INFECTION AND IMMUNITY, Nov. 1981, p. 461-468
0019-9567/81/110461-08$02.00/0

Vol. 34, No. 2

High-Molecular-Weight Polysaccharide Antigen from *Pseudomonas aeruginosa* Immunotype 2

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Received 11 May 1981/Accepted 7 July 1981

Previously, we isolated a high-molecular-weight immunogenic polysaccharide (designated PS) from *Pseudomonas aeruginosa* immunotype 1 (IT-1). The method which we used was modified to permit the isolation of a similar PS from *P. aeruginosa* IT-2. This antigen was composed primarily of carbohydrate, had a complex monosaccharide composition, including sugars not found in the lipopolysaccharide, and was nonpyrogenic in rabbits and nontoxic in mice at high doses. This material protected mice from challenges with live homologous cells. *P. aeruginosa* IT-2 PS gave a line of identity with the O side chain of the lipopolysaccharide, but differed from this polysaccharide in molecular weight, chemical composition, and ability to immunize mice actively. Lipopolysaccharide from *P. aeruginosa* IT-2 contained an immunological determinant not found on *P. aeruginosa* IT-2 PS, which was detected due to its stability during treatment with dilute alkali. Thus, we recovered a high-molecular-weight PS antigen from *P. aeruginosa* IT-2, which was serologically identical to the lipopolysaccharide O side chain but was chemically and physically distinct. Also, like *P. aeruginosa* IT-1 strains, *P. aeruginosa* IT-2 contains an alkali-stable immunodeterminant on the lipopolysaccharide that may represent a core-like antigen.

The high-molecular-weight polysaccharide designated PS which can be obtained from the outer cell surface or cultural supernatants of *Pseudomonas aeruginosa* immunotype 1 (IT-1) (7) actively protects mice from challenges with live homologous organisms (6). This antigen is composed principally of carbohydrate, has a molecular weight of approximately 1.5×10^5, is nonpyrogenic in rabbits at a dose of 25 μg/kg of body weight, and is nontoxic in mice at a dose of 400 mg/kg of body weight. The high molecular weight of PS, which is necessary for immunogenicity, and the low toxicity of this compound in animals suggest that it may be a candidate for a human vaccine.

PS appears to be a high-molecular-weight form of the polysaccharide side chain from *P. aeruginosa* IT-1 lipopolysaccharide (LPS) since PS and a polysaccharide which was obtained from LPS that had been hydrolyzed with acetic acid (O side chain) appeared to be immunologically identical (7). About 20% of the weight of *P. aeruginosa* IT-1 LPS can be accounted for by an O side chain fraction that elutes in the void volumes of Sephadex G-100 columns, and another 50% can be accounted for by an immunologically identical but smaller polysaccharide fraction. The LPS and PS from *P. aeruginosa* IT-1 differ chemically, since PS contains galactose and arabinose and LPS does not (7).

A systematic study of the PS antigens in different strains of *P. aeruginosa* led to the isolation and characterization of PS from *P. aeruginosa* IT-2. As described here, *P. aeruginosa* IT-2 PS is also immunogenic and nontoxic in mice, is nonpyrogenic in rabbits, and exhibits serological cross-reactions with *P. aeruginosa* IT-2 LPS.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* IT-2 (strain 05142) and IT-6 (strain 01544) were obtained from M. Fisher, Parke, Davis & Co., Detroit, Mich.

Preparation of PS. *P. aeruginosa* IT-2 PS was prepared from 10-liter cultures of *P. aeruginosa* grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) containing 3% glycerol. Each culture was grown in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) for 72 h with stirring (200 rpm) and aeration (5 liters of air per h). Cetavalon (hexadecyltrimethylammonium bromide; final concentration, 0.5%; Fisher Scientific Co., Medford, Mass.) was added to the culture before centrifugation to remove the organisms. The supernatant was concentrated with a stacked ultrafiltration cell (Amicon

Corp., Lexington, Mass.) to a final volume of 800 ml by using PM-30 membranes. After precipitation of this concentrated supernatant with alcohol (80%, vol/vol), the crude material was collected by centrifugation and dissolved in phosphate-buffered saline, as described previously (7). This solution was then heated to 60 to 80°C, and Cetavalon (final concentration, 1.0%) was added. The precipitate was removed by centrifugation, and the supernatant was precipitated with alcohol (80%, vol/vol). The alcohol precipitate was collected by centrifugation and dissolved in phosphate-buffered saline; then it was subjected to a second series of heating, Cetavalon addition, centrifugation, alcohol precipitation of the supernatant, and centrifugation to collect the PS-containing material.

The addition of Cetavalon to hot preparations of crude concentrated culture supernatants allowed the elimination of the subsequent nuclease digestion steps described previously (7). Thus, after the two Cetavalon steps, the PS was purified by treating it with 1% acetic acid at 90°C for 18 h and extracting it with chloroform; this was followed by phenol extraction, and the compound was finally purified by Sephadex G-100 column chromatography, as described previously (7).

Preparation of LPS. LPS was isolated by the phenol-water extraction procedure of Westphal et al. (9). Low yields of LPS from the aqueous layer led us to investigate the phenol layer as a source of LPS. After the separation of the phenol and water layers, the phenol layer was dialyzed against running deionized water for 2 days, residual cells and denatured proteins were removed by centrifugation at 10,000 rpm for 30 min, and the LPS was recovered by ultracentrifugation at 33,000 rpm for 3 h. Further processing of the LPS recovered from the phenol phase was performed as described previously (7). We also attempted to extract the *P. aeruginosa* IT-2 LPS by the phenol-chloroform-petroleum ether method of Galanos et al. (3).

Preparation of O-specific polysaccharide. Isolated LPS was suspended in 1% acetic acid at a concentration of 10 mg/ml and then heated at 90°C for 6 h. The resulting lipid A precipitate was removed by centrifugation, and the supernatant was applied to a Sepharose CL-6B column (1.6 by 60 cm) by using phosphate-buffered saline as the eluant. The column eluate was monitored at 206 nm, and the serological activities of the optically active fractions were determined by the capillary precipitin reaction.

Alkali treatment of antigens and preparation of antisera. *P. aeruginosa* IT-2 PS and LPS were treated with 0.1 N NaOH at a concentration of 2 mg/ml at 56°C for 12 h. These solutions were neutralized with acid and buffer when they were used in immunological studies. Antisera to *P. aeruginosa* IT-2 cells, LPS, and PS were prepared as described previously (7).

Antisera to IT-2 alkali-treated LPS (A-LPS) were raised in New Zealand white rabbits that weighed 3 to 4 kg by an initial intramuscular injection of 0.5 mg of A-LPS in complete Freund adjuvant, followed 1 week later by a series of four intravenous injections of 0.5 mg of A-LPS in saline every 4 days. Blood was collected by cardiac puncture 6 days after the last injection.

Serological methods. Ouchterlony double diffusion and immunoelectrophoresis were performed as previously described (7). Hemagglutination of sheep erythrocytes (SRBC) coated with either LPS or A-LPS was performed in microtiter plates by using 25-μl volumes of serum dilutions and cells. SRBC were coated by suspending 0.12 ml of packed SRBC in 1.0 ml of saline containing 0.5 mg of antigen, followed by dropwise addition of 1.0 ml of a 0.01% chromium chloride solution. Titers were read as the reciprocals of the highest serum dilutions that caused agglutination of the SRBC. Each hemagglutination inhibition test was performed by incubating 25 μl of a serum dilution four times more concentrated than the titer with 25 μl of a solution containing the inhibitor to be tested; then the preparation was incubated at 37°C for 30 min, 25 μl of coated cells was added, and the preparation was incubated further at 37°C.

Chemical analysis. The chemical constituents and the monosaccharide and lipid compositions of the PS, LPS, and O side chain polysaccharide antigens were determined as described previously (7).

Molecular weight determinations. The molecular weights of the PS and O side chain polysaccharides were determined with a Waters Associates high-performance liquid chromatography system by using two protein I-250 sizing columns in series and 0.1 M phosphate buffer as the eluant. Dextran polymers of known molecular weights (Pharmacia Co., Uppsala, Sweden) were used as standards. The molecular weights of unknowns were determined by calculating linear regression plots of the elution volumes versus $\log_{10}$ molecular weights of the standards. The coefficient correlation for this formula was 0.95 over the molecular weight range from 10,000 to 250,000.

Animal studies. ICR mice obtained from the Walter Reed Army Institute of Research animal colony and CD-1 mice obtained from Charles River Laboratories, Wilmington, Mass., were used for active immunization and passive transfer experiments, as previously described (6). The pyrogenicity in rabbits and toxicity in mice of *P. aeruginosa* IT-2 PS were also determined as described previously (7).

Adsorption of antisera for passive transfer. Rabbit antisera to *P. aeruginosa* IT-2 PS, LPS, and A-LPS were adsorbed with these antigens by incubating 1 ml of antiserum with 1 mg of the absorbing antigen at 37°C for 2 h; this was followed by a 48-h incubation at 4°C and the hemagglutination removal of any precipitate by centrifugation. The sera were then tested with the hemagglutination assay and readsorbed as described above if any hemagglutination activity was still present.

RESULTS

Isolation of *P. aeruginosa* IT-2 PS, LPS, and O-side chain polysaccharide. The use of the procedures described above for isolating PS antigen from supernatants of *P. aeruginosa* IT-2 cultures consistently yielded a product which had a high carbohydrate content, eluted in the void volume of a Sephadex G-100 column, and gave a single precipitin line in Ouchterlony dou-

ble-diffusion gels. Attempts to isolate PS antigen from agar cultures of *P. aeruginosa* IT-2 were generally unsuccessful, indicating that production of the high-molecular-weight PS was facilitated by growth in liquid media. The yields of *P. aeruginosa* IT-2 PS were lower than the yields of *P. aeruginosa* IT-1 PS; the average yield was 5 mg of PS per liter of medium. To eliminate detectable LPS from PS preparations, it was necessary to hydrolyze the LPS into its lipid and polysaccharide components with acetic acid. As found previously with *P. aeruginosa* IT-1 PS (7), *P. aeruginosa* IT-2 LPS could not be eliminated from the PS preparation by ultracentrifugation, column chromatography in the presence or absence of disaggregating buffers, or ion-exchange chromatography. *P. aeruginosa* IT-2 PS was serologically active after this treatment and remained stable to acetic acid hydrolysis at 95°C for up to 72 h.

Attempts to isolate LPS from *P. aeruginosa* IT-2 by phenol-water extraction (9) of cells generally gave poor yields of antigen in the aqueous phase. The LPS of this strain was best recovered from the phenol phase. The presence of *P. aeruginosa* IT-2 LPS in the phenol phase is unique to the LPS of this *P. aeruginosa* immunotype (unpublished data). The use of the phenol-chloroform-petroleum ether method of Galanos et al. (3) for extracting LPS from *P. aeruginosa* IT-2 was unsuccessful.

O-side chain polysaccharide was obtained from acetic acid-hydrolyzed LPS as a single serologically active peak which eluted from a Sepharose CL-6B column (1.6 by 60 cm) with a K_{av} of 0.52. Two other peaks were also detected in column eluates of acetic acid-hydrolyzed LPS. One of these was a very small peak which eluted in the void volume and was presumably nonhydrolyzed LPS; this peak was not characterized further. The second peak was a peak of carbohydrate-containing material that eluted in the bed volume and was neither serologically active nor precipitable with alcohol but was dialyzable; this peak had a molecular weight of less than 10,000, as determined by high-performance liquid chromatography.

Characterization of *P. aeruginosa* IT-2 PS and LPS. Table 1 shows the chemical compositions of the PS and LPS isolated from *P. aeruginosa* IT-2. The PS was composed almost exclusively of carbohydrate but did contain low levels of nucleic acids and protein. The water content of PS was 18.4%, as determined by a Karl Fischer titration (7). No lipid was detected in *P. aeruginosa* IT-2 PS by quantitative gas-liquid chromatography (limit of detection, 1 part in 1,000). On the other hand, LPS contained lipid, protein, nucleic acid, phosphate, and carbohydrate. The lipid and carbohydrate contents of our *P. aeruginosa* IT-2 LPS preparation were consistent with the contents reported by other investigators (4, 10).

TABLE 1. *Compositions of P. aeruginosa IT-2 PS and LPS*

Component	% By weight in:	
	PS	LPS
Carbohydrate	83.4	72
Lipid	0	12.2
Nucleic acid	1.2	2.2
Protein	0.8	5.8
Phosphate[a]	0	2.4
Water	18.4	

[a] The material for phosphate determinations was freed of nucleic acid by passage over an ion-exchange column.

Monosaccharide compositions of *P. aeruginosa* IT-2 PS, LPS, and O-side chain polysaccharide. Gas-liquid chromatographic and colorimetric analyses of the monosaccharide compositions of *P. aeruginosa* IT-2 PS, intact *P. aeruginosa* IT-2 LPS, and the O-side chain polysaccharide portion of the LPS showed that the LPS contained rhamnose, mannose, glucose, 2-acetamido-2,6-dideoxygalactose, galactosamine, glucosamine, heptose, and 2-keto-3-deoxyoctulosonic acid, whereas PS contained mannose, glucose, rhamnose, glucosamine, xylose, galactose, and arabinose; the latter three sugars were not detected in LPS (Table 2). The O-side chain polysaccharide portion of the LPS contained only rhamnose, mannose, and glucose.

Molecular weights of *P. aeruginosa* IT-2 PS and O-side chain polysaccharide from *P. aeruginosa* IT-2 LPS. High-performance liquid chromatography of *P. aeruginosa* IT-2 PS on a series of two I-250 protein-sizing columns showed that this preparation eluted as a single homogeneous peak with a molecular weight of 2.25×10^5. The serologically active O-side chain polysaccharide had a molecular weight of 0.3×10^5.

Chromatography of *P. aeruginosa* IT-2 PS in the presence of 3% sodium deoxycholate on a Sephadex G-100 column (2.6 by 100 cm) did not change the elution pattern of the PS.

Animal toxicity of *P. aeruginosa* IT-2 PS. Injection of up to 25 μg *of P. aeruginosa* IT-2 PS per kg into rabbits elicited no pyrogenic response(<0.1°C increase in 3 h). Similarly, 400 mg of *P. aeruginosa* IT-2 PS per kg injected intraperitoneally into mice elicited no signs of toxicity or symptoms of endotoxin poisoning, and the mice gained weight normally.

Serological relationship of *P. aeruginosa* IT-2 PS and LPS. Immunoelectrophoresis of *P.*

TABLE 2. *Molar ratios of sugars in P. aeruginosa IT-2 PS and LPS*

Prepn	Molar ratio of:[a]										
	Arabinose	Rhamnose	Xylose	Mannose	Galactose	Glucose	KDO	Gluam	Galam	Hep	Dideoxygal
PS	0.74	0.19	0.10	0.34	2.23	1.00	0	0.13	0	0	P[b]
LPS	0	0.34	0	0.15	0	1.00	0.05	0.05	0.05	0.04	P
O-side chain polysaccharide from LPS	0	0.36	0	0.34	0	1.00	0	0	0	0	0

[a] Molar ratio per mole of glucose. Abbreviations: KDO, 2-keto-3-deoxyoctonoate; Gluam, 2-acetamido-2-deoxyglucose; Galam, 2-acetamido-2-deoxygalactose; Hep, L-glycero-D-mannoheptose; Dideoxygal, 2-acetamido-2,6-dideoxygalactose.

[b] P, Present. Lack of a standard prohibited assignment of a molar value.

aeruginosa IT-2 PS showed that this antigen migrated toward the cathode when it was electrophoresed for 45 min (Fig. 1). This is the opposite polarity of migration shown by *P. aeruginosa* IT-1 PS. *P. aeruginosa* IT-2 LPS did not migrate after 2 h of electrophoresis.

In immunodiffusion gels, *P. aeruginosa* IT-2 PS gave a single precipitin line when it was tested against antiserum to intact *P. aeruginosa* IT-2 cells (Fig. 2). Acetic acid hydrolysis of *P. aeruginosa* IT-2 LPS released into the solution an antigen that gave a reaction of identity with *P. aeruginosa* IT-2 PS in immunodiffusion gels. A similar line of identity was generated with *P. aeruginosa* IT-2 LPS solutions that stood in phosphate-buffered saline at room temperature for 1 to 2 weeks (Fig. 2).

Table 3 shows the hemagglutination assay titers of antisera prepared against *P. aeruginosa* IT-2 cells, *P. aeruginosa* IT-2 LPS, *P. aeruginosa* IT-2 A-LPS, and *P. aeruginosa* IT-2 PS when these preparations were tested versus SRBC coated with either LPS or A-LPS. Attempts to coat SRBC with *P. aeruginosa* IT-2 PS by using either chromium chloride linking or steroyl chloride derivitization of PS (7) were unsuccessful. However, since acid-hydrolyzed LPS and PS gave a line of identity in immunodiffusion gels, it was possible to use LPS-sensitized SRBC to measure antibodies to PS. The specificity of this reaction was confirmed by using PS as an inhibitor in a hemagglutination inhibition assay.

We found that alkali treatment (0.1 N NaOH, 37°C, 2 h) destroyed the serological activity of *P. aeruginosa* IT-1 PS and that the determinants shared with *P. aeruginosa* IT-1 LPS were also destroyed by this treatment. We did not observe this phenomenon with *P. aeruginosa* IT-2 LPS, even after alkali treatment at 56°C for 12 h. Antiserum to *P. aeruginosa* IT-2 PS reacted at the same titer with A-LPS-coated

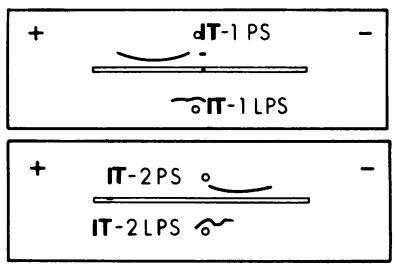

FIG. 1. *Immunoelectrophoretic patterns of PS and LPS from P. aeruginosa IT-1 and IT-2.*

SRBC and with intact LPS-coated SRBC (Table 3). However, alkali treatment of *P. aeruginosa* IT-2 PS at 56°C for 12 h did alter the inhibitory activity of this PS in serological assays specific for the PS determinants, indicating that when the PS determinants were freed from the LPS, they became alkali labile (Table 4). Antiserum to *P. aeruginosa* IT-2 A-LPS (56°C, 12 h) reacted with SRBC coated with A-LPS (Table 3). This reactivity could be inhibited by both intact LPS and A-LPS but not by PS, acetic acid-hydrolyzed LPS, or alkali-treated PS (Table 4). These results indicated that although *P. aeruginosa* IT-2 A-LPS and *P. aeruginosa* IT-2 PS had serologically active determinants in common, the immunogenicity of these determinants was destroyed by alkali treatment. This allowed us to detect alkali-stable determinants on the LPS by using an assay in which antisera to A-LPS and A-LPS-coated SRBC were used.

Active immunization of mice with *P. aeruginosa* IT-2 PS, LPS, O-side chain polysaccharide, alkali-treated PS, and A-LPS. *P. aeruginosa* IT-2 PS induced protective immunity in mice that were given 10 to 50 μg of *P. aeruginosa* IT-2 PS intraperitoneally and challenged 7 days later with 2.6 100% lethal doses of live *P. aeruginosa* IT-2 (Table 5). These immunizations were not effective in protecting

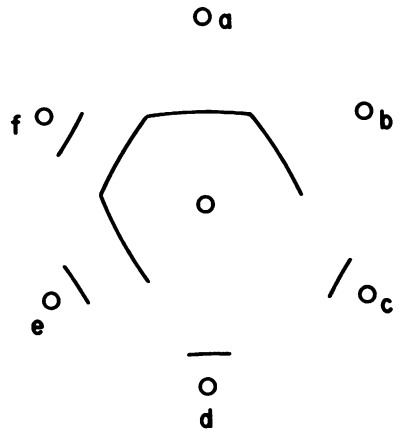

FIG. 2. *Immunodiffusion gel patterns obtained with P. aeruginosa IT-2 PS, acetic acid-hydrolyzed LPS, and LPS rehydrated and left to stand at room temperature versus antiserum to P. aeruginosa IT-2 (center well). The antigens in the outer wells were as follows: well a, PS (1 mg/ml); well b, acetic acid-hydrolyzed LPS (1 mg/ml); well c, freshly rehydrated LPS (1 mg/ml); well d, LPS rehydrated in solution for 3 days (1 mg/ml); well e, LPS in solution for 7 days (1 mg/ml); well f, LPS in solution for 14 days (1 mg/ml).*

TABLE 3. *Hemagglutination titers of antisera to P. aeruginosa IT-2 cells, PS, LPS, and A-LPS when tested versus SRBC coated with either LPS or A-LPS*

Antiserum to:	Titer when tested against SRBC coated with:	
	LPS	A-LPS
Cells	1,024[a]	2,048
PS	64	64
LPS	1,024	2,048
A-LPS	64	1,024

[a] Reciprocal of the twofold serum dilution that produced positive agglutination of antigen-coated SRBC.

mice against challenge with *P. aeruginosa* IT-6. LPS from *P. aeruginosa* IT-2 was effective in protecting mice at a level of 0.10 μg/mouse against a similar challenge (Table 5); again, no protection against challenge with *P. aeruginosa* IT-6 was observed. Isolated O-side chain polysaccharide was not effective in inducing protection at doses of up to 500 μg/mouse. Alkali treatment of *P. aeruginosa* IT-2 PS (56°C, 12 h) destroyed the protective efficacy of this compound at doses of up to 500 μg/mouse, whereas alkali treatment of *P. aeruginosa* IT-2 LPS raised the minimum effective protective dose of this compound to 1.0 μg/mouse (Table 5).

Table 6 shows the enhancement of nonspecific resistance to challenges with live cells 24 h after immunization with *P. aeruginosa* IT-2 PS and LPS. Doses of PS of up to 500 μg/mouse provided no protection against challenge with live cells, whereas doses of 1 and 10 μg of LPS per mouse provided a minimal level of nonspecific resistance. Higher doses of LPS caused severe endotoxin poisoning symptoms in the animals.

Passive transfer studies. Confirmation of the presence of cross-reactive determinants on *P. aeruginosa* IT-2 PS and LPS was obtained by passively transferring rabbit antisera raised

TABLE 4. *Inhibitory activities of PS, alkali-treated PS, LPS, A-LPS, and LPS O side chain in serological assays*

Inhibitor	Serum inhibited	Activity of antigen on SRBC	
		LPS	A-LPS
PS	Anti-LPS	9[a]	7
Alkali-treated PS	Anti-LPS	—[b]	—
LPS	Anti-A-LPS	10	8
A-LPS	Anti-A-LPS	7	9
PS	Anti-A-LPS	—	—
O side chain	Anti-A-LPS	—	—
Alkali-treated PS	Anti-A-LPS	—	—

[a] Reciprocal of the $\log_2$ dilution of a 1-mg/ml solution that produced inhibition of 4 hemagglutination units of serum.

[b] —, Inhibitory activity at 1 mg/ml.

TABLE 5. *Active immunization of mice with P. aeruginosa IT-2 PS, alkali-treated PS, LPS, O side chains, and A-LPS*

Immunogen	Amt (μg)	Challenge strain	No. of LD_{100}[a]	% Protected[b]	P value[c]
PS	0	IT-2	2.6	0	
	10	IT-2	2.6	50	0.016
	25	IT-2	2.6	70	0.002
	50	IT-2	2.6	80	<0.001
	50	IT-6	1.8	0	
LPS	0	IT-2	2.9	0	
	0.01	IT-2	2.9	30	0.105
	0.10	IT-2	2.9	100	<0.001
	0.10	IT-6	1.6	0	
O side chains	0	IT-2	2.6	0	
	10	IT-2	2.6	0	
	100	IT-2	2.6	0	
	500	IT-2	2.6	0	
A-LPS	0	IT-2	2.9	0	
	0.10	IT-2	2.9	10	0.500
	1.0	IT-2	2.9	70	0.002
	10.0	IT-2	2.9	60	0.005
Alkali-treated PS	500	IT-2	2.9	0	

[a] LD_{100}, 100% lethal dose. The 100% lethal doses for *P. aeruginosa* IT-2 and IT-6 were 10^8 and 10^7 cells per mouse, respectively.

[b] Ten mice were used in each experiment.

[c] *P* values were calculated by the Fisher exact test.

TABLE 6. *Enhancement of nonspecific resistance to live cell challenge 24 h after injection of PS and LPS*

Immunogen	Amt (μg)	Challenge dose (no. of cells)[a]	% Protected[b]
PS	1	2.0×10^8	0
	100	2.0×10^8	0
	500	2.0×10^8	0
LPS	0.01	2.0×10^8	10
	1.0	2.0×10^8	30
	10.0	2.0×10^8	20
Saline		2.0×10^8	0

[a] Mice were challenged with live *P. aeruginosa* IT-2 cells; the 100% lethal dose was 10^8 cells per mouse.
[b] Ten mice were used in each experiment.

to these antigens to mice; 0.1-ml volumes of antiserum to *P. aeruginosa* IT-2 PS, LPS, or A-LPS given intraperitoneally to mice 3 h before challenge with live homologous *P. aeruginosa* IT-2 cells provided protection for 90 to 100% of the mice (Table 7). Adsorption of antiserum to *P. aeruginosa* IT-2 PS with *P. aeruginosa* IT-2 PS or LPS abrogated the protective effect of the serum (Table 7). Adsorption of *P. aeruginosa* IT-2 LPS serum with *P. aeruginosa* IT-2 PS did not remove the protective effect of this serum; however, a second adsorption of this serum with *P. aeruginosa* IT-2 A-LPS did remove the passive protective effect. Adsorption of antiserum to A-LPS with PS had no effect on the passive protective capacity of this serum (Table 7). Adsorption of antiserum to A-LPS with intact *P. aeruginosa* IT-2 LPS or A-LPS removed the passive protection conferred by this serum.

DISCUSSION

The use of our previously described procedure for isolating the high-molecular-weight PS antigen produced by *P. aeruginosa* IT-1 was extended to *P. aeruginosa* IT-2. Like *P. aeruginosa* IT-1 PS, *P. aeruginosa* IT-2 PS is composed primarily of carbohydrate and contains low levels of contaminating nucleic acids and protein; this compound has a chemically complex monosaccharide composition, containing sugars (notably galactose and arabinose) which are not found in the corresponding LPS, and it gives a reaction of identity in immunodiffusion gels with the O-side chain polysaccharide from the homologous *P. aeruginosa* IT-2 LPS. *P. aeruginosa* IT-2 PS has a molecular weight of 2.25×10^5, suggesting that it is large enough to be immunogenic in humans. *P. aeruginosa* IT-2 PS also has no detectable pyrogenic activity in rabbits at a dose of 25 μg/kg and no toxicity in mice at a dose 400 mg/kg, suggesting that it is safe for human use.

The successful isolation of PS from *P. aeruginosa* IT-2 has helped define the nature of the PS antigens in *P. aeruginosa*. In the PS preparation procedure we used hydrolysis of crude preparations in 1% acetic acid to cleave LPS into its lipid A and polysaccharide components. In immunodiffusion gels PS from *P. aeruginosa* IT-1 and PS from *P. aeruginosa* IT-2 both gave reactions of identity with their homologous O-side chain polysaccharides. Thus, we thought that PS might be a mixture of LPS O side chains and other molecules containing carbohydrates. Although PS appeared to be homogeneous in gel filtration and immunoelectrophoresis analyses, neither of these techniques insured that the PS preparations were homogeneous chemical entities. However, both *P. aeruginosa* IT-1 and *P. aeruginosa* IT-2 cultures yielded PS antigens which contained galactose and arabinose, had molecular weights five to eight times larger than the molecular weights of the O side chains, and were effective in eliciting protective immunity in mice. Furthermore, like *P. aeruginosa* IT-1 PS (7), *P. aeruginosa* IT-2 PS did not disaggregate when it was chromatographed in the presence of 3% sodium deoxycholate; this indicated that these antigens are not molecular aggregates of smaller monomers. These data support the hypothesis that the preparation of PS antigens by our procedure yields a high-molecular-weight, immunogenic polysaccharide material containing an immunological determinant in common with the LPS O side chain.

TABLE 7. *Protection of mice after passive transfer of rabbit antisera raised against PS, LPS, and A-LPS and absorption of these sera with PS, LPS, and A-LPS from P. aeruginosa IT-2*

Antiserum to:	Adsorbed with:	Challenge		No. of survivors/total no.	% Protected
		Strain	No. of LD_{100}[a]		
PS		IT-2	2.7	10/10	100
	PS	IT-2	2.7	1/10	10
	LPS	IT-2	2.7	0/10	0
	A-LPS	IT-2	2.7	2/10	20
		IT-6	2.1	0/10	0
LPS		IT-2	2.7	10/10	100
	PS	IT-2	2.7	8/10	80
	LPS	IT-2	2.7	0/10	0
	PS + A-LPS	IT-2	2.7	1/10	10
		IT-6	2.1	0/10	0
A-LPS		IT-2	2.7	9/10	90
	PS	IT-2	2.7	10/10	100
	LPS	IT-2	2.7	0/10	0
	A-LPS	IT-2	2.7	1/10	10
		IT-6	2.1	0/10	0

[a] LD_{100}, 100% lethal dose. See Table 5 for 100% lethal dose values.

The vaccine potential of PS for humans is suggested by the data of Pollack and Young (8), Young et al. (11), and Crowder et al. (2). Pollack and Young (8) found a high level of survival from *P. aeruginosa* bacteremia in patients who had high preexisting LPS antibody titers. Young et al. (11) and Crowder et al. (2) showed that persons surviving *P. aeruginosa* bacteremias and other deep infections developed LPS serotype-specific antibodies. These data indicate that human immunity to *P. aeruginosa* is primarily LPS serotype specific. The protection which we observed in mice based on active and passive immunization does not necessarily reflect the parameters of human susceptibility and immunity to *P. aeruginosa*. However, the presence of the same type-specific serological determinant on LPS O side chains and PS suggests that PS may induce an appropriate type-specific immune response in humans. A preliminary evaluation of a *P. aeruginosa* IT-1 PS vaccine in healthy volunteers has indicated that this vaccine is effective in inducing high-titer serotype-specific opsonic antibody (Pier, Abstr. Clin. Res., p. 576A, 1981).

Further evaluations of relevant serological determinants on *P. aeruginosa* antigens showed that like *P. aeruginosa* IT-1 LPS, *P. aeruginosa* IT-2 LPS contains an immunological determinant that is not shared with PS and is detected due to its stability to dilute alkali treatment. The shared determinants of the *P. aeruginosa* IT-2 PS and LPS were serologically active on the LPS after alkali treatment, but were destroyed on *P. aeruginosa* IT-2 PS. *P. aeruginosa* IT-2 PS required a hotter and longer alkali treatment than *P. aeruginosa* IT-1 PS before its serological activity was destroyed. However, this treatment did destroy the immunogenicity of the PS shared determinants on the LPS. The reactivities of rabbit antisera to A-LPS and A-LPS-coated SRBC were not inhibited by PS, but these reactivities were inhibited by both intact LPS and A-LPS. Furthermore, absorption of antiserum to A-LPS with PS had no effect on the passive protective effect of the antiserum, whereas adsorption of this serum with LPS or A-LPS removed the passive protective effect. Since PS and the O side chains of the LPS from *P. aeruginosa* IT-1 and *P. aeruginosa* IT-2 lack heptose and 2-keto-3-deoxyoctulosonic acid, whereas these sugars are found in intact LPS and A-LPS (unpublished data), it is tempting to speculate that the alkali-stable determinant is associated with a core-like polysaccharide region of the *P. aeruginosa* LPS analogous to the region found in *Salmonella* LPS (5). Some workers have suggested that *P. aeruginosa* LPS has a core unit (1) and that it may share serological activity with members of the *Enterobacteriaceae* (12), but definitive serological and structural proof is lacking.

These observations on the isolation and characterization of a high-molecular-weight PS antigen from *P. aeruginosa* IT-2 extend our original work on *P. aeruginosa* IT-1 PS to another strain of *P. aeruginosa*. Our data support the general applicability of the previously described method (7) for obtaining high-molecular-weight, immunogenic, nontoxic PS antigens from strains of *P. aeruginosa*. Furthermore, our data also indicate that the production of PS antigens that are serologically identical but chemically and physically different from the O-side chain polysaccharide of the LPS may be a general phenomenon in *P. aeruginosa* strains. Other data (Pier, submitted for publication) have indicated serological cross-reactivity of the PS from *P. aeruginosa* IT-1 and IT-2, as well as cross-protective efficacy in mouse immunization challenge experiments. Finally, the presence of an alkali-stable, immunogenic determinant on the LPS that is not shared with PS or the O side chain of the LPS may indicate a core-like structure for LPS in *P. aeruginosa*; antibody to this LPS determinant protects mice.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service grant AI-15835 from the National Institutes of Health and by a grant from the Cystic Fibrosis Foundation.

We thank Diane Thomas and Robert Gillette for technical assistance and Loreen Carr and Barbara Chamberlain for assistance in the preparation of the manuscript.

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