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Cross-Protection by *Pseudomonas aeruginosa* Polysaccharides

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High-molecular-weight polysaccharide from *Pseudomonas aeruginosa* immunotypes 1 and 2 gave cross-protection in outbred CD-1 mice challenged with the heterologous immunotype organism. Both active immunization with 50 μg of polysaccharide, as well as passive transfer of immune serum were effective. The basis for this cross-protection is the ability of high doses of polysaccharide to induce antibody formation to both homologous and heterologous immunotype determinants.

Previous studies have shown that high-molecular-weight polysaccharide (PS)-type antigen can be isolated from cultural supernates or from the extracellular slime of *Pseudomonas aeruginosa* Fisher immunotypes 1 (IT-1) and 2 (IT-2) (11, 12). PS antigen appears to be a nontoxic, immunogenic form of the lipopolysaccharide (LPS) immunotype determinant. The PS antigens are composed principally of carbohydrates, with a molecular weight in the range of 1.5×10^5 to 2.5×10^5. They are nonpyrogenic in rabbits and nontoxic in mice (11, 12). PS antigen protects mice challenged with live organism 7 days after immunization with a single dose (10, 11).

It has also been shown that the PS antigens from IT-1 or IT-2 *P. aeruginosa* are cross-reactive with the respective O side chain PS of their lipopolysaccharide (LPS) (10–12). O side chains contain the immunotype-specific serological determinant for these strains of *P. aeruginosa* (1–3, 7, 8). To determine whether the protective immunity induced by PS antigens was based upon the immunotype-specific determinant shared between PS and LPS or whether the PS antigens showed cross-immunotype protection, we examined the ability of PS antigens from *P. aeruginosa* IT-1 and IT-2 to protect mice challenged with the live, heterologous organism. In addition, immune responses of rabbits and inbred mice to these two PS antigens was determined. Finally, the ability of intact LPS antigens to protect mice against homologous and heterologous strain challenge was also studied to compare the immunizing efficacy of PS and LPS antigens from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* IT-1, IT-2, and IT-4 strains were used as previously described (5, 8, 10).

Antigens. High-molecular-weight PS and LPS were prepared as previously described (9, 11, 12).

Antisera. Rabbit antisera employed in serological assays and passive protective studies were prepared as previously described (11, 12). Murine antisera were obtained by retroorbital bleeding of ether-anesthetized mice 5 to 7 days after intraperitoneal immunization with a single dose of antigen in 0.5 ml of saline.

Adsorption of antisera. Antisera were adsorbed with the homologous or heterologous immunotype organism. Organisms used for adsorptions were grown in tryptic soy broth for 24 h, recovered by centrifugation, and killed by suspension in phosphate-buffered saline containing 1% Formalin for 24 h at room temperature. The organisms were washed three times with water and then lyophilized before their use as adsorbing antigens. Adsorptions with lyophilized cells (final concentration, 10 mg/ml of serum) were carried out at 4°C for 24 h with mixing.

Animal studies. Outbred CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Inbred C3H/ANF and BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn. Animal studies for immunization-challenge experiments of active and passive protection were as previously described (10, 11). The challenge doses used here were chosen to routinely kill 90 to 100% of unimmunized mice.

Serological methods. The measurement of serum antibody levels to IT-1 and IT-2 were performed in a radioactive-antigen-binding assay as described by Farr (4). The antigen used in these assays was intrinsically labeled ^{14}C-PS, which was prepared from IT-1 and IT-2 organisms as follows. A 2-liter volume of Davis minimal media (Difco Laboratories, Detroit, Mich.) containing 1% sodium acetate instead of glucose as the carbon source was inoculated with growth from a tryptic soy agar culture of either the IT-1 or the IT-2 organisms. To this medium was added 20 mCi of [^{14}C]sodium acetate. The organisms were grown at 37°C with stirring for 48 h. The culture was centrifuged to remove the organisms and the supernates concentrated to a volume of 50 ml on an Amicon ultrafiltration TCF apparatus (Amicon Corp., Danvers, Mass.) utilizing PM 30 membranes. Crude PS antigen was precipitated from the concentrate by the addition of alcohol (80% vol/vol), and preparation of PS continued as previously described (9, 11). Specific activities for

TABLE 1. Ability of immunization with IT-1 and IT-2 PS antigens to afford protection in mice to challenge with live *P. aeruginosa* IT-1 and IT-2

Immunogen	Amt (μg)	Challenge		Survivors/total challenged[a]		
		Organism	No. ($\times 10^8$)	Expt 1	Expt 2	Expt 3
IT-1 PS	1	IT-1	3.2	0/10	1/10	1/10
	10			3/10	4/10	1/10
	50			9/10	10/10	10/10
	1	IT-2	3.8	0/10	0/10	1/10
	10			2/10	3/10	3/10
	50			5/10	5/10	6/10
	50	IT-4	1.2	1/10	0/10	1/10
IT-2 PS	1	IT-1	3.2	0/10	0/10	0/10
	10			6/10	4/10	3/10
	50			8/10	8/10	10/10
	1	IT-2	3.8	1/10	2/10	1/10
	10			7/10	7/10	5/10
	50			10/10	9/10	8/10
	50	IT-4	1.2	0/10	2/10	0/10
Saline		IT-1	3.2	0/10	0/10	1/10
		IT-2	3.8	1/10	0/10	0/10
		IT-4	1.2	0/10	0/10	1/10

[a] *P* value for protection of 50% or greater is ≤0.016 by Fisher exact test when there are no survivors in the control group; with one survivor in control group, *P* value for protection of 60% or greater is ≤0.027. Lower survival rates have *P* values > 0.05.

the PS antigens obtained were approximately 1.04 and 0.98 cpm/ng for the IT-1 PS and IT-2 PS, respectively.

Calculation of the concentration of specific antibody was as described (9).

RESULTS

Table 1 shows the results of three experiments examining the efficacy of cross-protection in outbred CD-1 mice actively immunized with the PS antigen from *P. aeruginosa* IT-1 and IT-2. Doses lower than 50 μg of IT-1 PS generally did not routinely protect a significant number of the outbred CD-1 mice from homologous IT-1 challenges. At a 50-μg dose, IT-1 PS was effective in eliciting 50 to 60% protection against challenge with the *P. aeruginosa* IT-2 cells. After a 50-μg dose of IT-2 PS, 80 to 100% protection against challenge with IT-1 cells was noted. IT-2 PS also had some cross protective capabilities at a 10-μg dose. Both IT-1 and IT-2 PS gave 90 to 100% protection after a 50-μg dose against challenge with homologous cells. Neither PS was capable of protecting against challenge with *P. aeruginosa* IT-4.

When LPS was used in cross-protection experiments, an LPS dose of 0.01 μg per mouse was effective in affording protection to the live homologous immunotype organism only (Table 2). Higher doses of LPS (10 to 100 μg) gave cross-protection similar to that seen with the PS antigens. These high doses of LPS were sublethal. Symptoms of endotoxin shock were noted in the animals at approximately 8 to 30 h postimmunization. These symptoms had completely subsided by the time of challenge 7 days later. The lack of protection to challenge with IT-4 organisms after immunization with these high doses of LPS indicates the specificity of this high-dose LPS cross-protection.

We next looked at the ability of rabbit antisera raised to the PS antigens from *P. aeruginosa* IT-1 and IT-2 to passively protect mice against live organism challenge. A 0.1-ml portion of rabbit antisera to the purified IT-1 and IT-2 PS antigens provided 100% protection to challenge with either the homologous or heterologous immunotype organisms (Table 3). Adsorption of antiserum raised to IT-1 PS with IT-1 cells removed the protective efficacy of this serum to *P. aeruginosa* IT-1 live cell challenge. However, protection against challenge with the heterologous strain of *P. aeruginosa* IT-2 remained.

When the antiserum raised to IT-1 PS was

adsorbed with IT-2 cells, passive protection against IT-2 *P. aeruginosa* was removed. However, there was no effect on passive protection against challenge with the homologous IT-1 strain. Similarly, adsorption of antiserum raised to the IT-2 PS with IT-2 cells eliminated passive protection to *P. aeruginosa* IT-2 live cell challenge. It did not significantly affect the protective efficacy against IT-1 live cell challenge. Antiserum raised to IT-2 PS adsorbed with IT-1 cells still conferred passive protection against IT-2 live cell challenge in mice, but had lost passive protection against *P. aeruginosa* IT-1 live cell challenge. When rabbit antisera raised to the LPS antigens from *P. aeruginosa* IT-1 and IT-2 were used in passive protection studies identical to those described above for antisera raised to the PS antigens, identical results were obtained (data not shown).

Serological studies. A radioactive-antigen-binding assay was employed to further define the nature of the cross-protective component of the PS antigens from *P. aeruginosa* IT-1 and IT-2. These studies used both hyperimmune rabbit antisera raised to the PS and LPS antigens and murine antisera raised to single doses of these antigens. Table 4 shows the concentration of specific antibody against the IT-1 and IT-2 PS in hyperimmune rabbit antisera raised to the IT-1 and IT-2 PS and LPS antigens. Antisera raised to PS and LPS from both strains contained antibody to both the IT-1 and IT-2 PS antigens. Adsorption of antisera raised to IT-1 PS and LPS antigens with the homologous IT-1 cells reduced the specific IT-1 PS antibody levels by 90% or more. This adsorption had little effect on the antibody level to IT-2 PS (Table 4). Similarly, adsorption of antisera raised to IT-2 PS and LPS with IT-2 cells reduced the specific IT-2 PS antibody below the level of detection in the radioactive-antigen-binding assay, but left intact appreciable amounts of antibody directed at the IT-1 PS (Table 4). IT-2 cells were able to remove some of the antibody to IT-1 PS from antisera raised to IT-2 PS and IT-2 LPS.

Further elucidation of the serological relatedness of IT-1 and IT-2 PS and LPS antigens was obtained by using mouse antisera raised to single immunizations of these antigens. It has been found (G. B. Pier and R. B. Markham, submitted for publication) that mice of the C3H/ANF strain are high responders to a single 1-μg dose of the IT-1 PS antigen, making both binding and protective antibodies. Mice of the BALB/c strain, on the other hand, make a minimal antibody response after a 25- to 50-μg dose of IT-1 PS. Data regarding the immune response of these two strains to high- and low-dose immunizations with the IT-1 and IT-2 PS and LPS antigens are shown in Table 5. After immunization with either 1 or 50 μg of the IT-1 PS, the C3H/ANF strain made primarily an immunotype-specific response. After immunization with 50 μg of IT-1 PS the BALB/c strain made almost equivalent antibody responses to the IT-1 and IT-2 PS antigens. Both mouse strains made a type-specific response to immunization with IT-1 LPS at a dosage of 0.10 μg, whereas at the high dose of 100 μg of IT-1 LPS, antibody to the IT-2 PS was elicited. After immunization with IT-2 PS both mouse strains produced antibody to IT-2 PS and antibody specific for IT-1 PS. Immunization with IT-2 LPS again produced an immunotype-specific response at low LPS doses and cross-immunotype antibody at high doses. Immunization with LPS generally gave a higher antibody level than did immunization with PS, except in C3H/ANF mice in which 50 μg of IT-1 PS induced a higher level of IT-1-specific antibody than did IT-1 LPS.

TABLE 2. Protection of mice after immunization with IT-1 and IT-2 LPS antigens and challenge with live *P. aeruginosa* IT-1 and IT-2

Immunogen	Amt (μg)	Challenge		Survivors/ total challenged[a]
		Organism	No. ($\times 10^8$)	
IT-1 LPS	0.01	IT-1	4.1	10/10
	0.01	IT-2	4.7	0/10
	0.10			0/10
	1.0			0/10
	10.0			3/10
	50.0			6/10
	100.0			7/10
	100.0	IT-4	1.1	0/10
IT-2 LPS	0.01	IT-2	4.7	10/10
	0.01	IT-1	4.1	0/10
	0.10			0/10
	1.0			0/10
	10.0			7/10
	50.0			9/10
	100.0			8/10
	100.0	IT-4	1.1	0/10
Saline		IT-1	4.1	0/10
		IT-2	4.7	1/10
		IT-4	1.1	1/10

[a] See Table 1, footnote *a* for *P* values.

DISCUSSION

These data indicate that the high-molecular-weight PS antigens obtained from *P. aeruginosa*

TABLE 3. Passive protection of mice given rabbit antisera raised to the PS antigens of *P. aeruginosa* IT-1 and IT-2

Antisera to:	Amt (ml)	Adsorbed with:	Challenge		Survivors/total challenged[a]
			Organism	No. ($\times 10^8$)	
IT-1 PS	0.1	—[b]	IT-1	1.6	10/10[a]
		—	IT-2	4.7	10/10
		—	IT-4	1.6	0/10
		IT-1 organisms	IT-1	1.6	0/10
		IT-1 organisms	IT-2	4.7	8/10
		IT-2 organisms	IT-1	1.6	10/10
		IT-2 organisms	IT-2	4.7	0/10
IT-2 PS	0.1	—	IT-1	1.6	9/10
		—	IT-2	4.7	10/10
		—	IT-4	1.6	0/10
		IT-1 organisms	IT-1	1.6	0/10
		IT-1 organisms	IT-2	4.7	10/10
		IT-2 organisms	IT-1	1.6	10/10
		IT-2 organisms	IT-2	4.7	0/10
NRS[c]	0.1	—	IT-1	1.6	0/10
		—	IT-2	4.7	0/10
		—	IT-4	1.6	0/10

[a] See Table 1, footnote *a* for *P* values.
[b] —, Not adsorbed.
[c] NRS, Normal rabbit serum.

IT-1 and IT-2 elicit cross-protective immunity when used as active immunogens in outbred mice. Antibodies raised to these PS antigens in rabbits are also cross-protective when passively transferred to mice. The basis for this cross-protection appears to reside in the generation of antibodies to the heterologous immunotype determinant that is present on the PS and shared with the O side chain portion of the LPS (11, 12). Antibody induced to the heterologous PS (heterotype antibody) could not be adsorbed out with cells from which the PS was isolated, whereas the antibody directed to the homologous PS was readily removed. Thus, high-molecular-weight PS and LPS antigens from *P. aeruginosa* IT-1 and IT-2 are cross-immunogenic at high doses, but do not appear to be antigenically cross-reactive. A similar cross-immunogenicity without cross-reactivity of capsular PS antigens from *Neisseria meningitidis* has been described (6). The generation of cross-protective antibodies by PS antigens suggests that a limited number of these antigens may be needed to produce a comprehensive multivalent vaccine to *P. aeruginosa*.

TABLE 4. Binding antibody in single pools of hyperimmune rabbit antisera raised to *P. aeruginosa* IT-1 and IT-2 PS antigens

Antisera raised to:	Binding antibody (μg/ml)	
	IT-1 PS	IT-2 PS
IT-1 PS	43.9	10.7
Adsorbed with IT-1 organisms	<4[a]	10.6
Adsorbed with IT-2 organisms	30.2	<4
IT-1 LPS	105.7	82.6
Adsorbed with IT-1 organisms	9.7	82.4
Adsorbed with IT-2 organisms	108.9	<4
IT-2 PS	55.2	138.3
Adsorbed with IT-1 organisms	<4	143.2
Adsorbed with IT-2 organisms	32.3	<4
IT-2 LPS	80.2	214.2
Adsorbed with IT-1 organisms	4.5	166.8
Adsorbed with IT-2 organisms	28.8	<4

[a] Lower limit of detection of radioactive-antigen-binding assay for animal sera is 4.0 μg/ml.

The induction of heterotype antibodies after immunization with PS appears to be dependent on antigen dosage, the genetic constitution of the responding animal, and the particular PS antigen used. CD-1 mice require a high dose (50

TABLE 5. Binding antibody to the *P. aeruginosa* IT-1 and IT-2 PS antigens in mouse antisera after a single immunization with IT-1 and IT-2 PS and LPS

Mouse strain	Immunogen	Amt (μg)	Antibody (μg/ml) to[a]:	
			IT-1 PS	IT-2 PS
C3H/ANF	IT-1 PS	1	11.0 ± 0.8	<4
		50	63.0 ± 15.8	<4
	IT-1 LPS	0.1	38.4 ± 5.3	<4
		100.0	51.0 ± 6.2	8.7 ± 1.4
	IT-2 PS	1	5.2 ± 0.4	8.5 ± 1.1
		50	13.1 ± 1.3	29.2 ± 6.0
	IT-2 LPS	0.1	<4	48.2 ± 4.1
		100	13.3 ± 2.1	57.1 ± 8.3
BALB/c	IT-1 PS	1	<4	<4
		50	7.1 ± 1.3	6.5 ± 0.3
	IT-1 LPS	0.1	84.2 ± 4.3	<4
		100.0	68.5 ± 4.1	14.2 ± 3.8
	IT-2 PS	1	9.4 ± 1.9	12.1 ± 2.6
		50	16.2 ± 3.1	21.3 ± 5.8
	IT-2 LPS	0.1	<4	51.3 ± 9.4
		100	11.5 ± 2.3	46.9 ± 10.2

[a] Represents average concentration of antibody in five individual animal sera immunized with the indicated antigen dosage ± the standard error of the mean.

μg) of IT-1 and IT-2 PS for cross-protection. BALB/c mice and rabbits require a relatively high dose of IT-1 PS for antibody formation. C3H/ANF mice, however, required a low dose (1 μg) of IT-1 PS to induce specific antibody. Furthermore, the C3H/ANF mice responded to IT-1 PS immunization with only an IT-1 type-specific response, whereas BALB/c mice and hyperimmunized rabbits generated IT-2 PS-specific antibody after immunization with high doses of IT-1 PS. Thus, the dosage of PS and the genetic composition of the animals affected the threshold and specificity of the immune response. On the other hand, immunization with IT-2 PS produced both serotypic and heterotypic responses in all immunized animals, suggesting that this antigen may be a more broadly protective immunogen, and responses may be less restricted genetically. The antibody with specificity for IT-1 PS in rabbit hyperimmune serum that was raised to IT-2 PS may be more representative of the typical cross-reactive antibodies one would normally expect, since IT-2 organisms could adsorb out about 60% of the IT-1 PS antibody.

High doses of LPS were also found to elicit heterotype antibodies in mice. These doses of LPS were 10^3 to 10^4 times greater than the dose of LPS needed for type-specific antibody induction. The requirement for nearly equivalent amounts of LPS and PS for cross-protective immunity and antibody induction indicates that the clones of lymphocytes that recognize and respond to the heterologous immunotype determinant do so only at a certain dosage threshold. Furthermore, the chemical composition of PS (11, 12) shows only a low level (<1%) of potential LPS contamination of PS. Thus, for contaminating LPS to be responsible for the cross-immunogenicity of PS, the PS preparation would need to be 50 to 100% LPS, a possibility precluded by the chemical data. The ability of high doses of *P. aeruginosa* PS and LPS antigens to induce heterotype antibody may have a correlate in patients infected with *P. aeruginosa*. It has been shown (13) that in the convalescent serum from some patients infected with a single serotype of *P. aeruginosa*, antibody increases to heterologous serotypes of *P. aeruginosa* can be documented.

A possible explanation for cross-protection with IT-1 and IT-2 PS antigens is that these two serological determinants are closely related structural entities. PS antigens and the O PS side chain from LPS are serologically identical antigens (11, 12). PS antigens contain 5 to 7 individual monosaccharides, whereas O side chains from IT-1 and IT-2 LPS contain principally

rhamnose, glucose, and dideoxyhexosamines (11, 12). Since rhamnose, glucose, and dideoxyhexosamines are also part of PS antigens, the structural configuration of these monosaccharides most likely determines the epitope principally found on IT-1 and IT-2 PS. The differences in either the arrangement or linkages between glucose, rhamnose, and the dideoxyhexosamines must account for the serological distinction of the IT-1 and IT-2 strains of *P. aeruginosa*. After immunization with high doses of PS, antibodies to these closely related structures are induced, some with specificity for the PS inducing homologous antibody and some with specificity for the PS inducing heterologous antibody. The antibodies with specificity for the heterologous PS probably have a low affinity for the homologous PS antigenic determinant. This would explain the poor ability of organisms homologous to the inducing antigen to adsorb antibodies with specificity for the heterologous PS.

These experiments provide further insight into the nature of the serological determinants on *P. aeruginosa* antigens. It was shown that the specificity of mouse protection against live organism challenge with *P. aeruginosa* is dependent on the dosage of the immunizing antigen, the animal strain used to assess immunogenicity, and the PS antigen used for immunization. At low doses of LPS, only type-specific antibody responses and protection are seen. At high doses of PS and LPS, cross-protection and heterotype antibody responses occur in rabbits and some mouse strains. It required equivalent doses of intact LPS and PS to elicit heterotype protection in outbred CD-1 mice and cross-immunotype antibody in inbred BALB/c mice. Thus, low levels of contaminating LPS in the PS preparations cannot be held accountable for the efficacy of PS vaccination in mice.

PS antigens appear to be immunogenic forms of the *P. aeruginosa* LPS immunotype determinant. PS have previously been shown to differ from LPS O side chains, which bear the immunotype determinant (3, 7), by molecular size, monosaccharide constituents, and immunogenicity (10–12). Here it was shown that PS differs from intact LPS by its ability to elicit cross-protection in mice at doses equivalent to those required for homologous immunotype protection. Whether PS vaccines will have the same immunogenicity properties in humans as has been shown for mice will depend on the nature of the human immune response to this vaccine. PS from IT-1 *P. aeruginosa* has been shown to be immunogenic in healthy adult volunteers (9). The nature of the immunotype specificity of the human immune response elicited is currently being determined.

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