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Protection Against Fatal *Pseudomonas aeruginosa* Burn Wound Sepsis by Immunization with Lipopolysaccharide and High-Molecular-Weight Polysaccharide

S. J. CRYZ, JR.,* E. FÜRER, AND R. GERMANIER
Swiss Serum and Vaccine Institute, 3001 Berne, Switzerland

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A murine burn wound model was employed to evaluate the relative efficacy of purified *Pseudomonas aeruginosa* lipopolysaccharide (LPS) and high-molecular-weight polysaccharide as protective immunogens. LPS was found to be both highly immunogenic and protective. As little as three 0.001-μg doses elicited good immunoglobulin M and G titers and increased the mean lethal dose more than 1,000-fold. The level of protection against a live challenge correlated with antibody titers and was found to be serotype specific. An immunizing regimen which evoked only an immunoglobulin M response was still found to offer substantial protection. Immunization with a high-molecular-weight polysaccharide was also found to be protective. However, approximately 1,000-fold more high-molecular-weight polysaccharide, as compared with LPS, was needed to protect mice to an equivalent degree. Immunization with LPS was found to promote bacterial clearance and prevent establishment of bacteremia. A multivalent LPS vaccine conferred high levels of protection (110- to 53,000-fold) against eight different challenge strains of various serotypes.

Pseudomonas aeruginosa is a leading cause of morbidity and mortality in patients with a variety of underlying conditions, most notably cystic fibrosis (20), burn wounds (18), and neutropenia (22). Even with significant advances in supportive and antibiotic therapy, the mortality rate for *P. aeruginosa* bacteremia remains high (5, 27). Most *P. aeruginosa* bacteremic isolates are serum resistant even in the presence of specific antibacterial antibody (26). Removal of the invading bacteria is dependent upon their phagocytosis and subsequent killing, a process greatly facilitated by opsonic antibody (25, 26). Human antibody directed against lipopolysaccharide (LPS) and high-molecular-weight polysaccharide (PS) has been shown to promote phagocytosis (13, 25). Elevated anti-LPS titers at the onset of *P. aeruginosa* bacteremia have been correlated with increased survival rates (17).

Immunization of mice with very low doses of purified LPS provides substantial protection against a live challenge administered intraperitoneally (14–16). PS has also been shown to elicit a protective immune response but is much less immunogenic than LPS (14–16). Studies aimed at determining the protection afforded by immunization with LPS against *P. aeruginosa* infections in an animal model relevant to human disease are limited (10, 11, 21). Only one report to date has employed a burn wound sepsis model (21). PS has been evaluated as a protective antigen only against an intraperitoneal challenge (14–16).

The present study was performed to determine the protective capacity of LPS and PS when used as active immunogens in a burn wound sepsis model. Various immunizing doses and schedules were tested, and the antibody response was analyzed in relation to protection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain PA220 was provided by B. Wretlind, Karolinska Institute, Stockholm, Sweden. *P. aeruginosa* PA103 and PA53 were obtained from B. H. Iglewski, University of Oregon Health Sciences Center, Portland. Strains 8505, 6510, and 6511 were gifts from T. L. Pitt, Public Health Laboratory, London, England. Strains IT-1, IT-2, IT-3, IT-5, IT-6, and IT-7 (Fischer immunotype strains) were provided by M. Fisher, Parke, Davis & Co., Detroit, Mich. *P. aeruginosa* M-2 was provided by I. A. Holder, Shriners Burns Institute, Cincinnati, Ohio. Cultures were maintained lyophilized. Cultures for LPS isolation were grown on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 1% (vol/vol) glycerol in a 50-liter fermentor at 37°C to stationary phase. Challenge inocula were grown to mid-log phase at 37°C on the dialyzed deferrated TSB medium of Bjorn et al. (1).

Purification of LPS. LPS was purified as previously described (4). Briefly, the cell wall fragments from ca. 85 g (wet weight) of cells were suspended in 120 ml of distilled water to which 154 ml of 80% (wt/vol) phenol (at 75°C) was added. After stirring for 5 min at 70°C, the mixture was cooled and centrifuged at 20,000 × *g* for 20 min. The water and phenol phases (which were collected and processed separately) were dialyzed extensively to remove residual phenol. Initial experiments showed that, depending upon the strain, LPS was found predominantly in the water phase (PA220, IT-5, IT-6, IT-7, 8505, and 6510) or in the phenol phase (IT-1, IT-2, and 6511) or was dispersed more or less equally between the two phases (IT-3 and PA53). Although it was possible to obtain LPS of sufficient purity (≥95%) from the water phase material simply by repeated rounds of ultracentrifugation, this was not adequate for the phenol phase material. Therefore, to maintain uniformity, all lots of LPS (phenol and water phase derived) were digested with pronase, RNase, and DNase (Boehringer Mannheim Biochemicals, Mannheim, West Germany) as follows. LPS (5 to 10 mg/ml) was suspended in phosphate-buffered saline, pH 7.4 (PBS). Nucleases were added to a final concentration of 20 μg/ml, and the mixture was incubated at 37°C for 3 h. Pronase was added to a final concentration of 100 μg/ml, and incubation continued for an additional 24 h at 22°C. The digested material was then subjected to three rounds of ultracentrifugation at 100,000 × *g*, and the pellet was suspended in water

* Corresponding author.

TABLE 1. Protection against *P. aeruginosa* PA220 (serotype G) by active immunization in a burn wound sepsis model: effect of immunizing dose and source of LPS

Immunogen (source)	Serotype	Immunizing dose (μg)[a]	Anti-LPS ELISA[b] titer[c]		LD_{50}[d]	Fold protection
			IgM	IgG		
None [$Al(OH)_3$ only]		0	<10	<10	$<0.5 \times 10^1$	1
PA220 LPS (water phase)	G	0.1	130	270	5.5×10^5	110,000[e]
		0.01	55	90	3.6×10^5	72,000[e]
		0.001	32	50	2.2×10^4	4,400[e]
IT-1 LPS (phenol phase)	G	0.1	80	90	3.9×10^5	78,000[e]
		0.01	36	70	4.2×10^5	84,000[e]
		0.001	30	38	1.7×10^4	3,400[e]

[a] Mice were immunized intramuscularly at days 0, 14, and 28 with the indicated dose of LPS in 100-μl volumes with $Al(OH)_3$. Challenges were performed 14 days after the final immunization.
[b] Enzyme-linked immunosorbent assay.
[c] At time of challenge.
[d] Expressed as number of viable PA220.
[e] $P < 0.01$.

and lyophilized. The final preparations contained less than 2% protein (wt/wt) as determined by the method of Lowry et al. (7). Nucleic acid content was less than 1% based on an absence of ribose from the various materials as determined by paper chromatography.

Escherichia coli LPS was purchased from Difco Laboratories, Detroit, Mich., and used without further purification.

Purification of PS. PS was prepared from *P. aeruginosa* IT-1 by the method of Pier et al. (16) which yields an LPS-free preparation. The final preparation contained (by dry weight) 60.2% carbohydrate, 2.7% protein, and 0.4% nucleic acids and eluted in the void volume when chromatographed on Ultrogel AcA44 (LKB Produkter, Bromma, Stockholm, Sweden).

Enzyme-linked immunosorbent assay. An enzyme-linked immunosorbent assay for measurement of antibody to LPS was performed as follows. Microtiter plates (Linbro; Flow Laboratories, Inc., Hamden, Conn.) were coated by placing 200 μl of a 20-μg/ml solution of LPS in 5 mM sodium phosphate buffer (pH 7.2) in each well. Plates were incubated at 37°C for 3 h and stored at 4°C. Serum dilutions were done in PBS containing 0.02% Tween 20 (PBS-T), and 200 μl of each dilution was added per well. Plates were incubated for 6 h at 22°C and washed three times with PBS-T. Peroxidase-labeled goat anti-mouse immunoglobulin M (IgM) and IgG were obtained from Biogenzia Lemania, Lausanne, Switzerland, and used at a 1:1,000 dilution in PBS-T. Each well received 200 μl of conjugate, and the plates were incubated overnight at 4°C. After three washes with PBS-T, 200 μl of substrate solution [10 mg of 2,2'-azino-di(3-ethylbenzylthiozoline) sulfonic acid 6 (Boehringer Mannheim) dissolved in 50 ml of 0.1 M NaH_2PO_4-HCl (pH 4) to which 0.125 ml of 10% H_2O_2 was added just before use] was placed in each well. Color was allowed to develop at room temperature and was measured at 405 nm with a Titertek Multiscan (Flow Laboratories, Inc.). Titers were expressed as the highest dilution of serum which gave an absorbancy at 405 nm of 0.4.

Immunization of mice. Outbred Swiss Webster white mice, each weighing 18 to 20 g, were immunized intramuscularly with 100-μl volumes containing the antigen in 0.5% aluminum hydroxide gel. Control groups were immunized only with aluminum hydroxide gel. Immunization schedules are described below. Animals were challenged 14 days after the last immunization.

Burn wound sepsis model. The burn wound sepsis model described by Stieritz and Holder (23) was employed with slight modification (3). The challenge inocula were diluted in cold PBS and kept on ice. The number of viable organisms per challenge dose was determined for each experiment by plate counts. Animals were observed for a minimum of 5 days postchallenge before mortality rates were tabulated. To determine the mean lethal dose required to kill 50% of animals (LD_{50}), groups of six animals per challenge dose were used.

Bacterial quantitation in tissues and blood. Mice were challenged with approximately 10^2 bacteria. At various times postchallenge, groups of three mice were sacrificed, and the number of bacteria per milliliter of blood, per liver, and per gram (wet weight) of skin at the challenge site was determined as previously described (3).

Serological typing. O antigen-specific typing sera were obtained from Toshiba Kagaku Co., Ltd., Tokyo, Japan. Typing was performed by slide agglutination.

Statistical analysis. The LD_{50} was calculated by the method of Reed and Muench (19). Protection against a live *P. aeruginosa* challenge, expressed as fold protection, was obtained by dividing the LD_{50} value for immunized groups by that of the control group. Significance between LD_{50} values was calculated by a two-tailed Student's *t* test.

Multivalent vaccine. A nonavalent LPS vaccine was made by combining equal amounts of LPS purified from the following strains (serotypes) of *P. aeruginosa*: PA220 (G), IT-2 (E), IT-3 (B), PA-53 (I), IT-5 (H), IT-6 (C), IT-7 (B), 6510 (A), and 6511 (F). For immunization of mice, the vaccine was appropriately diluted in PBS and mixed (1:1) with a 1% (wt/vol) $Al(OH)_3$ suspension to yield a final concentration of 0.5% $Al(OH)_3$ in the vaccine.

RESULTS

Preliminary experiments demonstrated that enzymatic digestions of PA220 LPS with pronase and nucleases had no appreciable effect on either its immunogenicity or protective capacity as compared with untreated starting material ($LD_{50} = 1.2 \times 10^6$ PA220 after three biweekly immunizations with 0.01 μg of LPS). Therefore, all later experiments were performed with enzyme-treated LPS preparations.

The partition of LPS into the water or phenol phase after extraction was found to be strain dependent (see above).

TABLE 2. Active immunization of mice with LPS: effect of dose schedule on protection against *P. aeruginosa* PA220 in a burn wound sepsis model

Amt of PA220 LPS administered (μg)	No. of immunizations[a]	Anti-LPS ELISA[b] titer[c]		LD_{50}[d]	Fold protection
		IgM	IgG		
None [$Al(OH)_3$ only]	3	<10	<10	$<0.5 \times 10^1$	1
0.1	1	55	<10	5×10^5	10,000[e]
0.1	2	55	55	2.4×10^6	48,000[e]
0.1	3	70	360	5×10^6	100,000[e]

[a] Immunizations were given intramuscularly in 100-μl volumes with $Al(OH)_3$ at 14-day intervals. Challenges (PA220) were performed 14 days after the final immunization.
[b] Enzyme-linked immunosorbent assay.
[c] At the time of challenge.
[d] Expressed in terms of viable PA220.
[e] $P < 0.01$.

Dispersion is most likely dependent upon the relative proportion (length) of the hydrophilic O-polysaccharide side chains in comparison with the hydrophobic lipid A moiety. Since prior studies have shown that protection against *P. aeruginosa* mediated by anti-LPS is predominantly serotype specific (3, 14–16), we compared LPS of the same serotype (G) isolated from either the phenol or water phase for its ability to evoke a protective immune response (Table 1). Both LPS preparations were found to be comparably immunogenic, as gauged by antibody response and protection afforded against a live challenge. The level of protection correlated with anti-LPS titers, which in turn were dose dependent. LPS from either phase was extremely immunogenic, with doses as low as 0.001 μg eliciting good IgM and IgG titers. Furthermore, immunization with such a small amount of LPS increased the LD_{50} value over 1,000-fold as compared with control mice. Protection was specific for *P. aeruginosa* LPS. The LD_{50} value for mice immunized in an identical manner with *E. coli* LPS was comparable to that for control mice when challenged with PA220. Additionally, the protection observed was serotype specific. Immunization of mice with 0.1 μg of PA220 LPS (serotype G) provided no protection compared with control mice when the challenge strain was of a different serotype (M-2, serotype B).

The protective immune response evoked by LPS vaccination was next studied as a function of the number of immunizing doses. Groups of mice each received either one, two, or three immunizations with 0.1 μg of PA220 LPS, and the antibody response and protection were determined (Table 2). Comparable levels of IgM were found in all groups, whereas an IgG response was noted only when two or three immunizations were given. Maximal antibody titers and protection were seen with the three-dose schedule. However, the decrease in protection was not dramatic even when a single dose was administered and the antibody response was limited to the IgM class.

PS was also tested for its ability to evoke a protective immune response (Table 3). Although all three doses of PS employed were capable of increasing the LD_{50} (6- to 600-fold) in a dose-dependent manner, the protection afforded was far less than that seen when comparable quantities of LPS were used (Table 1). Whereas 1-μg immunizing doses of PS could increase the LD_{50} approximately 600-fold, a comparable level of protection could be obtained with a 1,000-fold-lower dose of LPS.

The effect of prior immunization with LPS on the course of infection was studied by monitoring the multiplication and

TABLE 3. Protection against *P. aeruginosa* PA220 by immunization with PS[a]

Immunogen[b]	Dose (μg)	LD_{50}[c]	Fold protection
PS	1	8.9×10^3	570[d]
	0.1	1.55×10^3	100[d]
	0.01	8.9×10^1	5.7[e]
None [$Al(OH)_3$ only]		$<1.55 \times 10^1$	

[a] PS was purified from *P. aeruginosa* IT-1 (serotype G).
[b] Mice were immunized at days 0, 14, and 28. Challenge was performed on day 42.
[c] Expressed as the number of viable PA220.
[d] $P < 0.01$.
[e] Not significant.

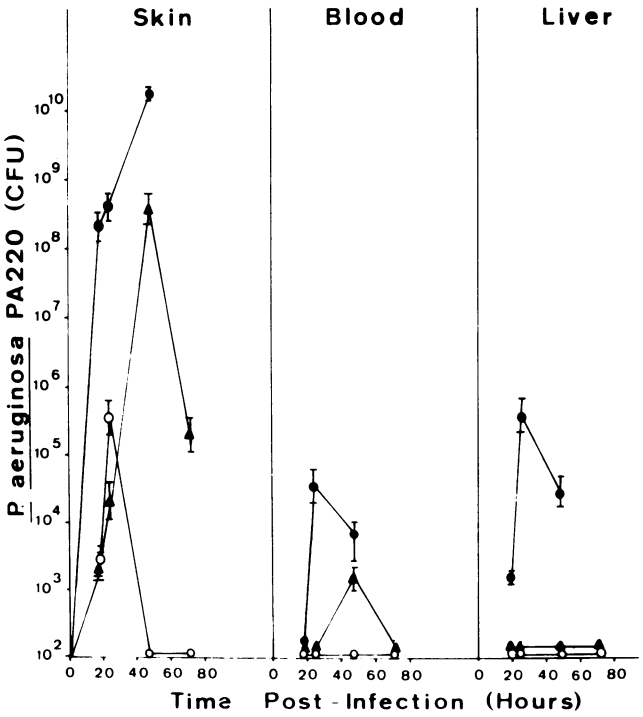

FIG. 1. Growth and dissemination of PA220 after burning and challenge. Mice were immunized on days 0, 14, and 28 with: ●, $Al(OH)_3$; ○, 0.01 μg of PA220 LPS; or ▲, 0.0001 μg of PA220 LPS. Mice were challenged on day 42 with approximately 10^2 organisms. Each data point represents the average ± standard error of the mean for groups of three mice. Skin, Number of bacteria per gram (wet weight) of skin at the challenge site. Blood, Number of bacteria per milliliter of blood. Liver, Number of bacteria per liver.

TABLE 4. Protection afforded against *P. aeruginosa* challenge by vaccination with a polyvalent LPS vaccine[a]

Challenge strain (serotype)	LD_{50}[b]		Fold protection[c]
	Control	Immunized	
PA220 (G)	$<0.8 \times 10^1$	4.3×10^5	5.3×10^4
IT-3 (B)[d]	2.7×10^2	1.1×10^5	4×10^2
PA-53 (I)	1.9×10^4	2.2×10^6	1.1×10^2
IT-5 (H)	$<1.3 \times 10^1$	1.3×10^5	1×10^4
IT-6 (C)	2.4×10^2	2.4×10^6	1×10^4
IT-7 (B)[e]	$<1 \times 10^1$	3.5×10^4	3.5×10^3
6510 (A)	5.5×10^2	3.9×10^6	7×10^3
8505 (F)	2.4×10^2	1.3×10^6	5.4×10^3

[a] Mice were vaccinated with the 9-valent LPS vaccine (a total of 0.09 μg of LPS, 0.01 μg of each component) on days 0, 14, and 28. Challenges were performed on day 42.
[b] Expressed as the number of viable PA220.
[c] All were significant ($P < 0.01$).
[d] Corresponds to Fisher immunotype 3.
[e] Corresponds to Fisher immunotype 7.

dissemination of the bacterial challenge. Mice which received a three-dose immunization schedule (either with 0.01 or 0.0001 μg of PA220 LPS per dose) were subsequently challenged with 10^2 PA220, and the bacterial counts in the skin, liver, and blood were determined at various times postchallenge (Fig. 1). Bacterial growth in the skin of control mice was extremely rapid, reaching levels of greater than 10^8 CFU/g of tissue. Bacteremia (2.5×10^2 CFU/ml of blood) and liver colonization were first noted at 19 h postinfection. Immunization with 0.01 μg of LPS resulted in a marked reduction of bacterial multiplication in the skin and clearing of the infecting organisms by 48 h postchallenge. Bacteremia and organ colonization were absent. Immunization with 0.0001 μg of LPS resulted in lower bacterial numbers present in the skin at early time points. However, by 48 h the bacterial load was comparable with that seen in control mice. Transient bacteremia, without liver infection, was seen at 48 h, corresponding to peak levels of numbers in the skin. The reduction of bacterial numbers in the skin at 72 h correlated with the absence of bacteremia.

The above studies demonstrated that a monovalent LPS vaccine was both highly immunogenic and protective against a live challenge. Since protection was found to be serotype specific, we were interested in expanding these studies to serotypes other than G. To achieve this, a multivalent vaccine composed of nine serotypes of LPS was developed and used to immunize mice (Table 4). Protection was found to vary depending on the challenge strain, ranging from 110-fold (PA-53) to 53,000-fold (PA220). In most instances (six of eight strains), the LD_{50} for mice immunized with the polyvalent vaccine was increased by more than 1,000-fold compared with control mice. It is interesting to note that the protection afforded against PA220 was comparable when either multivalent vaccine or monovalent PA220 LPS vaccine was employed (53,000- and 100,000-fold, respectively).

DISCUSSION

In the present study, the efficacy of LPS and PS as active immunogens for the prevention of fatal *P. aeruginosa* sepsis was evaluated in a murine burn model. Although vaccination with LPS has been shown to be protective against either an intraperitoneal (14–16) or pulmonary (10–12) challenge in several studies, only one report to date has evaluated the benefit of prior immunization with LPS in a burn model (21). The latter study was limited to protection against a single strain of *P. aeruginosa* and used only one challenge dose and immunization schedule. Vaccination with PS has been evaluated for protection only against intraperitoneal challenge (14–16).

LPS isolated from several strains of *P. aeruginosa*, derived either from the phenol or water phase, was found to be highly immunogenic and protective in mice. The level of protection correlated with anti-LPS antibody titers. Maximal protection was achieved when IgG titers were highest. However, an immunization schedule which evoked only an IgM response still afforded a high degree of protection. It would appear, therefore, that anti-LPS antibody of both immunoglobulin classes is highly protective. This is somewhat surprising in view of the previous findings of Bjornson and Michael (2) that antimucopolysaccharide IgG passively administered intraperitoneally is far more effective than IgM at preventing lethal infection. However, this difference can most likely be attributed to the comparatively greater ability of IgG to transverse the peritoneum, enter the bloodstream, and diffuse to various tissues rather than to a specific protective function (opsonization, etc.). The production of antibody by active immunization would, in all likelihood, circumvent this problem.

Anti-LPS antibody was found to limit bacterial multiplication at the initial foci of infection (skin) and to limit or prevent bacteremia. Low immunizing doses (0.0001 μg) resulted in a substantial delay in the onset of bacteremia and bacterial multiplication in the skin. Bacteremia was transient, observed at only one time point. Since no fatalities were noted in this group, survival is consistent with sepsis of a short duration. When a higher immunizing dose was employed (0.01 μg), the bacterial challenge was completely cleared by 48 h postchallenge, and bacteremia, with accompanying organ colonization, was prevented.

The level of protection afforded by vaccination with PS or LPS is considerably higher than that reported after immunization with other *P. aeruginosa* antigens, such as toxin A toxoids or detoxified proteases, evaluated in a similar burn wound (8, 9, 24; I. A. Holder, C. B. Saelinger, C. G. Haidaris, and M. Michael., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B94, p. 29). Substantial levels of protection (minimum, 110-fold) were obtained by immunization with serotype-specific LPS against several challenge strains. This finding correlates well with prior studies showing that passively transferred anti-LPS provided uniformly high protection against challenge strains of the same serotype (3).

In contrast, several investigators have noted considerable variation in protection afforded after vaccination with several different antigens, depending upon the challenge strain employed. Okada et al. (8) found that although multivalent vaccines (consisting of somatic antigens [OEP], toxin A toxoid, detoxified alkaline protease, and detoxified elastase in various combinations) could increase the LD_{50} 10,000-fold for one challenge strain, little or no protection was seen against two other strains. Similarly, immunization with purified flagellar antigens afforded protection ranging from 40 to 100%, depending on the challenge strain (6).

Results from the present study demonstrate that vaccination with LPS or PS can provide substantial protection against highly virulent strains of *P. aeruginosa* in a burn wound model relevant to human disease. In this model system, opsonic antibody appears to be of critical importance in preventing lethal sepsis, acting to clear the bacterial challenge so as to prevent or limit bacteremia.

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