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Further Purification and Characterization of High-Molecular-Weight Polysaccharide from *Pseudomonas aeruginosa*

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Previously published reports on high-molecular-weight polysaccharides from immunotype 1 and 2 of *Pseudomonas aeruginosa* indicated the presence of high levels of mannose in these preparations. This mannose has been found to be due to the presence of a yeastlike mannan in high-molecular-weight polysaccharide preparations. The source of the mannan was found to be the tryptic soy broth used to grow the bacteria. Mannan could be removed from the polysaccharide preparations by chromatography over columns of concanavalin A-Sepharose. The resulting polysaccharides had the same serological reactivity against rabbit antisera and the same immunogenic properties in mice as did the mannan-containing polysaccharides. Comparison of mannan-depleted polysaccharide with preparations of high-molecular-weight polysaccharide obtained from either ultrafiltered tryptic soy broth or a chemically defined medium showed that these polysaccharides were immunologically and chemically similar. Human immune responses to mannan-depleted polysaccharide from the immunotype 1 strain of *P. aeruginosa* were comparable with those previously seen in humans receiving mannan-containing polysaccharides. Thus, we found that *P. aeruginosa* high-molecular-weight polysaccharides prepared in either tryptic soy broth and then subjected to concanavalin A-Sepharose chromatography, ultrafiltered tryptic soy broth, or a chemically defined medium were immunologically and chemically comparable.

The safety and immunogenicity of high-molecular-weight polysaccharide (PS) from immunotype 1 *Pseudomonas aeruginosa* has been established in humans (9). The basis for this trial was the finding that high-molecular-weight PS could be isolated from culture supernates of *P. aeruginosa* and that this material was immunogenic in animals (12, 13) and protected mice from intraperitoneal challenge with live organisms (11, 12). High-molecular-weight PS appears to be an immunogenic, nontoxic form of the lipopolysaccharide (LPS) "O" side chain. A hallmark that distinguishes PS from LPS and LPS O side chains has been the chemical composition of PS. One of these chemical distinctions has been the presence of high levels of mannose in PS preparations, levels not seen in LPS (2, 16). Investigations into the structure of the high-molecular-weight PS from immunotype 1 *P. aeruginosa* led to the identification of a yeastlike mannan in these preparations. This mannan was found by methylation analysis (1, 5) to be a highly branched polymer of alpha (1-2)- and alpha (1,6)-linked mannopyranosyl residues. Exploration of the role of the mannan in the serological and immunogenic activity of high-molecular-weight PS from both immunotype 1 and 2 *P. aeruginosa* led to the finding that mannan was separable from the serologically active and immunogenic components. This report compares the immunochemical properties of immunotype 1 and 2 PS lacking mannan with the previously described properties of PS containing mannan from these strains of *P. aeruginosa*. In addition, we examined the human immune response to mannan-depleted PS from the immunotype 1 strain of *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria. Immunotype 1 and 2 strains of *P. aeruginosa*, originally obtained from M. Fisher, Detroit, Mich., were used throughout the study.

Antigens. For immunochemical analyses and animal studies, high-molecular-weight PS from immunotype 1 and 2 strains of *P. aeruginosa* were prepared from three different media as previously described (9, 12, 13). The media employed were unmodified tryptic soy broth (TSB) with 3% sodium acetate, ultrafiltered tryptic soy broth (UF-TSB) with 3% sodium acetate, prepared by passage of TSB through 10,000-molecular-weight cutoff membranes, and the chemically de-

fined medium (CDM) described by Terleckyj et al. (15). To remove the medium mannan component, materials obtained from TSB cultures were further purified by chromatography over columns of concanavalin A covalently linked to Sepharose (ConA-Sepharose) (Pharmacia Fine Chemicals, Piscataway, N.J.) in buffer containing 0.1 M acetate, 0.15 M NaCl, 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$, and 0.1% Merthiolate, pH 6.5.

Human immunizations. High-molecular-weight PS from immunotype 1 *P. aeruginosa* was prepared from culture supernates of bacteria grown in unmodified TSB then depleted of mannan by ConA-Sepharose chromatography. Two separate lots (number 3 and 4) were made, and then each was bottled by the Massachussetts State Biological Laboratories for injection into humans as previously described (9). These materials were tested for safety and toxicity in laboratory animals as outlined by the Food and Drug Administration regulations (Title 21, Section 610.11). Informed consent was obtained from all volunteers before the administration of the vaccine. Approval for this study was obtained from the Committee for the Protection of Human Subjects from Research Risks, Brigham and Women's Hospital.

Mitogenesis assays. Mitogenic activity of PS preparations on mouse splenocytes was assayed as previously described (10). Purified ConA for use in these experiments was obtained from Pharmacia Fine Chemicals. Stimulation indices were calculated by dividing the counts per minute of [^{3}H]thymidine incorporated into stimulated cultures by the counts per minute incorporated into unstimulated cultures.

Serological assays. Antigen preparations were tested for serological identity in Ouchterlony immunodiffusion gels, as previously described (12, 13). These gels were also used to assess the reactivity of antigen preparations with ConA. Solutions of ConA (5 mg/ml) were substituted for serum when indicated. Analysis of antibody binding levels in mouse and human sera was accomplished using a radioactive antigen binding assay as previously described (8, 9). Statistical comparison of antibody titers after immunization of animals and humans was done with a *t* test. Testing of the opsonic activity in human serum, pre- and post-immunization, was done using an opsonophagocytic assay that was previously described (9). Determination of antibodies to *Candida albicans* mannan was kindly performed by Michael Lew using an enzyme-linked immunosorbent assay (2).

Mouse immunizations. C3H/ANF mice were immunized by injection with 10 μg of antigen in 0.5 ml of saline. Mice were bled before and 7 days after the immunization from the retroorbital plexus while under ether anesthesia. These sera were then tested in the radioactive antigen binding assay. Mouse data are expressed as increases in the antigen binding capacity (counts per minute bound × specific activity of antigen) per 100 μl of serum minus the preimmune antigen binding capacity.

Chemical components. Determination of the protein level in PS preparations was done by the method of Lowry et al. (6). Determination of nucleic acids was done as previously described (12, 13). Determination of the 2-keto-3-deoxyoctulosonic acid concentrations was done by the method of Osborn (7). Quantitative analysis of the monosaccharide constituents in PS preparations was done using the alditol acetate method of Sawardeker et al. (14). Analysis was carried out on a column (2 mm by 3 ft [ca. 91 cm]) of SP 2340 (Supelco, Inc., Bellefonte, Pa.) in a Packard 421 gas-liquid chromatograph. Analysis conditions were as follows: nitrogen carrier gas flow, 20 ml/min; initial temperature, 160°C held for 3 min, temperature rise of 5°C/min to a final temperature of 255°C; injector temperature, 210°C, detector temperature, 260°C. Sugars were identified based on their retention times (as compared with authentic standards) and cochromatography with known standards, except for the dideoxyhexoseamines. These were identified based on the retention times of known dideoxyhexoseamines found in the *P. aeruginosa* LPS (1) and the capsular PS of *Bacteroides fragilis* 23745 (3). The former materials were prepared by us as previously described (12, 13), and the latter material was kindly supplied by Dennis L. Kasper, Channing Laboratory. Quantitation was performed using a Hewlett Packard 3388 integrator. The total amounts of each sugar were calculated by the integrator, which had been previously programmed with the response factors to individual monosaccharides by using known amounts of authentic standards. The total carbohydrate content of each antigen preparation was determined by dividing the sum of the total amount of the sugars identified by the total amount of antigen injected into the gas-liquid chromatograph. The latter amount was determined by use of an internal standard of inositol added to the antigen preparation before the preparation of the alditol acetate derivative.

RESULTS

Comparison of mannan-containing and mannan-depleted PS. After identification of a mannan component in high-molecular-weight PS preparations, we prepared mannan-depleted PS for immunochemical analyses from *P. aeruginosa* immunotype 1 and 2 and compared the serological properties of these two antigens. Figure 1 shows the immunodiffusion reaction of immunotype 1 and 2 PS before and after chromatography on ConA-Sepharose against rabbit antisera raised to whole organisms and against solutions of ConA. This chromatography step separated the mannan component from the serologically active component in high-molecular-weight PS preparations. As a test for the potential source of the mannan in the PS preparations, we looked at the reactivity of unmodified TSB media with solutions of ConA. We found that TSB contained a material giving a precipitin line in gel diffusion against solutions of ConA (Fig. 1).

Table 1 compares the immunogenicity of 10 μg of the mannan-containing and mannan-depleted high-molecular-weight PS preparations in inbred C3H/ANF mice. Both mannan-containing and mannan-depleted immunotype 1 and 2 PS induce significant ($P < 0.01$) increases in binding antibody against homologous PS. The mannan-depleted immunotype 1 and 2 PS were also cross-immunogenic in outbred mice (not

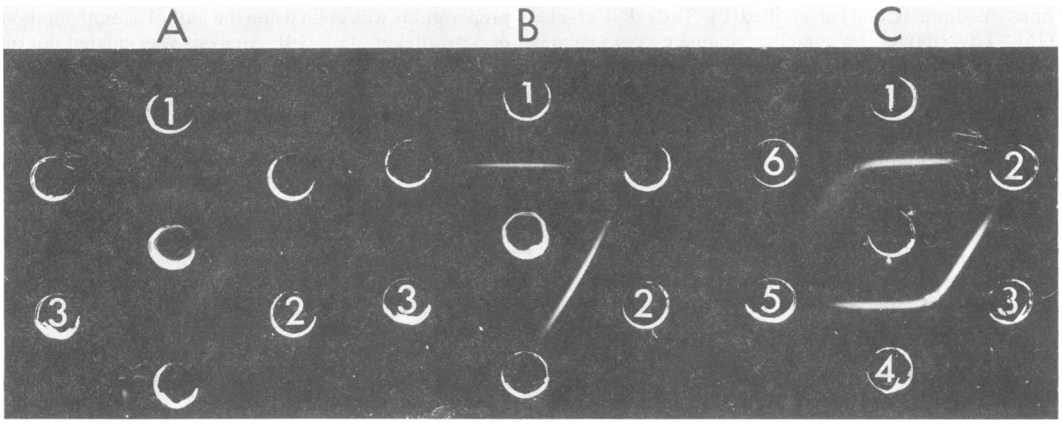

FIG. 1. Immunodiffusion analysis of the reaction of antisera raised to whole immunotype 1 and 2 *P. aeruginosa* organisms and solutions of ConA against preparations of high-molecular-weight PS either containing mannan or depleted of mannan by ConA-Sepharose chromatography. (A) Center well, antiserum to immunotype 1 *P. aeruginosa*, 20 μl; outer wells—(1) mannan-containing immunotype 1 PS, 1 mg/ml; (2) immunotype 1 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan (eluted from ConA-Sepharose column after binding), 1 mg/ml. (B) Center well, antiserum to immunotype 2 *P. aeruginosa*, 20 μl; outer wells—(1) mannan-containing immunotype 2 PS, 1 mg/ml; (2) immunotype 2 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan, 1 mg/ml. (C) Center well, ConA, 5 mg/ml, 20 μl; outer wells—(1) immunotype 1 PS before ConA-Sepharose chromatography, 1 mg/ml; (2) immunotype 1 PS after ConA-Sepharose chromatography, 1 mg/ml; (3) purified mannan, 1 mg/ml; (4) immunotype 2 PS before ConA-Sepharose chromatography, 1 mg/ml; (5) immunotype 2 PS after ConA-Sepharose chromatography, 1 mg/ml; (6) tryptic soy broth, 20 μl.

shown), as has been previously reported for the mannan-containing PS (8).

We next prepared high-molecular-weight PS from UF-TSB and from the CDM of Terleckyj et al. (15). The yield of PS from UF-TSB was quite low (0.5 mg/liter) compared with the yield from regular TSB after mannan removal (5 mg/liter) or from CDM (4 mg/liter), making the use of UF-TSB for routine production of PS impractical. High-molecular-weight PS obtained from both UF-TSB and CDM gave reactions of identity in immunodiffusion gels with both mannan-containing and mannan-depleted PS preparations. These materials were also comparably immunogenic in mice (Table 1). The PS preparation from the CDM did not react with ConA in immunodiffusion gels, but PS from UF-TSB had a faint line versus solutions of ConA (not shown). This reactivity could be removed by ConA-Sepharose chromatography and indicates that ultrafiltration of the TSB did not successfully remove all of the media mannan component. However, these results do indicate that preparation of high-molecular-weight PS from either UF-TSB or CDM resulted in an antigen that was immunologically similar to previously described high-molecular-weight PS preparations.

Chemical composition and monosaccharide constituents of mannan-containing and mannan-depleted PS. Table 2 shows the chemical composition of high-molecular-weight PS obtained from the various media described above. All of these PS preparations contained similar levels of contaminating protein and nucleic acids, and all were negative for 2-keto-3-deoxyoctulosonic acid in the thiobarbituric acid assay. Chromatography of mannan-containing PS over ConA-Sepharose columns did not affect the protein content, indicating that ConA did not leach from these affinity gels into the PS preparations. The total amount of each preparation identifiable as carbohydrate by quantitative gas-liquid chromatography was similar for all preparations.

Table 3 shows the monosaccharide constituents of PS obtained from immunotype 1 and 2 *P. aeruginosa*. These materials were obtained from TSB before and after ConA-Sepharose chromatography, from UF-TSB, or from the CDM. As expected, PS obtained from TSB before ConA-Sepharose chromatography had a high level of mannose. PS from UF-TSB had a reduced content of mannose compared with PS isolated from TSB and not chromatographed. (The mannose content in PS from UF-TSB could be reduced even further by ConA-Sepharose chromatography.) The PS preparations obtained from the CDM had a similar quantitative and qualitative monosaccharide composition to mannan-depleted PS. This fact indicates that ConA-Sepharose chromatography yielded PS that was composed principally of bacterial products. Interestingly, all preparations of high-molecular-

TABLE 1. Immunogenicity of 10 μg of various high-molecular-weight PS preparations in C3H/ANF mice

Immunogen	Prepared in/by:	Increase over preimmune serum in antigen binding capacity[a] vs homologous PS
Immunotype 1 PS	TSB	55 ± 39[b]
	After ConA-Sepharose	66 ± 30
	UF-TSB	51 ± 20
	CDM	72 ± 22
Saline		0
Immunotype 2 PS	TSB	150 ± 22
	After ConA-Sepharose	140 ± 28
	UF-TSB	147 ± 33
	CDM	136 ± 18
Saline		0

[a] Antigen binding capacity is counts per minute of antigen bound by 100 μl of serum × the specific activity of the radiolabeled antigen.

[b] Average of four mice ± standard deviation.

weight PS studied here contained high levels of galactose and arabinose, two monosaccharides not found in *P. aeruginosa* LPS (2, 16).

Safety and immunogenicity of mannan-depleted immunotype 1 PS in humans. The safety and immunogenicity of immunotype 1 PS depleted of mannan by ConA-Sepharose chromatography was assessed in eight humans. Toxicity testing in mice and guinea pigs and pyrogenicity testing in rabbits were performed by the Massachusetts State Biological Laboratories on two lots (3 and 4) of mannan-depleted immunotype 1 PS, as described previously (9). Before administration of this material to humans, we tested the PS for mitogenicity on murine splenocytes. This was to determine whether residual ConA was present in the vaccine. Table 4 shows that mannan-depleted lots 3 and 4 both had less mitogenic activity than mannan-containing immunotype 1 PS. This suggests that the mannan may have some mitogenic properties. At the highest doses of mannan-depleted PS tested (500 μg/ml) there was little to no mitogenic response. ConA was, of course, a potent mitogen for murine spleen cells, and addition of high levels (500×) of PS to mitogenic doses of ConA did not affect the mitogenic properties of ConA. Thus, no detectable ConA could be found in the PS preparations by the murine splenocyte mitogenesis assay.

Five humans received 100 μg of lot 3 immunotype 1 PS, and three received 100 μg of lot 4. The geometric mean antibody levels preimmunization and at 14 and 28 days post-immunization are shown in Table 5. A significant ($P < 0.001$) increase in binding antibody was observed in vaccinates at both 14 and 28 days post-immunization. No reaction to the vaccine up to 72 h post-immunization was noted in any vaccinate either locally or systemically, except a slightly sore and tender arm at the injection site in one of eight vaccinates, confirming our previous observation (9) that reactions are unusual with *P. aeruginosa* PS vaccines. Increases in opsonic titers of fourfold or greater were observed for seven of the eight vaccinates (Table 5). Thus, the mannan-depleted high-molecular-weight PS possessed similar immunogenic properties in humans as had previously described PS preparations. In addition, this vaccine elicited only a mild local reaction in one of eight vaccinates.

Response of humans to *C. albicans* mannan. Because 42 persons had been previously immunized with immunotype 1 PS containing mannan (9), we selected 10 sera from persons given this vaccine and tested their antibody responses to *C. albicans* mannan, as described previously (4). This mannan is structurally identical to the mannan found in our PS preparations. No increase in enzyme-linked immunosorbent assay binding titers was seen in the pre- and post-vaccination sera of persons receiving mannan-containing PS vaccines, indicating that the mannan component was not immunogenic in these vaccinates.

DISCUSSION

The identification of a mannan component, likely from the medium, in high-molecular-

TABLE 2. Chemical composition of various high-molecular-weight PS preparations

Preparation	% [a]			
	Protein	Nucleic acid	KDO[b]	Carbohydrate
Immunotype 1 PS from:				
TSB	2.7	0.7	ND[b]	73
After ConA-Sepharose	1.8	0.6	ND	71
UF-TSB	0.1	0.3	ND	82
CDM	0.4	0.7	ND	78
Immunotype 2 PS from:				
TSB	3.7	0.6	ND	75
After ConA-Sepharose	3.4	0.4	ND	73
UF-TSB	2.0	0.8	ND	77
CDM	1.6	0.9	ND	79

[a] Percentage is calculated as total amount of material detected divided by total amount of material measured. Unaccounted for material is moisture (13).

[b] KDO, 2-Keto-3-deoxyoctulosonic acid; ND, none detected.

TABLE 3. Monosaccharide constituents of various high-molecular-weight PS preparations

Preparation	Monosaccharide[a] (% total identified)						
	Rham	Ara	Xyl	Man	Gal	Glu	Dideoxy[b]
Immunotype 1 PS from:							
TSB	3.2	5.8	ND[c]	54.4	17.6	7.2	ND
After ConA-Sepharose	24.5	24.0	3.1	ND	30.0	9.0	11.0
UF-TSB	23.2	20.1	4.5	tr	30.9	17.0	7.3
CDM	22.4	20.9	tr	ND	31.1	15.4	10.2
Immunotype 2 PS from:							
TSB	4.8	5.0	7.3	45.1	22.5	10.7	3.7
After ConA-Sepharose	1.2	7.2	tr	ND	45.6	26.1	20.0
UF-TSB	2.8	8.2	2.7	tr	40.4	22.0	24.0
CDM	2.4	7.9	2.1	ND	41.2	23.1	23.3

[a] Rham, rhammose; ara, arabinose; xyl, xylose; man, mannose; gal, galactose; glu, glucose; dideoxy, dideoxyhexoseamine.

[b] Amount of dideoxyhexoseamine calculated from area of glucoseaminitol hexaacetate standard. The lack of a dideoxyhexoseamine standard prohibits determining an accurate amount.

[c] ND, None detected.

weight PS preparations from *P. aeruginosa* necessitated a determination of the role of this material in the serological activity and immunogenicity of PS. We found that removal of mannan by ConA-Sepharose chromatography resulted in a material that contained similar serological activity and immunogenicity as those of mannan-containing PS. The mannan-depleted PS from immunotype 1 was also immunogenic in humans, inducing significant increases in binding and opsonic antibody after immunization with a single 100-μg dose. There were no serious untoward reactions to the vaccine among eight vaccinates. Preparation of high-molecular-weight PS in a CDM resulted in a material immunologically identical and chemically similar to PS prepared in TSB and depleted of mannan by ConA-Sepharose chromatography. Thus, these two methods likely yield a PS preparation consisting solely of bacterial products.

TABLE 4. Mitogenic stimulation indices of immunotype 1 *P. aeruginosa* PS human vaccines on murine splenocytes

Immunotype 1 vaccine	Dose (μg/ml)	Stimulation index
Lot 3	500	0.7
	250	0.5
	100	0.8
	50	0.7
Lot 4	500	1.5
	250	0.9
	100	1.2
	50	1.3
Lot 3 before mannan depletion	500	3.7
	250	3.9
	100	2.0
	50	1.7
ConA	1	4.9
Plus lot 3[a]	500	4.6
Plus lot 4[a]	500	5.4

[a] ConA at 1 μg/ml plus indicated PS vaccine at 500 μg/ml.

The use of ultrafiltered or dialyzed media for the production of bacterial vaccines is generally employed to avoid media contamination of the final product. Out initial attempts to produce PS in UF-TSB, before preparing any material for human use, showed that bacterial growth and antigen production in UF-TSB were severely reduced. Also, before human studies, we isolated high-molecular-weight components of TSB and could not identify any carbohydrate in this fraction. The presence of mannan from the medium in our high-molecular-weight PS preparations was, therefore, unexpected. However, because PS are prepared from large culture volumes (20 to 30 liters), low levels of mannan (<1%) in TSB could go undetected but end up in high proportion in the final PS product, if the mannan copurifies with the PS, which appears to be the case. Although UF-TSB is a poor medium for PS production, the CDM seems to be adequate for antigen production and is the preferable medium for producing high-molecular-weight PS. Recent attempts to produce PS from both ultrafiltered Todd-Hewitt broth or ultrafiltered Columbia broth indicate that these media may be superior to the CDM because of greater yields of material.

Particularly important is the finding of chemical similarity among PS depleted of mannan by ConA-Sepharose chromatography, PS obtained from UF-TSB, and PS obtained from the CDM. Thus, the components of PS in these preparations are likely to be bacterial products and not media or other contaminants. High-molecular-

TABLE 5. Antibody levels and opsonic titers in sera from eight human vaccinates before and after a 100-μg dose of immunotype 1 PS[a]

Serum sample	Antibody level[b] (μg/ml)	Opsonic titer[c]
Preimmune	6.02 ± 1.5 (3.9–14.1)	2.6 ± 0.8 (2–8)
Day 14	39.9 ± 2.8[d] (8.9–158.9)	26.3 ± 2.4[d] (8–128)
Day 28	48.8 ± 3.0[d] (11.5–180.7)	26.3 ± 3.3[d] (8–128)

[a] Five vaccinates received lot 3 and three received lot 4.

[b] Determined in the radioactive antigen binding assay as described previously (9). Reported as $\log_{10}$ geometric mean ± standard deviation. Range is shown within parentheses.

[c] The titer is the reciprocal of the serum dilution showing ≥90% kill of the input inoculum as described previously (9). Results are expressed as $\log_2$ geometric mean ± standard deviation. Range is shown within parentheses.

[d] $P < 0.001$.

weight PS obtained from TSB after a ConA-Sepharose chromatography step, from UF-TSB, or from the CDM, all contained the monosaccharides galactose and arabinose. These two monosaccharides are absent from LPS (2, 16). Thus, high-molecular-weight PS remains chemically distinct from both intact LPS and O side chains by virtue of its monosaccharide constituents. Since galactose and arabinose appeared in all PS preparations made here, they must be bacterial components of PS preparation. Whether galactose and arabinose are covalently linked to the monosaccharides shared by PS and LPS O side chains remains to be seen. Attempts to separate the galactose and arabinose components from the serologically active monosaccharides that are shared between LPS and PS thus far have been unsuccessful. However, it is possible that an arabinogalactan-like molecule is present in the PS preparations, along with a high-molecular-weight form of the LPS O side chain. Structural studies are now under way to determine whether this is the case. Currently, we are able to prepare nontoxic PS that is immunologically active in humans, giving rise to binding and opsonic antibodies directed at the serotype determinant located on the O polysaccharide side chain of the LPS.

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