine preparation. Low-molecular-weight PS precipitating in agar gel against homologous rabbit anti-LPS was pooled. Gel filtration on a Sephadex G-100 column demonstrated that oxidized and unoxidized PS had similar molecular weight distributions.

**Conjugate vaccine.** By reductive amination with sodium cyanoborohydride, the amino groups of aminobutyl-BSA were coupled to the aldehyde groups of periodate-oxidized PS. The PS content of 2 lots of conjugate vaccine were 11.5

**FIG. 1.** Chromatographic profiles of LPS from *P. aeruginosa* 1369 after hydrolysis with acetic acid (8). The material was eluted from Bio-Gel A-5m (2.6 by 100 cm) with distilled water. Fractions (6.9 ml) were collected, and portions were assayed for total carbohydrate content. HMWPS, High-molecular-weight PS; LMWPS, low-molecular-weight PS.
and 12.5% by weight. Covalent linkage was demonstrated by comigration of protein and carbohydrate on a Sephadex G-100 column (Fig. 2) and high-pressure liquid chromatography (Fig. 3). In high-pressure liquid chromatography, the conjugate eluted after 10 min, whereas the aminobutyl-BSA eluted after 18 min, indicating a higher molecular weight for the PS-protein conjugate.

Toxicity studies. The first lot of low-molecular-weight immunotype 1 PS exhibited 1,000-fold less activity than native LPS in the Limulus assay, whereas the second lot of PS was 16,000-fold less reactive (Table 1). BSA and aminobutyl-BSA had essentially no activity in the Limulus assay. On a weight basis, each lot of the conjugate vaccine was 4,094-fold less active than native LPS in the Limulus assay. Mice immunized with each lot of the conjugate vaccine showed no evidence of toxicity or inflammation at the site of injection. Immunized mice gained weight at the same rate as control mice injected only with saline.

Immunogenicity of immunotype 1 vaccine. Mice were immunized on days 1, 7, 14, and 19 with PS, aminobutyl-BSA, a mixture of PS and aminobutyl BSA, conjugate vaccine (lot 2654-3), or saline. Mice were bled for ELISA and passive protection studies on day 24. On day 27, immunized mice were challenged with 10 times the LD$_{50}$ of P. aeruginosa 1369. Only the conjugate vaccine protected mice and stimulated serum IgG reactive in the ELISA against native LPS (Table 2). Serum from mice immunized with the first lot of conjugate vaccine was protective in normal mice challenged intraperitoneally with 10 times the LD$_{50}$ of P. aeruginosa 1369 immunotype 1. A second lot of conjugate vaccine (lot 2654-14) was administered on days 7, 14, and 21. Mice were bled on days −1, 6, 13, 20, 29, and 37. The ELISA titers of pooled sera against LPS exceeded 1:1,000, and sera were marginally protective after administration of the second dose of vaccine (Table 3). After the third injection, the ELISA titer exceeded 1:2,000, and sera were highly protective in both burned and normal mice challenged with lethal inocula of P. aeruginosa 1369 immunotype 1. Mice immunized with conjugate vaccine and saline controls were challenged with 10 times the LD$_{50}$ of P. aeruginosa 1369

![Figure 2](http://iai.asm.org/)  
**FIG. 2.** Sephadex G-100 (1.0 by 100 cm) chromatographic profiles of conjugate PS-aminobutyl-BSA (Conj. PS-AMBBSA; lot 2654-4) (A) and aminobutyl-BSA (AMBBSA) (B). Fractions of 1.3 ml were collected, and portions were assayed for carbohydrate (○) and protein (○).

![Figure 3](http://iai.asm.org/)  
**FIG. 3.** High-pressure liquid chromatographic profiles of aminobutyl-BSA (AMBBSA) (A) and conjugate PS-aminobutyl-BSA (PS-AMBBSA; lot 2654-4) (B) in an Altex spherogel TSK-3000 (7.5 mm by 60 cm) column.

<table>
<thead>
<tr>
<th>TABLE 1. Activity of 2 lots of P. aeruginosa immunotype 1 conjugate vaccine in the Limulus amoebocyte lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance tested</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Native LPS</td>
</tr>
<tr>
<td>Molecular weight PS</td>
</tr>
<tr>
<td>BSA</td>
</tr>
<tr>
<td>Aminobutyl-BSA</td>
</tr>
<tr>
<td>Aminobutyl-BSA-PS conjugate</td>
</tr>
</tbody>
</table>

$^a$ Dilution values represent the mean of duplicate twofold titrations.  
$^b$ 70 µg of protein: 10 µg of PS per ml.
TABLE 2. Immunogenicity of the components of \textit{P. aeruginosa} immunotype 1 PS-protein conjugate vaccine

<table>
<thead>
<tr>
<th>Immunizing substance</th>
<th>Dose (µg/mouse)$^a$</th>
<th>ELISA titer of IgG$^b$</th>
<th>Cumulative mortality after 3 days (no. of dead/total) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Active immunity</td>
</tr>
<tr>
<td>Free PS</td>
<td>5.0</td>
<td>&lt;1:1,400</td>
<td>10/10</td>
</tr>
<tr>
<td>Aminobutyl-BSA</td>
<td>38.4</td>
<td>1:1,239</td>
<td>3/10$^d$</td>
</tr>
<tr>
<td>PS–aminobutyl-BSA conjugate (lot 2654-3)</td>
<td>5.0:38.4</td>
<td>1:2,239</td>
<td>10/10</td>
</tr>
<tr>
<td>PS–aminobutyl-BSA mixture</td>
<td>5.0:38.4</td>
<td>&lt;1:1,400</td>
<td>8/10</td>
</tr>
<tr>
<td>Saline</td>
<td>&lt;1:1,400</td>
<td></td>
<td>8/10</td>
</tr>
</tbody>
</table>

$^a$ Mice were immunized by subcutaneous injection on days 1, 7, 14 and 19.
$^b$ Titters were determined by linear regression of absorbance of twofold dilutions of sera. Serum was obtained 5 days after the fourth immunization.
$^c$ Mice were passively immunized with 0.1 ml of serum.
$^d$ Values are statistically significant ($P < 0.05$ Fisher exact test) compared with saline controls.

Immunotype 1 72 days after the fourth injection. Mice that received the conjugate were highly resistant to infection, for only 1 of 19 died after challenge versus the death of 8 of 9 saline-treated control mice ($P < 0.005$).

Fractionation of mouse serum collected and pooled after the third and fourth injection of conjugate vaccine on a Sephadex G-200 column indicated that the passively protective antibody was IgG, not IgM (Table 4). IgA was not detected by immunodiffusion in either the IgG or IgM fraction.

**DISCUSSION**

There is convincing evidence that antibody to serotype-specific LPS is highly effective in preventing \textit{P. aeruginosa} infection in experimental animals (6, 23). Purified polyvalent LPS vaccine induces increased levels of humoral antibody in patient populations; however, endotoxicity inherent in the lipid A region of LPS limits dosage (25, 35).

The low-molecular-weight PS studied in this report had 1,000-fold less activity in the \textit{Limulus} assay than did native LPS. This suggests that the 5.0-µg dose of PS used to immunize mice could have contained approximately 0.005 µg of intact LPS. Immunotype 1 LPS in aequous environments exists as complexes with molecular weights of $\geq 10^{5}$ (17). Hence, LPS would have eluted from the Bio-Gel A-5m column (Fig. 1) with or before the high-molecular-weight PS fraction ($\geq 1.5 \times 10^{6}$), not with the low-molecular-weight fraction ($\leq 4 \times 10^{5}$). In a recent study, Pier (26) has demonstrated that high-molecular-weight PS ($\geq 7 \times 10^{6}$) derived from acetic acid hydrolysis of immunotype 1 LPS also had 1/1,000 the activity of LPS in the \textit{Limulus} assay. These findings suggest that PS of \textit{P. aeruginosa} might itself have low-level activity in the \textit{Limulus} assay (Table 1). It has been reported that peptidoglycan from gram-positive bacteria (34) and polynucleotides (10) also have low-level activity in the \textit{Limulus} assay. Abolishment of \textit{Limulus} assay activity by enzymatic hydrolysis of these polymers indicates that their activity was not due to endotoxin contamination. The most convincing evidence that low-molecular-weight PS is free of LPS is its lack of immunogenicity. If approximately 1 to 5 ng of immunotype 1 LPS was present in the PS, then four injections should have stimulated resistance to infection in mice, as this dose of LPS has been shown to be immunogenic (7, 27). This was not seen. Only when the PS was conjugated to protein was immunogenicity demonstrated (Table 2).

BSA was used as the protein carrier for two reasons. It is available free of endotoxin contamination (Table 1), and numerous workers, beginning with Goebel (15), have demonstrated its effectiveness as a carrier for weakly antigenic microbial PS.

The conjugate vaccine described in this report has several desirable properties. The methodology used in its production is reproducible. Two lots of vaccine were made. Each lot had similar protein:carbohydrate ratios, and each was highly immunogenic (Tables 2 and 3). Activity in the \textit{Limulus} assay was minimal, and no toxicity was seen in mice on repeated injection. Adjuvant was not needed to stimulate passively protective IgG, although the enhancing effect of adjuvant on the antigenicity of the conjugate vaccine is presently not known. The protective antibody stimulated by the vaccine was IgG (Table 4), the antibody class best correlated with resistance to infection by \textit{P. aeruginosa} (2). Finally, active immunity lasted at least 72 days after the last injection.

Before a conjugate vaccine of the type described in this study can be employed in human trials, several questions must be answered. More than 90% of \textit{P. aeruginosa} infections in humans are caused by only seven immunotypes (13). A protective vaccine thus would have to be polyvalent. It is not presently known whether the antigenicity of PS of immunotypes 2 through 7 would be destroyed by periodate oxidation. These studies are in progress.

**TABLE 4. Protective antibody stimulated by \textit{P. aeruginosa} immunotype 1 conjugate vaccine (lot 2654-14) is IgG**

<table>
<thead>
<tr>
<th>Burned mice passively immunized with:</th>
<th>Cumulative mortality (no. dead/total)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10/10</td>
</tr>
<tr>
<td>Immune serum</td>
<td>0/10$^b$</td>
</tr>
<tr>
<td>Immune serum IgG fraction</td>
<td>1/9$^b$</td>
</tr>
<tr>
<td>Immune serum IgM fraction</td>
<td>8/11$^c$</td>
</tr>
</tbody>
</table>

$^a$ Mice were given 0.05 ml of serum or immunoglobulin fraction 3 h before burning and challenge with 788 CFU.
$^b$ Statistically significant ($P < 0.001$) protection compared with serum from mice immunized with saline.
$^c$ Not significant.

**TABLE 3. Induction of passively protective antibody with \textit{P. aeruginosa} conjugate vaccine (lot 2654-14); effect of booster injections**

<table>
<thead>
<tr>
<th>Day of immunization$^a$</th>
<th>Day bled after first injection</th>
<th>ELISA titer of IgG</th>
<th>Cumulative mortality (no. dead/total) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal Mice</td>
</tr>
<tr>
<td>Preimmune</td>
<td>–1</td>
<td>&lt;1:400</td>
<td>9/10</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>&lt;1:400</td>
<td>9/10</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>1:1,313</td>
<td>6/10$^d$</td>
</tr>
<tr>
<td>14</td>
<td>20</td>
<td>1:2,481</td>
<td>0/10$^d$</td>
</tr>
<tr>
<td>21</td>
<td>29</td>
<td>1:3,808</td>
<td>1/10$^d$</td>
</tr>
<tr>
<td>37</td>
<td>37</td>
<td>1:2,482</td>
<td>4/10$^d$</td>
</tr>
</tbody>
</table>

$^a$ Mice were immunized with vaccine (see Table 2 for dosage) and bled on the day indicated.
$^b$ Normal and burned mice were given 0.05 ml of serum 3 h before infection. Passively immunized normal mice were challenged with 10 times the LD$_{50}$, and burned mice were challenged with 100 times the LD$_{50}$.
$^c$ Values are statistically significant ($P < 0.05$) compared with preimmune serum.
$^d$ $P < 0.001$.  

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LITERATURE CITED