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Protective Immunity Induced in Mice by Immunization with High-Molecular-Weight Polysaccharide from *Pseudomonas aeruginosa*

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A high-molecular-weight alkali-labile polysaccharide (PS) isolated from the slime of immunotype 1 *Pseudomonas aeruginosa* was tested for its ability to protect mice from lethal challenge with the live, homologous organism. Intraperitoneal (i.p.) injection of 10 to 25 μg of the PS protected 60 to 70% of the mice against challenge with up to 50 50% lethal dose units. Although single immunization of mice with up to 250 μg of PS effected protective levels of only 70%, two successive immunizations provided 100% protection. Subcutaneous and intravenous immunization with PS also provided protection to i.p. challenges with immunotype 1 *P. aeruginosa*, but not to i.p. challenge with immunotype 4 *P. aeruginosa*. Although lipopolysaccharide (LPS) was found to be more immunogenic than PS in our studies, contamination of the alkali-labile PS with LPS did not account for the protection seen. Alkali treatment (0.1 N NaOH, 37°C, 2 h) of the PS destroyed its protective effectiveness, while similarly treated LPS retained its capacity for inducing immunity in mice. Adsorption and passive protection studies with sera raised to either PS or a mixture of PS and LPS indicated that antibody directed to the alkali-labile PS antigen was capable of contributing to the protection of mice against challenge with *P. aeruginosa*.

Death from septicemic *Pseudomonas aeruginosa* infections remains a major problem for the compromised host (5, 17, 19, 20). Studies in animals (1, 14) and humans (8, 11) have suggested that immunization with either killed organisms or purified bacterial antigens has some efficacy in reducing the incidence of mortality associated with *P. aeruginosa* infection. Recently a heptavalent preparation of the lipopolysaccharide (LPS) from the seven Fisher immunotype strains (9) of *P. aeruginosa* has been tested in burn patients (2), patients with malignancies (22), and children with cystic fibrosis (15). Results of these trials were equivocal because of the lack of statistical significance in the parameters being tested (22) or the lack of a simultaneous control group to compare the incidence of *P. aeruginosa* infections with those of the immunized group (2). Moreover, vaccination with this preparation was associated with a high incidence of side effects, especially in children, thus limiting its use (15).

Since safe and effective vaccines have been prepared from high-molecular-weight polysaccharide capsules from a number of pathogenic bacteria (3, 4, 6), we tested a similar, recently described (16) high-molecular-weight polysaccharide (PS) antigen we isolated from the slime layer of immunotype 1 (IT-1) *P. aeruginosa* for its ability to induce protection in a mouse model. Previous studies (21) have shown this model to be an economical and convenient bioassay of the immunological potential of *P. aeruginosa* antigens in inducing protective immunity. PS is composed of carbohydrate (glucose, galactose, mannose, rhamnose, and arabinose) and water, has a molecular weight of between 150,000 and 350,000 as determined by molecular sieve chromatography, and did not contain the characteristic chemical components of LPS such as lipid, 2-keto-3-deoxyoctonoate, or heptose. In addition, PS is nontoxic in mice at high doses and nonpyrogenic in rabbits, suggesting it does not possess the toxic properties of the previously tested LPS vaccine (15, 16).

IT-1 PS shares antigenic determinants with the polysaccharide portion of the LPS of IT-1, but differs from the LPS polysaccharide because IT-1 PS contains galactose and arabinose and IT-1 LPS does not. These antigenic determinants on both PS and LPS are destroyed by treatment with 0.1 N NaOH at 37°C for 2 h. LPS remains capable of binding antibody after this treatment, while PS does not, indicating

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that LPS contains alkali-stable determinants not shared with PS (16). The following data describe the results obtained by both active immunizations with PS, and passive transfer of immune sera against PS, in protecting mice from lethal challenge with live, IT-1 *P. aeruginosa* organisms.

MATERIALS AND METHODS

Bacterial strain, antigens, antisera, and serological analysis. IT-1 *P. aeruginosa*, PS and LPS antigens, polysaccharide from LPS obtained by acetic acid hydrolysis and column chromatography, alkali treatment of PS and LPS, and preparation of antisera were all as previously described (16). IT-4 *P. aeruginosa* (05141) was kindly provided by M. Fisher of Parke-Davis Corp., Detroit, Mich. Serological assay of antisera in the indirect solid phase radioimmunoassay (SPRIA) and inhibition of the SPRIA were also as previously described (16, 23, 24). Hemagglutination assays of sera from immunized mice were performed by sensitizing Formalin-fixed human O-negative erythrocytes with 0.3 mg of stearoyl chloride-derivitized PS (16) or 0.1 mg of LPS per 4 ml of 2% cells for 2 h at 37°C. Serial dilutions of mouse sera were made in phosphate-buffered saline by the microtiter dilution method, after which 0.05 ml of 0.5% sensitized cells was added, and the hemagglutination titer was determined after overnight incubation at room temperature. 2-Mercaptoethanol (2-ME) treatment of sera was performed by adding 2-ME to a final concentration of 0.1 N to the sera and incubating at 37°C for 1 h.

Active immunization of mice. Two- to four-week-old ICR mice, weighing 12 to 17 g at the time of immunization and 22 to 30 g at the time of challenge, were obtained from the Walter Reed Army Institute of Research animal colony and employed throughout this study. Mice were immunized by either the intraperitoneal (i.p.), subcutaneous (s.c.), or intravenous (i.v.) route with 0.1 ml of the appropriate amount of antigen in saline. Seven days later, all challenges were performed by i.p. inoculation of the appropriate dose of live organisms in 0.1 ml of saline. Challenge doses were prepared from 18-h cultures of either IT-1 *P. aeruginosa* or IT-4 *P. aeruginosa* grown on Trypticase soy agar with 3% glycerol at 37°C, and suspended in saline to an optical density of 1.2 at 650 nm. Mice were observed for 96 h.

Determination of LD_{50}. Fifty percent lethal dose (LD_{50}) values were determined from the results of i.p. administration of graded doses of live organisms and calculated by the method of Reed and Muench (18). Variability in the LD_{50} value of a given dose necessitated a separate LD_{50} determination for each different immunization-challenge experiment.

Passive transfer of immune sera. Rabbit antisera raised to either IT-1 *P. aeruginosa* PS or a mixture of PS and LPS were diluted 1:10 for use in passive transfer experiments. Aliquots of this sera were adsorbed with either 5 mg of PS, 5 mg of LPS, 5 mg of alkali-treated LPS, or a mixture of 5 mg of PS and 5 mg of alkali-treated LPS and subsequently tested for its ability to confer protection. Adsorptions were done for 1 h at 37°C and then overnight at 4°C, after which any precipitate was removed by centrifugation. These sera were then tested in the SPRIA against PS, LPS, and alkali-treated LPS antigens to determine the completeness of the adsorption process. Readsorptions were performed with smaller amounts of absorbing antigens until all antibody against the absorbing antigen was removed. Sera were further tested by inhibition of the SPRIA to determine that the concentration of free antigen in the sera was below 10 μg/ml. Mice were then given 0.25 ml of the diluted sera at various times pre- and postchallenge. Challenges were with live organisms resuspended in saline as per the active immunization experiments.

Statistics. *P* values for significant levels of protection were calculated by the chi-square method.

RESULTS

Active immunizations. Table 1 shows the results of two experiments immunizing mice with various amounts of PS by the i.p. route and subsequent challenge. A dose of 10 to 25 μg per mouse was needed to afford a protective level of 60 to 70%. All nonimmunized mice given an equivalent challenge dose in this and all subsequent experiments were dead by 30 h postchallenge. Higher doses of PS (Table 2) did not

TABLE 1. *Protection of mice by i.p. immunization with PS and i.p. challenge with IT-1 P. aeruginosa*[a]

Expt.	Amt of PS given (μg)	Challenge dose (LD_{50})	No. of organisms	No. of survivors/total	Protection (%)	*P* value
1	100	30	1.0×10^8	6/10	60	0.997
	50	30	1.0×10^8	6/10	60	0.997
	25	30	1.0×10^8	7/10	70	0.999
	10	30	1.0×10^8	6/10	60	0.997
	0	30	1.0×10^8	0/10	0	
2	100	50	1.7×10^8	8/10	80	>0.999
	50	50	1.7×10^8	8/10	80	>0.999
	25	50	1.7×10^8	6/10	60	0.997
	10	50	1.7×10^8	5/10	50	0.990
	1	50	1.7×10^8	2/10	20	0.864
	0	50	1.7×10^8	0/10	0	

[a] Mice were given the indicated amount of antigen in 0.1 ml of saline i.p. and challenged i.p. 7 days later with the indicated number of organisms in 0.1 ml of saline.

increase the protection level beyond 70%, but protective levels of 100% could be achieved if two immunizing doses were given 5 days apart, with challenge 7 days after the second immunization. Also, challenging mice with 5 LD_{50} units (Table 3) required only 0.1 μg of PS antigen for 70% protection, while still killing 90% of the control mice.

To show that PS was not non-specifically activating an inflammatory response in the peritoneum of the immunized mice, the animals were inoculated with PS s.c. on the back of the neck or i.v. in the tail vein and then challenged i.p. These two routes of immunization elicited a 90 to 100% protection against 30 LD_{50} units (Table 4). Further evidence that a specific immune response had occurred in the immunized mice was shown by the inability of immunized mice to survive challenge with a low amount of IT-4 *P. aeruginosa* organisms (Table 4). Finally, pooled sera obtained from 10 mice immunized with 50 μg of PS each contained a hemagglutinating titer of 8 directed against the PS antigen as well as against the LPS antigen. Although this hemagglutinating titer was low, pooled sera from unimmunized mice were unable to agglutinate sensitized cells at the highest concentration testable (1:2 dilution). The hemagglutinating titer was destroyed after treatment of the mouse sera with 2-ME, indicating the antibody was most likely immunoglobulin M. Attempts to show 2-ME-resistant antibody present in these sera by also adding antimouse gamma globulin antibody to the hemagglutinating test after incubation of the treated sera with the erythrocytes were unsuccessful.

LPS was next tested as an immunogen, and the results of two experiments are shown in Table 5. LPS was much more immunogenic on a weight basis than PS, with as little as 0.001 μg of LPS giving 87.5% protection in one of the experiments. This led us to suspect that LPS contamination might be the cause of protection seen with PS immunizations. Since we had previously noted that alkali-treated PS lost all serological activity, whereas alkali-treated LPS retained some serological activity, we immunized mice with alkali-treated PS or alkali-treated LPS to test the ability of these preparations to confer protection. Table 6 shows that neither 50 nor 250 μg of alkali-treated PS could induce protection in mice, whereas 0.05 and 0.01 μg of alkali-treated LPS are able to induce 90 to 100% protection. A mixture of 50 μg of alkali-treated PS and 0.01 μg of alkali-treated LPS retains the capacity to induce protection in 80% of the mice, indicating that the presence of alkali-treated PS in alkali-treated LPS preparations had little to no significant interference with the ability of LPS to induce immunity.

Since we had demonstrated that a polysaccha-

TABLE 2. *Effect of increased i.p. dosage and double i.p. immunizations of mice with PS on protective levels to IT-1 P. aeruginosa*[a]

Amt of PS given (μg)	Challenge dose (LD_{50})	No. of organisms	No. of survivors/total	Protection (%)	*P* value
500	50	1.7×10^8	6/10	60	0.997
250	50	1.7×10^8	7/10	70	0.999
50, day 1 50, day 5	50	1.7×10^8	10/10	100	>0.999

[a] Mice were immunized i.p. with the indicated amount of antigen in 0.1 ml of saline and challenged i.p. 7 days later with the indicated number of organisms in 0.1 ml of saline. Double immunizations were two immunizations of mice as described but with a challenge 12 days after the first immunization.

TABLE 3. *Protection of mice to low-dose challenge with IT-1 P. aeruginosa after immunization with PS*[a]

Amt of PS given (μg)	Challenge dose (LD_{50})	No. of organisms	No. of survivors/total	Protection (%)	*P* value
10	5	1.7×10^7	8/10	80	0.954
1	5	1.7×10^7	10/10	100	0.977
0.5	5	1.7×10^7	9/10	90	0.968
0.1	5	1.7×10^7	7/10	70	0.934
0	5	1.7×10^7	1/10	10	

[a] Mice were immunized i.p. with the antigen in 0.1 ml of saline and challenged i.p. 7 days later with the indicated number of organisms in 0.1 ml of saline.

TABLE 4. *Protection of mice by s.c. and i.v. immunization with PS against i.p. challenge with IT-1 and IT-4 P. aeruginosa*[a]

Amt of PS given (μg)	Route	Challenge dose (LD_{50})	No. of organisms	No. of survivors/total	Protected (%)	*P* value
50	s.c.	IT-1 (8)	8.0×10^7	9/10	90	>0.999
50	i.v.	IT-1 (8)	8.0×10^7	10/10	100	>0.999
0	s.c.	IT-1 (8)	8.0×10^7	0/10	0	
0	i.v.	IT-1 (8)	8.0×10^7	0/10	0	
50	s.c.	IT-4 (5)	6.0×10^7	0/10	0	0
50	i.v.	IT-4 (5)	6.0×10^7	0/10	0	0

[a] Mice were immunized with the antigen in 0.1 ml of saline by the indicated route and challenged 7 days later i.p. with the indicated number of organisms in 0.1 ml of saline.

TABLE 5. *Protection of mice by i.p. immunization with LPS against i.p. challenge with IT-1 P. aeruginosa*[a]

Expt.	Amt of LPS given (μg)	Challenge dose (LD_{50})	No. of organisms	No. survivors/total	Protected (%)	*P* value
1	0.5	30	1.5×10^9	9/10	90	>0.999
	0.1	30	1.5×10^9	9/10	90	>0.999
	0.05	30	1.5×10^9	9/10	90	>0.999
	0.01	30	1.5×10^9	7/10	70	0.999
	0.005	30	1.5×10^9	4/10	40	0.978
	0.001	30	1.5×10^9	0/10	0	
	0			0/10	0	
2	1.0	10	1.2×10^8	8/8	100	0.986
	0.5	10	1.2×10^8	8/8	100	0.986
	0.1	10	1.2×10^8	8/8	100	0.986
	0.05	10	1.2×10^8	8/8	100	0.986
	0.01	10	1.2×10^8	7/8	87.5	0.980
	0.001	10	1.2×10^8	7/8	87.5	0.980
	0	10	1.2×10^8	0/8	0	

[a] Mice were immunized i.p. with the indicated amounts of LPS in 0.1 ml of saline and challenged i.p. 7 days later with the indicated number of organisms in 0.1 ml of saline.

TABLE 6. *Protection of mice by i.p. immunization with alkali-treated PS or alkali-treated LPS to i.p. challenge with IT-1 P. aeruginosa*[a]

Antigen	Amt given (μg)	Challenge dose (LD_{50})	No. of organisms	No. of survivors/total	Protected (%)	*P* value
Alkali-treated PS	250	10	1.2×10^8	0/8	0	0
Alkali-treated PS	50	10	1.2×10^8	0/8	0	0
PS	50	10	1.2×10^8	7/8	87.5	0.980
Saline	0	10	1.2×10^8	0/8	0	
Alkali-treated LPS	0.05	20	5.0×10^8	10/10	100	>0.999
Alkali-treated LPS	0.01	20	5.0×10^8	10/10	100	>0.999
LPS	0.01	20	5.0×10^8	10/10	100	>0.999
Alkali-treated LPS + alkali-treated PS	0.01 50	20	5.0×10^8	8/10	80	>0.999
Saline	0	20	5.0×10^8	0/10	0	

[a] Mice were immunized i.p. with the indicated amount of antigen in 0.1 ml of saline and challenged i.p. 7 days later with the indicated amount of organisms in 0.1 ml of saline.

ride antigen could be isolated from LPS by acetic acid hydrolysis and Sephadex G-100 column chromatography that was immunologically identical to PS but different in its lack of galactose and arabinose, we immunized mice with this antigen to judge its ability to confer protection. Immunization of mice with the PS portion of LPS that eluted as the void column of a G-100 column induced protective levels of 70% against a challenge of 1.5×10^9 organisms. Immunization of mice with the PS portion of LPS that eluted in the molecular weight range of 60,000 was much less effective and only protected 30% of the challenged mice. Alkali treatment of these antigens destroyed all of their immunogenic capabilities.

Passive transfers. Evidence that specific antibody directed against the PS determinant was responsible for protecting mice from lethal challenge was obtained by passive transfer of rabbit serum raised to either PS or a mixture of PS and LPS. These sera were also adsorbed with PS, LPS, and alkali-treated LPS antigens. The results of these adsorption experiments are shown in Table 7. LPS could absorb out all antibody activity directed against either the PS, LPS, or alkali-treated LPS antigens, whereas PS only completely absorbed out the activity directed against PS. Alkali-treated LPS absorbed out the activity against itself, but not to intact LPS or PS.

Table 8 depicts the protection seen by giving mice 0.25 ml of diluted sera 24 and 3 h before challenge, at the time of challenge, and 3 and 6 h postchallenge. Unadsorbed antiserum raised to a mixture of LPS and PS protected mice if given 0 to 24 h before challenge. No protection was observed with this or any other sera if given 3 h or more postchallenge. Adsorption of this antiserum with either PS or alkali-treated LPS did not abrogate its protective capabilities, whereas adsorption with either intact LPS or a

mixture of PS and alkali-treated LPS removed all protective antibody.

Antiserum to PS itself was also capable of conferring protection when passively transferred to mice (Table 8). This serum lacks antibody directed against the alkali-stable portion of LPS, so that all of the protective capability of this serum was absorbed out with either PS or intact LPS.

DISCUSSION

These results demonstrate the ability of high-molecular-weight PS from the slime of IT-1 *P. aeruginosa* to actively immunize mice against a lethal challenge with live cells. The model employed here was chosen for its ease and convenience as well as because of the fact that antibody levels in mice have been shown to correlate with immunity to *P. aeruginosa* (21). This model does not parallel the disease states most often associated with susceptibility to *P. aeruginosa* infections in humans, which often involves impaired leukocyte levels or functions. It did, however, provide a simple way of assessing the immunogenic potential of this PS vaccine candidate, and, as such, demonstrated its efficacy.

The results of the passive transfer experiments demonstrated that antibody directed against the PS could prevent mice from dying of a lethal *P. aeruginosa* infection. Furthermore, the previously described antigenic relationship of PS to LPS was confirmed by the passive transfer data. Polysaccharide that does not contain galactose and arabinose but is immunologically identical to PS is either part of or closely associated with the LPS molecule and can be released from LPS either by acetic acid hydrolysis of LPS or by simply allowing LPS to stand at room temperature for 4 days in buffered salt solution (16). The lack of galactose and arabinose in the LPS did not affect its ability to absorb out all of the protective antibody contained in antiserum to PS, nor did the lack of galactose and arabinose affect the ability of polysaccharide obtained from LPS that elutes as the void volume of a G-100 column to induce immunity similar to that seen with PS immunization. However, the polysaccharide antigen obtained from acetic acid hydrolyzed LPS that elutes in the 60,000-dalton range of the G-100 column and accounts for 50% of the weight of the LPS was a poor immunogen, most likely because of its small size. These data do show that all of the PS antigenic determinants are expressed on LPS.

The very low amount of purified LPS needed to obtain protection of mice (0.001 to 0.01 μg) raises the possibility that the protection seen

TABLE 7. *Counts per minute of ^{125}I-labeled goat anti-rabbit globulin (^{125}I-GARG) bound to the antibody (Ab) contained in a 1:10 dilution of adsorbed sera used for passive transfer studies*[a]

Antisera to:	Adsorbed with:	cpm of ^{125}I-GARG bound to Ab vs:		
		PS	LPS	Alkali-treated LPS
PS and LPS	None	14,230	28,930	8,090
	PS	257	6,169	7,223
	LPS	0	105	0
	Alkali-treated LPS	13,280	22,450	101
	PS + alkali-treated LPS	0	0	0
PS	None	12,921	12,859	218
	PS	362	412	0
	LPS	112	418	92
	Alkali-treated LPS	12,519	13,492	0

[a] Sera were diluted 1:10 and adsorbed with indicated antigen for 1 h at 37°C, then overnight at 4°C, and then tested for reactivity against the indicated antigen in the SPRIA.

TABLE 8. *Passive protection of mice when given rabbit antisera pre- and postchallenge with 1.5 × 10^9 IT-1 P. aeruginosa*

Antisera to:	Adsorbed with:	No. of survivors/total at 72 h				
		−24[a]	−3	0	+3	+6
PS and LPS	None	10/10	10/10	10/10	0/10	0/10
	PS	10/10	10/10	10/10	0/10	0/10
	LPS	0/10	0/10	0/10	0/10	0/10
	Alkali-treated LPS	10/10	10/10	10/10	0/10	0/10
	PS and alkali-treated LPS	0/10	0/10	0/10	0/10	0/10
PS	None	10/10	10/10	10/10	0/10	0/10
	PS	0/10	0/10	0/10	0/10	0/10
	LPS	0/10	0/10	0/10	0/10	0/10
	Alkali-treated LPS	10/10	10/10	10/10	0/10	0/10

[a] Mice were given i.p. 0.25 ml of a 1:10 dilution of rabbit antisera to the indicated antigens at the indicated times (hours) pre- and postchallenge.

with most *P. aeruginosa* vaccines is due to LPS contamination. Our results with immunizing mice with alkali-treated PS and LPS showed that contamination of PS with LPS could not account for the protection seen with PS immunizations, inasmuch as alkali-treated PS was not effective in inducing immunity, whereas alkali-treated LPS retained immunogenic activity. Lieberman (10) has found the sugars rhamnose and glucose present in a ribosomal vaccine preparation of Habs serotype 2 *P. aeruginosa* and speculated that an immunizing dose of 1 μg of ribosomal RNA contained 10 to 20 ng of LPS. Our results show this to be a sufficient amount of LPS to immunize mice, though the possibility of variation in the immunogenicity of LPS from various *P. aeruginosa* strains, as well as the immunological response of different mouse strains, may affect the immunogenic dosage level of LPS. Mates and Zand (12) used alcohol-precipitated slime as well as ribosomes to prepare *P. aeruginosa* vaccines, which were assayed in a mouse protection model. Full protection was seen with 100 μg of alcohol-precipitated slime and 40 μg of ribosomes, meaning that a contamination level of these preparations with 0.01 and 0.025% LPS, respectively, would contain 0.01 μg of LPS. Miller et al. (13) have recently reported on a polyvalent *P. aeruginosa* vaccine prepared simply by extracting the bacteria with a mixture of glycine and ethylenediaminetetraacetate. Almost assuredly this preparation contained sufficient quantities of LPS to be immunogenic, though ethylenediaminetetraacetate has been shown to have an adverse effect on the immunological properties of LPS from some strains of *P. aeruginosa* (7).

Since several studies have suggested that immunization with *P. aeruginosa* cells or antigens can reduce the mortality from infection with this organism, the ability of our PS preparation to immunize mice is a significant step towards development of a *P. aeruginosa* vaccine. Further encouragement is gained from the lack of toxicity and pyrogenicity in animal tests (16). The mechanism of protection in the mouse model is related to the presence of specific antibody, as evidenced by the rise in hemagglutinating titers of immunized mice and by the passive transfer studies. The specific functional role of this antibody is currently under investigation, because phagocytosis mediated by opsonic antibody is considered to be a primary host defense against *P. aeruginosa* infection (21). Further investigations on the ability of PS to inhibit opsonic antibody found in the sera of patients convalescing from *P. aeruginosa* infections needs to be performed. Finally, isolation of high-molecular-weight PS antigens from other *P. aeruginosa* strains and assessment of their immunological relatedness needs to be done before the feasibility of these antigens as vaccines can be fully evaluated.

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