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Immunogenic and Antigenic Properties of a Heptavalent High-Molecular-Weight O-Polysaccharide Vaccine Derived from *Pseudomonas aeruginosa*

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We investigated the chemical and immunologic properties of a heptavalent vaccine composed of high-molecular-weight polymers of the lipopolysaccharide (LPS) O polysaccharides representative of the most common clinical isolates of *Pseudomonas aeruginosa*. We also evaluated the serum antibody response to nonvaccine strains of *P. aeruginosa*, including strains expressing structural variants (subtype strains) of the O side chain of the vaccine strains. The polyvalent vaccine, prepared under conditions suitable for human use, contained low levels of contaminants and passed preclinical safety and toxicity tests required for human use. Chemical analyses indicated that individual polysaccharides were composed of both O-side chain and core sugars. Following immunization of C3H/HeN mice and New Zealand White rabbits, antibody titers against vaccine components increased between 32- and 200-fold. Antibodies reactive with LPS isolated from smooth and rough nonvaccine strains were also elicited. Analysis of the opsonic activity against the known LPS subtype variants of the vaccine strains revealed a variable pattern of killing, which ranged from opsonic killing of ≥69% of bacterial cells representing all subtype variants within a serogroup to opsonization of only a minority of the subtype variant strains. Mouse and rabbit immune sera showed different patterns of opsonic activity against subtype strains, indicating that different epitopes on these antigens are immunodominant in the representatives of these two animal species tested. The polyvalent vaccine was effective at eliciting antibodies to vaccine components in mice and rabbits, but it remains to be determined if the current heptavalent formulation contains sufficient components to provoke human antibodies reactive with a majority of clinical strains of *P. aeruginosa*.

Acute nosocomial infections due to *Pseudomonas aeruginosa* remain a persistent problem for a variety of hospitalized patients. According to data from the National Nosocomial Infections Surveillance System, between October 1986 and December 1990, *P. aeruginosa* was the fourth most common cause of nosocomial infections overall, the second most common pathogen isolated from the lower respiratory tract, and the third most common pathogen isolated from the urinary tract (12). In the intensive care unit, *P. aeruginosa* was the most common isolate (12.4%), principally from lower respiratory tract infections (12). In spite of therapeutic advances, mortality rates from *P. aeruginosa* bacteremia and pneumonia remain unchanged over the past two decades (25). Clearly, new strategies are needed to reduce the complications from *P. aeruginosa* infection.

A promising tool involves immunotherapy against *P. aeruginosa* (24, 25). The antigens expressed on the O side chains of the cell surface lipopolysaccharide (LPS) are probably the most effective targets for protective antibodies (3, 4, 33, 42, 49). In addition, only about 10 different serogroups of *P. aeruginosa* are isolated from the vast majority of clinical sources (40), an indication that limited serologic diversity would make it feasible to manufacture a comprehensive vaccine with a reasonable number of components. A number of studies over the past 25 years have looked at the vaccine potential of purified LPS itself, with some suggestion of efficacy engendered by active vaccination or passive therapy with immune serum globulin derived from immunized plasma donors (1, 13, 14, 23). However, most of these preparations are no longer being developed for clinical use. Less toxic derivatives of the O side chain have also been produced and include O side chains conjugated to *P. aeruginosa* exotoxin A (7, 8) and high-molecular-weight polysaccharide versions of O side chains (27–29, 38). The O-side-chain conjugate vaccines have elicited antibodies in a number of volunteer and patient groups (5, 6, 10, 46) and have been used to immunize donors from whose plasma a hyperimmune intravenous immunoglobulin G (IgG) was manufactured (6).

High-molecular-weight polysaccharides are large (>150 kDa), immunogenic preparations of *P. aeruginosa* O side chains devoid of 2-keto-3-deoxyoctulosonic acid (KDO) and lipids. Monovalent preparations of several individual antigens have demonstrated excellent immunogenicity in humans (26, 30), and elevated titers have been observed in some vaccinates for up to 10 years (unpublished observation). Antibodies elicited by these vaccines promote opsonic killing of *P. aeruginosa* in vitro and protect animals against *P. aeruginosa* infections arising from a variety of immunocompromising conditions (22, 35, 36, 39, 41, 43). However, these challenge studies have all been carried out with the bacterial strains that were used to produce the vaccines, thereby raising questions about whether the antibodies induced by these vaccines are specific for epitopes shared by all members of a *P. aeruginosa* sero-

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group or whether these antibodies show a narrower specificity for epitopes associated with O-side-chain structural variants, referred to as subtype epitopes, that can be quite extensive among strains from the same serogroup.

Strains of *P. aeruginosa* in a single serogroup share group-specific structural and serologic epitopes (16, 17, 21), while subtype strains in a given serogroup express structural variations in the O polysaccharide that often, but not always, will elicit subtype-specific antibodies (20, 21, 47). Recent studies have shown that human antibodies elicited by a monovalent high-molecular-weight polysaccharide preparation derived from the Fisher immunotype (IT)-1 variant of the International Antigenic Typing System (IATS) serogroup 06 strains will opsonize all five subtype strains of this serogroup. An important question that is not fully answered is whether a multivalent O-polysaccharide-based vaccine containing only a single representative antigen from each of the commonly occurring serogroup strains of *P. aeruginosa* will elicit broadly reactive, group-specific antibodies or whether these immunogens elicit more narrowly expressed subtype-specific antibodies. Additional questions related to the high-molecular-weight polysaccharide vaccines are whether a multivalent preparation that meets the U.S. Food and Drug Administration (FDA) safety requirements can be manufactured and whether there is an adequate immune response elicited by a product containing multiple high-molecular-weight polysaccharide antigens. In this study, we report the chemical and preclinical immunologic properties of a heptavalent high-molecular-weight polysaccharide vaccine intended for human use and characterize the opsonic activity of mouse and rabbit antibodies against both vaccine strains and related, nonvaccine strains of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. The following *P. aeruginosa* strains were used to prepare high-molecular-weight polysaccharide antigens: Fisher IT-1 (IATS serogroup 06) was ATCC 27312; Fisher IT-2 (IATS serogroup 011), IT-3 (IATS serogroup 02), IT-4 (IATS serogroup 01), IT-5 (IATS serogroup 010), and IT-7 (IATS serogroup 05) strains were clinical isolates whose serogroups were determined as described previously (40); IATS serogroup 03 was ATCC 33350. Subtype antigens expressed by these strains were determined previously by structural analysis (26, 30, 31, 34, 35) or by Y. Knirel, Moscow, Russia. Additional strains used to evaluate immune responses included a clinical isolate of Fisher IT-6 (IATS serogroup 07), ATCC 33351 and 33356 (IATS serogroup 04 and 09 strains, respectively), and the subtype strains of IATS serogroups 02/05, 03, 04, 06, 09, and 010 as defined by Lanyi and Bergan (21) and described in Table 1 of reference 17. These last subtype strains were kindly provided by B. Lanyi, Budapest, Hungary. Two O-side-chain-deficient strains of *P. aeruginosa* were used: AK1401, derived from strain PAO1 (18), was provided by A. Kropinski, Kingston, Ontario, Canada; PAC557, derived from strain PAC1R, was provided by P. Meadow, London, Great Britain (45). Cultures received in the laboratory were inoculated onto tryptic soy agar, and single colony picks were used to inoculate tryptic soy broth containing 10% glycerol. After overnight growth at 37°C, 1-ml aliquots were frozen as stocks and seed cultures. Cultures for antigen preparation and phagocytic assays were derived from these stocks.

LPS preparation. LPS was extracted from the *P. aeruginosa* strains by the phenol-water extraction procedure (48) and then precipitated from the extract solutions by addition of 4 volumes of 95% ethanol. The recovered precipitate was redissolved in phosphate-buffered saline and treated with nuclease and then protease enzymes, ultracentrifuged, and chromatographed over gels of Sepharose CL4B, as described before (11).

Fermentation procedure. Seed cultures of *P. aeruginosa* were provided to the Pennsylvania State University fermentation facility. One loopful of seed culture was inoculated into 1 liter of filter-sterilized (0.45-μm-pore-size membrane filters) modified Mian's medium (pH 7.2) containing 22 g of sodium gluconate, 17 g of L-glutamate, 1 g of sodium phosphate monobasic salt (anhydrous), 3 g of potassium phosphate dibasic salt (trihydrate), and 2.5 g of magnesium sulfate (anhydrous) per liter. This starter culture was incubated at 37°C ± 1°C on an environmental shaker (150 rpm) for 36 to 48 h. Samples from the starters were Gram stained and subcultured for 24 to 48 h to confirm culture identity and clonality; they were then used in immunodiffusion assays to verify the serogroup of the bacteria in the culture. The starter cultures were then used to inoculate 225 liters of filter-sterilized modified Mian's medium in a BioServices 300-liter industrial fermentor. Fermentation conditions were automatically maintained at 37°C ± 1°C, pH 7.0 ± 0.5, and agitation at 100 rpm. The pH was adjusted with 15% (vol/vol) glacial acetic acid and 10% (wt/vol) sodium hydroxide. Air flow and supplemental O_2 were maintained at 80 and 5 liters/min, respectively, and the pressure inside the vessel was maintained at 7 lb/in^2. Fermentation proceeded for 44 to 48 h, after which samples were removed to check the pH and A_{650} and to determine culture identity by plating onto LB agar. For all strains except Fisher IT-1, the pH of the culture was then dropped to 5.0 ± 0.05 by the addition of 15% acetic acid; the culture was heated to 95°C for 17 to 18 h to extract the high-molecular-weight polysaccharide antigen from the cells. The culture was transferred to a sterile feed tank, where it was processed through 0.22-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.) to remove bacterial cells. The filtrate was concentrated to 4 to 8 liters with 30,000-molecular-weight-cutoff ultrafiltration membranes (Millipore) and dialyzed via ultrafiltration (diafiltered) by the addition 300 to 400 liters of sterile deionized water to the initial concentrate and reconcentration to about 6 liters. The concentrate was filter sterilized through 0.45-μm-pore-size membranes, dispensed into sterile Nalgene bottles, and frozen at −20°C for shipment.

Production of high-molecular-weight polysaccharide vaccine antigens. The basic procedure used was from our previously described protocols (34) with some variations. Fermentation concentrates were thawed at 4°C and concentrated to 800 to 1,000 ml on a Pellicon ultrafiltration apparatus (Millipore) with 10,000-molecular-weight-cutoff membranes. A 10% solution of hexadecyldimethyl ammonium bromide (Cetavalon) was added to achieve a final concentration of 1% to precipitate nucleic acids, which were removed by centrifugation (10,000 × *g*, 10 min). The high-molecular-weight polysaccharides in the supernatant were precipitated by the addition of 4 volumes of 95% ethanol, recovered by centrifugation, and redissolved in sterile deionized water. This procedure was repeated twice. At this stage, the preparation obtained from the Fisher IT-1 strain was adjusted to a pH of 5.0 with glacial acetic acid and heated at 95°C for 18 h to separate O side chains from lipid A. The Fisher IT-1 strain is treated at this stage with acid and heat, as opposed to the other strains, to which this step is applied at the end of the fermentation period, because application of acid and heat at the earlier stage with this strain does not sufficiently cleave the O side chains from the lipid A (unpublished observation). After cooling, the precipitated lipid A was removed by centrifugation (5,000 × *g*),

TABLE 1. Characteristics of monovalent components of polyvalent *P. aeruginosa* high-molecular-weight polysaccharide vaccine

Antigen from serogroup	Final yield (mg)	Contaminants (%)			K_{av}[a]
		Protein	Nucleic acid	KDO	
01	458	0.8	0.35	<0.003	0.45
02	210	1.1	0.45	<0.003	0.48
03	68	0.5	0.30	<0.003	0.57
05	377	0.5	0.34	<0.003	0.50
06	166	1.0	0.53	<0.003	0.48
10	141	0.9	0.46	<0.003	0.47
11	370	0.7	0.40	<0.003	0.48

[a] K_{av} represents the fraction of the total volume of the gel (Sepharose CL4B) and buffer in the column (1.6 by 100 cm) that the antigen actually traversed through. The smaller the K_{av}, the larger the molecular size of the antigen.

and the supernatant from this preparation was treated identically to the others, which involved multiple (6 to 12) extractions with chloroform until no precipitate was visible at the chloroform-water interface and two extractions of the aqueous layer with an equal volume of 90% phenol. Except for the high-molecular-weight polysaccharide from the Fisher IT-2 strain, the desired material was recovered from the aqueous layer by the addition of 4 volumes of 95% ethanol. The high-molecular-weight polysaccharide from the IT-2 strain was recovered by alcohol precipitation from the phenol phase, into which it partitions (37). The precipitates were collected by centrifugation (5,000 × *g*) and redissolved in 150 to 200 ml of 0.2 M ammonium carbonate buffer, pH 8.6.

The antigen was applied to a Sephacryl S-300 HR gel (Pharmacia Biotech Inc., Piscataway, N.J.) in a K100/100 column (Pharmacia Biotech). The buffer was 0.2 M ammonium carbonate. Fractions (25 ml) were collected, and the column eluate was monitored at 206 nm (Uvicord S; Pharmacia Biotech). Fractions corresponding to the first absorbance peak were pooled, 4 volumes of 95% ethanol were added, and the precipitated antigen was stored at 4°C. The precipitate was recovered by centrifugation, redissolved in sterile deionized water, dialyzed against sterile deionized water at 4°C, and lyophilized in sterile vessels. When yields of >300 mg were obtained, the material was refractionated on the S-300 HR gel to ensure separation of the high-molecular-weight polysaccharide from smaller-sized polymers.

The high-molecular-weight polysaccharide antigen from the Fisher IT-2 strain was further purified by ion-exchange chromatography with DEAE-Sephadex gels. The antigen was applied in 0.01 M Tris (pH 8.6) and eluted with a gradient of NaCl from 0.05 to 1 M. The column eluate was monitored at 206 nm, and fractions containing the antigen were identified in double immunodiffusion gels with antisera raised to IT-2 *P. aeruginosa* cells. The fractions containing immunologically active antigen were pooled, 4 volumes of 95% ethanol were added, and the precipitated antigen was stored at 4°C. The precipitate was recovered by centrifugation, redissolved in sterile deionized water, dialyzed against sterile deionized water at 4°C, and lyophilized in sterile vessels.

Evaluation of individual high-molecular-weight polysaccharide antigens. After purification, each antigen was tested by double immunodiffusion against specific antisera to confirm serologic identity. The protein, nucleic acid, and KDO contents were determined as described before (34). The relative molecular sizes (K_{av}) were determined on a Sepharose CL4B column, 1.6 by 100 cm, in 0.2 M ammonium carbonate–0.01 M EDTA, pH 8.6. The column void volume was measured with blue dextran 2000, and the bed volume was measured with acetone. The K_{av} was calculated as described previously (2). Each antigen was also evaluated for pyrogenicity in three rabbits, and the minimum concentration that caused gelation of a *Limulus* amebocyte lysate (Cape Cod Associates, Woods Hole, Mass.) was determined. Any antigen that induced pyrogenicity in rabbits (≥0.6°C rise in temperature at any measured hourly point over a 4-h period in an individual animal or an aggregate rise of ≥1.4°C in the maximum temperature of all three animals) was dissolved in sterile deionized water and centrifuged at 100,000 × *g* for 18 h. The supernatant was then lyophilized, and the recovered antigen was retested for pyrogenicity. The individual antigens were also evaluated for immunogenicity by vaccinating groups of five C3H/HeN female mice with doses of 1, 10, or 50 μg of antigen per mouse, given intraperitoneally two times at biweekly intervals. Antibody responses in sera pooled from the individual animals were evaluated by enzyme-linked immunosorbent assay (ELISA), with homologous purified LPS as the coating antigen, and in an opsonic killing assay (26). The monosaccharide constituents of each preparation were analyzed at the Complex Carbohydrate Research Center at the University of Georgia.

Preparation of polyvalent vaccine. Between 59.42 and 68.14 mg of each purified high-molecular-weight polysaccharide antigen was supplied to Bell-More Laboratories, Hampsted, Md., for bottling for human use. Each antigen was dissolved to 10 mg/ml in sterile water, and 5.5 ml (55 mg) of each was combined and added to 461.5 ml of lactose phosphate buffer diluent (2 g of lactose; 1.725 g of sodium phosphate monobasic salt, monohydrate; and 10.05 g of sodium phosphate dibasic salt, heptahydrate, per liter of water), mixed for 1 h at 4°C, and filtered through 0.22-μm-pore-size sterile filters (Millipak 20; Millipore). A bulk sterility test was performed on 10 1-ml aliquots, each inoculated into 10 ml of fluid thioglycolate medium (incubated for 14 days at 35°C). Sterile 6-ml vials were then aseptically filled with 1.1 ml of the bulk preparation (110 μg of each antigen; 770 μg of total antigen per vial); the vials were partially stoppered and frozen in a lyophilizer. The product was lyophilized for 41 h and sealed under vacuum. Fifty samples were used for sterility tests and moisture assays, and the remainder was stored at −20°C. The average moisture content of 10 vials was 1.1% (range, 0.6 to 1.7%). Sterility testing in fluid thioglycolate and tryptic soy broth was satisfactory.

Evaluation of polyvalent antigen. The polyvalent antigen was chemically analyzed for total nucleic acid, protein, and KDO level. It was tested for safety by a commercial laboratory (Microbiological Associates, Inc., Rockville, Md.) that employed the general safety test in mice and guinea pigs (in accordance with FDA regulations [9a]), the rabbit pyrogen test (in accordance with FDA regulations [9b], and the United States Pharmacopeia procedure for the *Limulus* amebocyte lysate assay (in accordance with FDA Good Laboratory Practice Regulations [9c]).

Immunologic assays. Immunogenicity in mice was determined by injecting groups of 20 6- to 8-week-old female C3H/HeN mice (Charles River Breeding Labs, Wilmington, Mass.) intraperitoneally twice with doses of 7, 70, or 350 μg of antigen (corresponding to 1, 10, and 50 μg of each individual component). Serum samples were collected 7 days after the second antigen dose and pooled from the individual animals for evaluation. Immunogenicity in two New Zealand White rabbits (Milbrook Farms, Amherst, Mass.) was determined by injecting animals once subcutaneously with 350 μg of vaccine in complete Freund's adjuvant followed for 2 weeks by thrice-weekly intravenous injections of 350 μg of antigen in 0.5 ml of

TABLE 2. IgM and IgG antibody titers in sera of mice immunized with individual monovalent antigens that composed the high-molecular-weight polysaccharide polyvalent vaccine

Antigen from serogroup	IgG and IgM titer[a] obtained in immune serum[b] after immunization with the indicated dose of antigen					
	50 μg		10 μg		1 μg	
	IgM	IgG	IgM	IgG	IgM	IgG
01	499	533	510	495	543	557
02	1,020	1,040	1,000	540	330	460
03	740	730	730	640	640	320
05	1,520	670	40	720	290	250
06	430	720	730	460	720	740
10	870	570	750	980	1,030	920
11	280	190	340	380	290	2,720

[a] Titer is calculated by regression analysis to find the equation describing the best-fit line when the mean of triplicate OD values (x axis) is plotted against the serum dilutions (y axis); the resultant formula is used to solve the equation for y (serum dilution) when an OD value of 0.2 was achieved.
[b] All preimmune sera had titers of <20.

saline. One week after the last dose, animals were bled and serum from the individual animals was prepared.

Antibody responses were evaluated by the ELISA protocol described above, with use of secondary antibodies specific to murine μ or γ heavy chains or rabbit γ heavy chains. Results are expressed either as optical density (OD) values obtained for a given serum dilution or as a titer, calculated by regression analysis to find the equation describing the best-fit line when the mean of triplicate OD values (x axis) was plotted against the serum dilutions (y axis); the resultant formula was used to solve the equation for y (serum dilution) when an OD value of 0.2 was achieved. Three to six points along the linear portion of the curve were used to derive titers, and r^2 values of ≥0.7 were always obtained. Opsonic killing was measured as described previously (26) with both vaccine and nonvaccine strains. Under routine conditions employed for the opsonic assay, bacterial killing of ≥40% is significant at $P < 0.05$ (Mann-Whitney U test).

RESULTS

Properties of individual vaccine components. The yield, the presence of chemical contaminants, and the K_{av} of the individual high-molecular-weight polysaccharide antigens are shown in Table 1. In addition to these properties, each antigen showed the expected serologic identity in immunodiffusion with previous preparations of the same high-molecular-weight polysaccharide, and each passed the rabbit pyrogenicity test under the criteria defined in Materials and Methods. Following immunization with 1, 10, or 50 μg of individual high-molecular-weight polysaccharide antigens, mice responded with both IgG and IgM antibodies that bound to the homologous LPS antigen (Table 2). While all preimmunization sera had titers of <20, postimmunization sera had titers ranging from 40 to 2,720. Analyses of these sera in an opsonic killing assay showed that for each antigen at least one of the three doses elicited a greater than fourfold increase in opsonic titer against the homologous strain (not shown).

Chemical analysis of individual antigens. The monosaccharide constituents identified in each antigen are shown in Table 3. In addition to the sugars expected to be present on the basis of prior analyses of *P. aeruginosa* LPS O side chains (16, 17), each antigen contained detectable LPS core sugar (rhamnose, glucose, galactose, and hexosamines) (Table 3). In only two cases, serogroups 02 and 05, could the level of these monosaccharides be used to accurately quantify the amount of core sugars present in the individual high-molecular-weight polysaccharide antigen, since the other serogroup antigens utilize some of the same sugars in both the core and the O side chain. However, all of the high-molecular-weight polysaccharide antigens contained some sugar residues that are part of the core and not part of the O antigen, indicating that all of these preparations contained LPS core residues attached to O-antigen residues.

Safety and pyrogenicity testing of heptavalent vaccine. The polyvalent antigen contained <1% nucleic acid, protein, or KDO. It passed the general safety test in guinea pigs and mice, wherein toxicity is determined by overt signs of ill health, death, or weight loss during a 7-day period. All animals gained weight during the test period. At a dose of 10 μg/kg of body weight, the polyvalent vaccine did not elicit any temperature increase during a 3-h test period in three rabbits injected intravenously with the vaccine. At a concentration of 700 μg/ml, the vaccine contained 15.49 endotoxin units/ml as determined by the *Limulus* lysate assay, well below the limit of 350 endotoxin units/ml allowed by FDA regulations.

Immune response to the heptavalent vaccine. Pooled sera were obtained from groups of five mice immunized twice with

TABLE 3. Monosaccharide constituents of individual components of *P. aeruginosa* high-molecular-weight polysaccharide heptavalent vaccine

Antigen	Amt of sugar (% of total carbohydrate)[a]									
	N-Acetyl-fucosamine[b]	*N*-Acetyl-quinivosamine[b]	Diamino-uronic acid[c]	*N*-Acetyl-galacto-samine	*N*-acetyl-gluco-samine	*N*-acetyl-galacto-saminouronic acid[d]	Bacillosamine	Galactose	Rhamnose	Glucose
01	**12.2**	**24.7**	**13.1**	**47.7**					0.7	1.6
02	**28.9**		**46.6**	8.3				4.7	7.8	11.2
03					**30.6**	**27.2**	++[e]	1.9	**26.9**	5.2
05	**28.9**		**45.3**	9.8				2.0	4.6	4.8
06		**26.0**				**40.3**		0.5	**25**	4.4
10		**24.8**			1.5	**35.7**			**35.6**	2.3
11	**76.1**			0.7				1.2	0.5	**21.0**

[a] Boldface type indicates an O-side-chain-specific sugar.
[b] The amounts of *N*-acetylfucosamine and *N*-acetylquinivosamine are estimates based on the response factor to heptose.
[c] The amount of diaminouronic acid is an estimate based on the response factor to *N*-acetylaminohexoses.
[d] The amount of *N*-acetylgalactosaminouronic acid is an estimate based on the response factor to *N*-acetylaminohexoses. This column includes *N*-acetyl-L-galactosaminouronic acid (serogroups 03 and 10) and *N*-formyl-D-galactosaminouronic acid and *N*-acetyl-D-galactosaminouronamide (serogroup 06).
[e] A sugar with the mass spectrum characteristic of bacillosamine is detected but not quantifiable because of lack of a standard.

TABLE 4. IgM and IgG antibody titers of mice immunized with heptavalent high-molecular-weight polysaccharide vaccine against LPS antigens isolated from the same strain used to produce the vaccine components

Antigen from serogroup	IgG and IgM titer[a] obtained in immune serum[b] after immunization with the indicated doses[c] of vaccine					
	350 μg		70 μg		7 μg	
	IgM	IgG	IgM	IgG	IgM	IgG
01	2,528	2,688	3,143	1,810	2,250	1,423
02	2,500	2,692	2,947	1,381	2,000	966
03	2,771	2,286	3,148	640	3,704	1,375
05	2,394	2,917	1,952	2,158	1,436	1,550
06	2,300	3,231	3,125	1,538	3,652	1,759
10	2,394	3,636	1,929	1,795	1,436	1,500
11	2,700	2,571	3,714	914	3,000	4,000

[a] Titer is calculated by regression analysis to find the equation describing the best-fit line when the mean of triplicate OD values (x axis) are plotted against the serum dilutions (y axis); the resultant formula is used to solve the equation for y (serum dilution) when an OD value of 0.2 was achieved.

[b] All preimmune sera had titers of <20.

[c] Represents doses of individual antigens of 50, 10, or 1 μg per mouse.

doses of the heptavalent vaccine containing 7, 70, or 350 μg of total antigen, corresponding to 1, 10, or 50 μg of each component. ELISA responses were measured against LPS isolated from each of the vaccine strains (Table 4). Each vaccine component induced increases in the concentration of LPS-specific antibodies in the sera of the immunized mice following two injections at each of the three doses. The overall titers were somewhat higher with the polyvalent vaccine, thus suggesting enhanced immunogenicity due to combining the individual antigens into a polyvalent preparation. Similarly, rabbits injected multiple times with 350-μg doses of the polyvalent vaccine showed increased antibody binding to LPS isolated from the vaccine strains (Fig. 1).

Because of the presence of core sugars in the high-molecular-weight polysaccharide antigens, we also measured the antibody response by ELISA to LPS from three *P. aeruginosa* strains not included in the vaccine-IATS serogroups 04, 07, and 09 and to LPS isolated from the O-side-chain-deficient strains, AK1401 and PAC557. There was an increase in antibody binding to these antigens following immunization of rabbits, suggesting the presence of antibody to common determinants in the core and/or antibodies cross-reactive to similar determinants in the O side chains of the LPS smooth, nonvaccine strains (Fig. 2). An irrelevant control antigen, capsular polysaccharide from type III group B streptococci, showed only a slight increase in the binding of the postimmunization sera.

Opsonic killing levels measured against the vaccine strains in sera of rabbits and mice immunized with the polyvalent preparation are shown in Fig. 3 and 4, respectively. Rabbits developed opsonic activity against all vaccine strains, as did mice at most doses. For five of the seven vaccine strains, the highest dose (350 μg) of vaccine appeared optimal for inducing opsonic killing activity in mice.

We next analyzed the opsonic killing activity in immune rabbit and mouse sera against nonvaccine strains of *P. aerugi-*

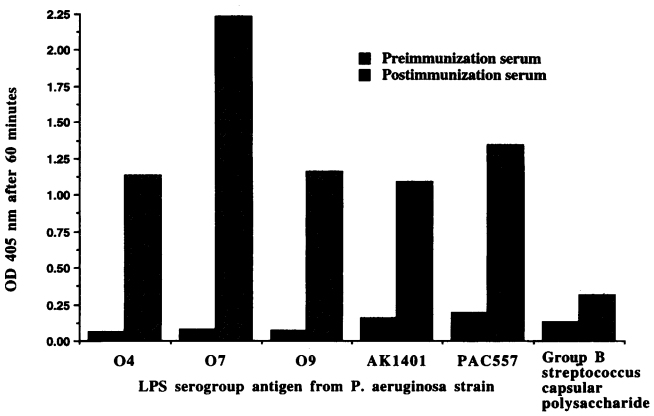

FIG. 2. Reactivity against *P. aeruginosa* LPS serogroup antigens extracted from nonvaccine strains of 1:100 dilutions of pre- and postimmunization sera from two rabbits immunized multiple times with 350-μg doses of the heptavalent high-molecular-weight polysaccharide vaccine. LPS from serogroups 04, 07, and 09 are smooth; LPS from strains AK1401 and PAC557 are rough. Bars represent means of four observations composed of duplicate determinations done on each rabbit serum sample.

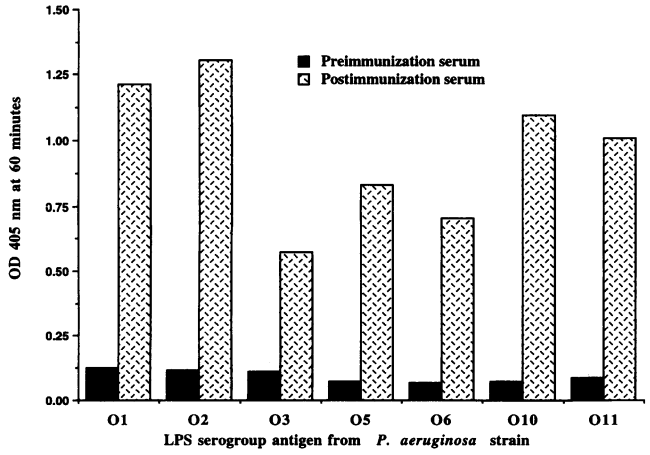

FIG. 1. Reactivity against *P. aeruginosa* LPS serogroup antigens extracted from vaccine strains of 1:100 dilutions of pre- and postimmunization sera from rabbits immunized multiple times with 350-μg doses of the heptavalent high-molecular-weight polysaccharide vaccine. Bars represent means of duplicate determinations.

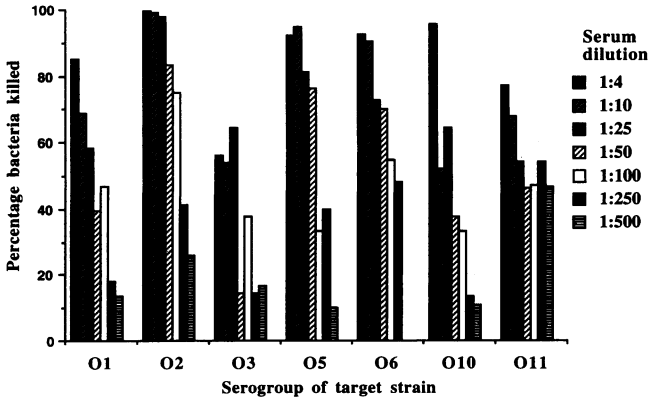

FIG. 3. Opsonic killing of *P. aeruginosa* vaccine strains by the dilution (indicated on the figure) of rabbit immune serum raised to the heptavalent high-molecular-weight polysaccharide vaccine. Preimmunization sera had no detectable opsonic killing activity. Bars represent means of four observations composed of duplicate determinations done on each rabbit serum sample.

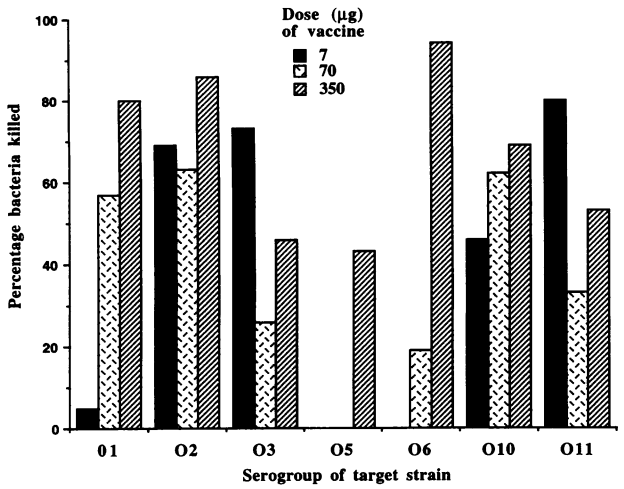

FIG. 4. Opsonic killing of *P. aeruginosa* vaccine strains by a 1:8 dilution of mouse immune serum raised to two doses (individual dose indicated on the figure) of the heptavalent high-molecular-weight polysaccharide vaccine. Preimmunization sera had no detectable opsonic killing activity. Bars represent means of duplicate determinations.

nosa. These included strains that were members of serogroups not included in the vaccine and strains that were members of serogroups included in the vaccine but distinguished from the vaccine strains by structural variation in the O side chains that can be identified serologically as distinct subtype antigens. The opsonic killing activity against these nonvaccine strains were variable, except in the case of serogroup 06 strains (Table 5), in which all subtype strains were effectively opsonized. The latter result is consistent with previous data that showed that animals and humans vaccinated with the Fisher IT-1 variant of the serogroup 06 strain contained antibodies effective at opsonizing all members of the serogroup 06 family of *P. aeruginosa* strains (32). In contrast to the serogroup 06 situation, it was noted that among the IATS 02/05 series of strains, in which seven related but variant O-side-chain structures have been described, immune rabbit sera opsonized only two of the related strains for phagocytic killing. Immune mouse sera had only low levels of opsonic activity against two nonvaccine serogroup 02/05 strains. Neither of the two subtype strains structurally related to serogroup 03 was opsonized by rabbit sera, but one of these subtype strains was opsonized by mouse sera. The single nonvaccine serogroup 010 variant strain was not killed by either rabbit or mouse immune sera.

Among strains representing nonvaccine serogroups, whose O-side-chain structures are chemically dissimilar to the ones contained in the vaccine, serogroup strains 04 and 09 were killed by rabbit sera, whereas mouse sera opsonized serogroup 09 well but serogroup 04 only poorly. Serogroup 07 was not opsonized by immune rabbit sera but was opsonized by immune mouse sera (Table 5), in spite of the presence of antibodies in rabbit sera to the serogroup 07 LPS (Fig. 2). We tried to determine whether the opsonic killing of these nonvaccine serogroup strains was due to antibodies specific to shared core structures by incubating sera with LPS purified from O-side-chain-deficient mutant strain AK1401 prior to its use in the opsonic assay. No effective inhibition was noted, but the possibility remains that opsonic killing of nonvaccine serogroup strains could be mediated by antibodies to epitopes expressed on LPS cores distinct from those on strain AK1401. The full extent of variation in LPS core structures in *P. aeruginosa* is not known.

DISCUSSION

The quest for an effective subunit vaccine against *P. aeruginosa* has been ongoing for over 25 years (9), with numerous, but incomplete, clinical trials initiated during this time (1, 5, 10, 14, 15, 44, 46, 50). Most of the vaccines that have been evaluated in humans have been based on antigenic determinants expressed on the LPS O side chain (1, 7, 23). Vaccines

TABLE 5. Opsonic killing of nonvaccine *P. aeruginosa* strains by immune rabbit or mouse sera raised to high-molecular-weight polysaccharide polyvalent vaccine[a]

Serogroup	Subtype antigens	Reference strain[b]	Corresponding subtype antigen in vaccine	% Bacteria killed by immune sera[c] from:	
				Rabbits	Mice
06	06a, 6b	170008	06a,? (Fisher IT-1)	85	97
	06a, 6c	170009		83	69
	06a, 6d	170010		75	81
	06a, ?	Habs 06		90	96
03	03a, 3b	170001	03a, 3b, 3c	0	70
	03a, 3d	Wokatsch 13		0	NT[d]
02/05	02a, 2b	170003	0(2a), 2c	5	0
	02a, 2d	170005	(Fisher IT-7)	5	0
	02a, 2d, 2e	170006		0	20
	0(2a), 2d, 2f	170007		60	15
	02a, 2b, 2e	Wokatsch 25		80	NT
04	04a, 4c	170040	None	50	21
07	07a, 7b, 7c	170011	None	0	90
09	09a, 9b	170020	None	75	90
10	10a, 10c	170002	10a, 10b	0	0

[a] Subtype antigens are designated by lowercase letters, and the letter "a" always refers to the determinant that defines the serogroup. ? indicates the potential for undefined subtype epitopes based on the presence of structural variation that has not yet been associated with an immunologically defined subtype determinant.

[b] Reference strains are defined in Table 1 of reference 17.

[c] Rabbit sera were used at a 1:40 final dilution; mouse sera were used at a 1:8 final dilution. Killing shown for mouse sera represents the maximal percentage of bacteria killed in any sera from animals immunized with either 7, 70, or 350 μg of polyvalent vaccine.

[d] NT, not tested.

containing intact LPS have mostly been abandoned at this time, whereas nontoxic vaccines composed of either conjugates of O side chains and exotoxin A (7) or lipid A-free, high-molecular-weight polysaccharide versions of the O side chains (28, 33) are still being developed. The choice of the components of these vaccines is based on numerous studies that indicate that O-side-chain antigens are the optimal targets for antibodies protective against *P. aeruginosa* (4, 42, 49, 51). In addition, a limited number (~10) of serogroups of *P. aeruginosa* account for >90% of clinical isolates (40). However, as the structures of the *P. aeruginosa* O antigens have been determined, it is clear that within immunologically defined serogroups there are strains that express variant versions of the O side chains containing epitopes in addition to those identified by serogroup-specific antibodies. The degree to which these variant epitopes, defined as subtype determinants (16, 17, 21), represent targets for protective antibody is only beginning to be explored.

Overall, little has been done to define whether protective antibodies to *P. aeruginosa* O side chains are specific to serogroup or subtype epitopes. This situation is further confused by a lack of understanding of whether there is variation in the ratio of the expression of serogroup and subtype epitopes by different *P. aeruginosa* strains and whether this affects binding of antibodies. In addition, it is not clear what the most effective laboratory method (i.e., antibody binding, opsonic activity, or in vivo animal protection) is for determining whether a single preparation of an O antigen will elicit antibodies protective against all strains expressing the same serogroup epitope. The data presented in this study indicate that evaluation of subtype strains within a serogroup will be necessary to characterize the degree of cross-reactivity elicited by any vaccine. The actual delineation of the components of a comprehensive, O-side-chain-specific *P. aeruginosa* vaccine may require a number of different clinical trials employing vaccines composed of different O antigens.

Our results indicate that the issues of antigenicity (the expression of serogroup and subtype epitopes on O antigens), immunogenicity (the elicitation of antibodies to these epitopes), and protective immunity (the biologic activity of the elicited antibodies) are related in a complex manner. No obvious pattern emerged relating expression of classes of epitopes to induction of opsonic antibodies, and the pattern observed in rabbits was distinct from that in mice. The decision to produce the high-molecular-weight polysaccharide vaccine from a limited set of strains was based primarily on expression by a strain of a serogroup determinant commonly encountered among clinical isolates of *P. aeruginosa*. Previous work has established that many of the vaccine strains produce a high-molecular-weight polysaccharide antigen that elicits protective immunity against challenge with the homologous bacterial strain in animals (34–37), and comparable preliminary studies were carried out with the remainder of the vaccine strains prior to production of this human vaccine (unpublished observations). There was no systematic evaluation of different strains to identify those that produce high-molecular-weight polysaccharide antigens that elicit broadly reactive serogroup-specific opsonic antibodies in animals.

Another problem that may be encountered in developing an O-side-chain-based vaccine for *P. aeruginosa* is that expression of serogroup and subtype antigenic determinants may vary considerably among clinical isolates. Decreased or altered expression of these determinants may provide a means whereby the bacterium escapes opsonic killing by antibodies. Kuzio and Kropinski (19) have shown that a *P. aeruginosa* bacteriophage can alter the expression of subtype antigens in a *P. aeruginosa* strain, and the studies of Knirel and coworkers demonstrate that a single strain can produce nonhomogeneous O-side-chain structures that could be associated with expression of different, and even variable, subtype epitopes (16, 17). Also, a number of these subtype epitopes likely are defined by the presence of acetyl, formyl, butyryl, and similar substituents on the O-side-chain sugars, and variation in the level of these substituents could alter the density of subtype epitopes on a given strain. Decreased epitope density could affect the strain's susceptibility to antibody-mediated opsonic killing. While no data have been produced to indicate that antigenic variation can occur in *P. aeruginosa* to avoid host immune effectors, on the basis of the documented variability in O-antigen structures, the possibility clearly exists.

A third major barrier in developing an O-side-chain-based vaccine for *P. aeruginosa* is that, even if a particular epitope is expressed by a clinical isolate and antibodies to it are elicited by the vaccine, the antibodies may not promote bacterial killing and hence immunity. Antibody binding is necessary, but not sufficient, for effective immunity. In the case of *P. aeruginosa* O antigens, additional data are needed to determine which serogroup and subtype epitopes are the best targets for opsonically active, protective antibodies.

Although these results in animals indicate that the current heptavalent formulation of the high-molecular-weight polysaccharide vaccine may not be sufficiently comprehensive, the applicability of these results to humans must await actual trials assessing human immune responses. Broadly reactive serogroup epitopes may be more immunodominant in humans than in animals. An additional reason for proceeding with human studies using the current heptavalent formulation is to evaluate vaccine safety. If the current formulation elicits unacceptable reactions in humans, then the addition of more components to the vaccine would be unwarranted, and alternative strategies would be needed.

Overall, our results document that a multicomponent, high-molecular-weight polysaccharide vaccine for *P. aeruginosa* that meets FDA requirements for safety and elicits high titers of antibodies in mice and rabbits against the individual vaccine components can be formulated. Our results also stress the importance of evaluating the immune response against both vaccine strains and nonvaccine strains. As we have emphasized previously (32), most *P. aeruginosa* vaccines tested in humans measure immune responses only to the isolated vaccine components and the strains from which the vaccine was derived. Given the complexity of serogroup and subtype epitopes on *P. aeruginosa* O antigens, it is clear that a broader class of test strains needs to be evaluated to ensure that the antibodies elicited by O-antigen-specific vaccines are broadly reactive against subtype strains within a serogroup. An appreciation of the complexity of epitope expression, immunogenicity, and biologic efficacy of antibodies in this situation should lead to the proper decisions regarding the development of active and passive immunotherapies against *P. aeruginosa* infections.

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REFERENCES

1. **Alexander, J. W., and M. W. Fisher.** 1974. Immunization against *Pseudomonas* infection after thermal injury. J. Infect. Dis. **130:** S152–S158.
2. **Andrews, P.** 1970. Estimation of molecular size and molecular weights of biological compounds by gel filtration. Methods Biochem. Anal. **18:**1–53.
3. **Cryz, S. J., Jr., E. Furer, and R. Germanier.** 1983. Passive protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. **40:**659–664.
4. **Cryz, S. J., Jr., E. Furer, and R. Germanier.** 1984. Protection against fatal *Pseudomonas aeruginosa* burn wound sepsis by immunization with lipopolysaccharide and high-molecular-weight polysaccharide. Infect. Immun. **43:**795–799.
5. **Cryz, S. J., Jr., E. Furer, J. U. Que, J. C. Sadoff, M. Brenner, and U. B. Schaad.** 1991. Clinical evaluation of an octavalent *Pseudomonas aeruginosa* conjugate vaccine in plasma donors and in bone marrow transplant and cystic fibrosis patients. Antibiot. Chemother. **44:**157–162.
6. **Cryz, S. J., Jr., E. Furer, J. C. Sadoff, T. Fredeking, J. U. Que, and A. S. Cross.** 1991. Production and characterization of a human hyperimmune intravenous immunoglobulin against *Pseudomonas aeruginosa* and *Klebsiella* species. J. Infect. Dis. **163:**1055–1061.
7. **Cryz, S. J., Jr., J. C. Sadoff, and E. Fürer.** 1989. Octavalent *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. Microb. Pathog. **6:**75–80.
8. **Cryz, S. J., Jr., J. C. Sadoff, and J. U. Que.** 1989. Conjugate vaccines against *Pseudomonas aeruginosa* and malaria. Contrib. Microbiol. Immunol. **10:**166–189.
9. **Fisher, M. W., H. B. Devlin, and F. Gnabasik.** 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. J. Bacteriol. **98:**835–836.

9a. **Food and Drug Administration.** 1987. Code of federal regulations, title 21, section 610.11. Food and Drug Administration, Rockville, Md.

9b. **Food and Drug Administration.** 1987. Code of federal regulations, title 21, section 610.13b. Food and Drug Administration, Rockville, Md.

9c. **Food and Drug Administration.** 1987. Code of federal regulations, title 21, section 58. Food and Drug Administration, Rockville, Md.

10. **Gottlieb, D. J., S. J. Cryz, Jr., E. Furer, J. U. Que, H. G. Prentice, A. S. Duncombe, and M. K. Brenner.** 1990. Immunity against *Pseudomonas aeruginosa* adoptively transferred to bone marrow transplant recipients. Blood **76:**2470–2475.
11. **Hatano, K., J. B. Goldberg, and G. B. Pier.** 1993. *Pseudomonas aeruginosa* lipopolysaccharide: evidence that the O side chains and common antigens are on the same molecule. J. Bacteriol. **175:** 5117–5128.
12. **Jarvis, W. R., and W. J. Martone.** 1992. Predominant pathogens in hospital infections. J. Antimicrob. Chemother. **29**(Suppl. A)**:**19–24.
13. **Jones, R. J., E. A. Roe, and J. L. Gupta.** 1978. Low mortality in burned patients in a Pseudomonas vaccine trial. Lancet **ii:**401–403.
14. **Jones, R. J., E. A. Roe, and J. L. Gupta.** 1979. Controlled trials of a polyvalent Pseudomonas vaccine in burns. Lancet. **ii:**977–982.
15. **Jones, R. J., E. A. Roe, and J. L. Gupta.** 1980. Controlled trial of Pseudomonas immunoglobulin and vaccine in burn patients. Lancet **ii:**1263–1265.
16. **Knirel, Y. A.** 1990. Polysaccharide antigens of *Pseudomonas aeruginosa*. Crit. Rev. Microbiol. **17:**273–304.
17. **Knirel, Y. A., E. V. Vinogradov, N. A. Kocharova, N. A. Paramonov, N. K. Kochetkov, B. A. Dmitriev, E. S. Stanislavsky, and B. Lányi.** 1988. The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa*. Acta Microbiol. Hung. **35:**3–24.
18. **Kropinski, A. M., L. C. Chan, and F. H. Milazzo.** 1979. The extraction and analysis of lipopolysaccharides from *Pseudomonas aeruginosa* strain PAO and three rough mutants. Can. J. Microbiol. **25:**390–398.
19. **Kuzio, J., and A. M. Kropinski.** 1983. O-antigen conversion in *Pseudomonas aeruginosa* PAO1 by bacteriophage D3. J. Bacteriol. **155:**203–212.
20. **Lam, J. S., M. Y. C. Handelsman, T. R. Chivers, and L. A. Macdonald.** 1992. Monoclonal antibodies as probes to examine serotype-specific and cross-reactive epitopes of lipopolysaccharides from serotype O2, serotype O5, and serotype O16 of *Pseudomonas aeruginosa*. J. Bacteriol. **174:**2178–2184.
21. **Lanyi, B., and T. Bergan.** 1978. Serological characterization of *Pseudomonas aeruginosa*. Methods Microbiol. **10:**94–168.
22. **Markham, R. B., and G. B. Pier.** 1983. Immunologic basis for mouse protection provided by high-molecular-weight polysaccharide from immunotype 1 *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5:**S957–S962.
23. **Miler, J. M., J. F. Spilsbury, R. J. Jones, E. A. Roe, and E. J. L. Lowbury.** 1977. A new polyvalent pseudomonas vaccine. J. Med. Microbiol. **10:**19–27.
24. **Pennington, J. E.** 1990. *Pseudomonas aeruginosa* immunotherapy. Eur. J. Clin. Microbiol. **9:**377–380.
25. **Pennington, J. E.** 1990. *Pseudomonas aeruginosa*. Vaccines and immunotherapy. Infect. Dis. Clin. North Am. **4:**259–270.
26. **Pier, G. B.** 1982. Safety and immunogenicity of a high molecular weight polysaccharide vaccine to immunotype 1 *Pseudomonas aeruginosa*. J. Clin. Invest. **69:**303–308.
27. **Pier, G. B.** Immunochemistry of *Pseudomonas aeruginosa* lipopolysaccharides and high-molecular weight polysaccharides. Rev. Infect. Dis. **5:**S950–S956.
28. **Pier, G. B.** 1985. *Pseudomonas aeruginosa* surface polysaccharide vaccines. New therapeutic approaches from basic research, p. 157–167. *In* D. P. Speert and R. E. W. Hancock (ed.), *Pseudomonas aeruginosa*: new therapeutic approaches from basic research., vol. 36. S. Karger, Basel.
29. **Pier, G. B.** 1988. Polysaccharide antigens of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **10:**S337–S340.
30. **Pier, G. B., and S. E. Bennett.** 1986. Structural analysis and immunogenicity of *Pseudomonas aeruginosa* immunotype 2 high molecular weight polysaccharide. J. Clin. Invest. **77:**491–495.
31. **Pier, G. B., M. Cohen, and H. Jennings.** 1983. Further purification and characterization of high-molecular weight polysaccharide from *Pseudomonas aeruginosa*. Infect. Immun. **42:**936–941.
32. **Pier, G. B., N. L. Koles, G. Meluleni, K. Hatano, and M. Pollack.** 1994. Specificity and function of murine monoclonal antibodies and immunization-induced human polyclonal antibodies to lipopolysaccharide subtypes of *Pseudomonas aeruginosa* serogroup 06. Infect. Immun. **62:**1137–1143.
33. **Pier, G. B., and R. B. Markham.** 1986. Serotypes and immune responses to *Pseudomonas aeruginosa*, p. 399–491. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
34. **Pier, G. B., and M. Pollack.** 1989. Isolation, structure, and immunogenicity of *Pseudomonas aeruginosa* immunotype 4 high-molecular-weight polysaccharide. Infect. Immun. **57:**426–431.
35. **Pier, G. B., M. Pollack, and M. Cohen.** 1984. Immunochemical characterization of high-molecular weight polysaccharide from Fisher immunotype 3 *Pseudomonas aeruginosa*. Infect. Immun. **45:**309–313.
36. **Pier, G. B., H. F. Sidberry, and J. C. Sadoff.** 1978. Protective immunity induced in mice by immunization with a high-molecular-weight polysaccharide from *Pseudomonas aeruginosa*. Infect. Immun. **22:**919–925.
37. **Pier, G. B., H. F. Sidberry, and J. C. Sadoff.** 1981. High-molecular-weight polysaccharide antigen from *Pseudomonas aeruginosa* immunotype 2. Infect. Immun. **34:**461–468.
38. **Pier, G. B., H. F. Sidberry, S. Zolyomi, and J. C. Sadoff.** 1978. Isolation and characterization of a high-molecular-weight polysaccharide from the slime of *Pseudomonas aeruginosa*. Infect. Immun. **22:**908–918.
39. **Pier, G. B., D. Thomas, G. Small, A. Siadak, and H. Zweerink.** 1989. In vitro and in vivo activity of polyclonal and monoclonal human immunoglobulins G, M, and A against *Pseudomonas aeruginosa* lipopolysaccharide. Infect. Immun. **57:**174–179.
40. **Pier, G. B., and D. M. Thomas.** 1982. Lipopolysaccharide and high molecular weight polysaccharide serotypes of *Pseudomonas aeruginosa*. J. Infect. Dis. **145:**217–223.
41. **Pollack, M., G. B. Pier, and R. K. Prescott.** 1984. Immunization with *Pseudomonas aeruginosa* high-molecular-weight polysacchar-

ides prevents death from *Pseudomonas* burn infections in mice. Infect. Immun. **43:**759–760.

42. **Pollack, M., and L. S. Young.** 1979. Protective activity of antibodies to exotoxin A and lipopolysaccharide at the onset of *Pseudomonas aeruginosa* septicemia in man. J. Clin. Invest. **63:**276–286.
43. **Powderly, W. G., G. B. Pier, and R. B. Markham.** 1986. T lymphocyte-mediated protection against *Pseudomonas aeruginosa* infection in granulocytopenic mice. J. Clin. Invest. **78:**375–380.
44. **Roe, E. A., and R. J. Jones.** 1983. Immunization of burned patients against *Pseudomonas aeruginosa* infection at Safdarjang Hospital, New Delhi. Rev. Infect. Dis. **5:**S922–S930.
45. **Rowe, P. S., and P. M. Meadow.** 1983. Structure of the core oligosaccharide from the lipopolysaccharide of *Pseudomonas aeruginosa* PACR1 and its defective mutants. Eur. J. Biochem. **132:**329–337.
46. **Schaad, U. B., A. B. Lang, J. Wedgwood, A. Ruedberg, E. Furer, J. U. Que, and S. J. Cryz, Jr.** 1990. Immunization of non-colonized cystic fibrosis patients with a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. Pediatr. Pulmonol. **5**(Suppl.)**:** 91–92.
47. **Vale, T. A., M. A. Gaston, and T. L. Pitt.** 1988. Subdivision of O serotypes of *Pseudomonas aeruginosa* with monoclonal antibodies. J. Clin. Microbiol. **26:**1779–1782.
48. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
49. **Young, L. S.** 1972. Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J. Infect. Dis. **126:**277–284.
50. **Young, L. S., R. D. Meyer, and D. Armstrong.** 1973. *Pseudomonas aeruginosa* vaccine in cancer patients. Ann. Intern. Med. **79:**518–527.
51. **Young, L. S., B. H. Yu, and D. Armstrong.** 1970. Agar-gel precipitating antibody in *Pseudomonas aeruginosa* infections. Infect. Immun. **2:**495–503.