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Specificity and Function of Murine Monoclonal Antibodies and Immunization-Induced Human Polyclonal Antibodies to Lipopolysaccharide Subtypes of *Pseudomonas aeruginosa* Serogroup 06

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Structural and antigenic heterogeneity has been noted among lipopolysaccharides (LPS) produced by *Pseudomonas aeruginosa* within serogroups previously considered to be serologically homogeneous. We characterized murine monoclonal antibodies (MAbs) and immunization-induced human polyclonal antibodies reactive with one or more of five structurally variant LPS subtypes belonging to serogroup 06 of the International Antigenic Typing System. Analyses of five different MAbs employing purified LPS or whole bacteria in enzyme-linked immunosorbent assays and Western blot (immunoblot) assays revealed five distinct patterns of subtype specificity, ranging from recognition of a single subtype to reactivity with all five. MAb-mediated opsonophagocytic killing and in vivo protection against live challenge in mice correlated, in general, with differential binding to various LPS subtypes. In comparison, sera from human vaccinees immunized with LPS-derived high-molecular-weight polysaccharide from *P. aeruginosa* Fisher immunotype 1, one of five serogroup 06 subtypes, exhibited LPS binding and opsonic activity against all five subtypes. Antibodies in the human sera effectively inhibited binding to all five LPS subtype antigens of the cross-reactive MAb, LC3-2H2, suggesting the existence of a common serogroup-related epitope. These findings emphasize the importance of defining subtype-associated variations in LPS antigenicity and corresponding differences in antibody specificity and function as a basis for designing immunoprophylactic or therapeutic strategies which target *P. aeruginosa* LPS.

Protective immunity against infections caused by *Pseudomonas aeruginosa* is mediated by antibodies to the O-polysaccharide portion of lipopolysaccharides (LPS) residing in the bacterial outer membrane. This has been documented in animal models (3, 4, 26, 33, 38, 50) and in humans (40, 47, 48). LPS-based vaccines can prevent human *Pseudomonas* infections (13, 14, 49), although the use of such vaccines may be associated with local and systemic toxicity. This toxicity is greatly reduced, however, through the use of high-molecular-weight polysaccharide derivatives of the LPS O-side chain (27, 29, 34) or by conjugates composed of LPS O-side chain and carrier proteins (6, 8, 9).

Variations in the monosaccharide composition of the O-polysaccharide portion of the LPS macromolecule provide an antigenic basis for serogrouping *P. aeruginosa*. The International Antigenic Typing System (IATS), which provides the most comprehensive serotyping scheme for *P. aeruginosa*, contains 20 major serogroups (22, 23). Almost all clinical isolates are classifiable with antibodies that recognize these 20 serogroups, and 7 to 10 of the serogroups cause >90% of clinical *P. aeruginosa* disease (35).

With detailed characterization of the O-side chain structures of LPS from different *P. aeruginosa* serogroups (15, 16) has come a growing recognition of structural and antigenic heterogeneity within individual serogroups, giving rise to immunologically distinct subgroups or subtypes (20). As many as eight variant, subtype-defining, O-polysaccharide structures have been identified in a single major serogroup.

Strains from IATS serogroup 06 are more commonly implicated in clinical *Pseudomonas* infections than those from any other major serogroup. This serogroup contains at least five subtypes whose LPS O-side chains share a common sequence of monosaccharide residues (Fig. 1) but which are distinguished by differences in the mode of substitution or anomeric configuration of one or more sugars making up otherwise identical tetrasaccharide repeat units (15, 16). Fisher immunotype 1 (IT-1), the most common serotype of the Fisher-Devlin-Gnabasek *P. aeruginosa* typing system, represents one of the five currently known IATS 06 subtypes.

The growing evidence for antigenic diversity among *P. aeruginosa* strains at the subtype level poses important practical questions concerning the coverage provided by LPS-directed immunoprophylaxis and immunotherapy. The relevance of these issues is emphasized by recent studies that have evaluated the functional activities of antibodies induced by LPS-derived *Pseudomonas* vaccines solely on the basis of testing carried out against vaccine strains. In some cases, these strains represent only one of multiple subtypes from a given serogroup (3, 8, 30–32, 38). It is unclear from such studies whether the O-antigen-based vaccines in question are capable of eliciting functional antibody responses to all or only some of the subtypes which make up particular major serogroups. In addition, little is known about the relative roles of subtype-

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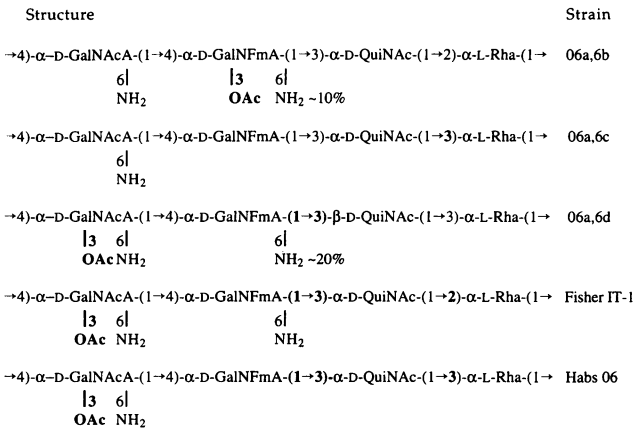

FIG. 1. Structure of the related antigens making up the *P. aeruginosa* LPS serogroup 06 strains. Boldface type indicates place in the structure that may be associated with subtype-specific determinants or distinguish the structure from a closely related one. GalNAcAN, 2-acetamido-2-deoxygalacturonic acid; GalNFmA, 2-formamido-2-deoxygalacturonic acid; QuiNAc, 2-amino-2,6-didexoyglucose (*N*-acetylquinovosamine); Rha, rhamnose; OAc, O-acetyl. Structures are according to Knirel et al. (15, 16).

specific and serogroup-specific antibodies in protective immunity, particularly whether antibodies directed to these different epitopes possess comparable levels of opsonic and protective activity. We explore these issues by characterizing murine monoclonal antibodies (MAbs) that recognize one or more of five structurally variant LPS subtypes belonging to *P. aeruginosa* IATS serogroup 06 and also investigate the specificities of immunization-induced human polyclonal antibodies against this serogroup antigen.

MATERIALS AND METHODS

***P. aeruginosa* strains.** The Fisher IT-1 strain was from the American Type Culture Collection, Rockville, Md. (ATCC 27312). The Habs 06 strain was kindly provided by T. L. Pitt, Colindale, Great Britain, and the PAC1R strain was provided by P. Meadows, London, Great Britain. The Lányi serogroup 06 strains were kindly provided by B. Lányi, Budapest, Hungary. These strains were originally designated 04a,b, 04a,c, and 04a,d by Lányi (17, 20) but have appeared in more recent publications as 06 serogroup strains (15, 16) (likely to conform to the IATS designations). Strain AK1401 was provided by Joe Lam, Guelph, Ontario, Canada (21).

LPS. LPS was extracted from LPS-smooth *P. aeruginosa* strains by the phenol-water method of Westphal and Jann (46) to obtain principally smooth LPS molecules. LPS from rough strains AK1401 and PAC557 was extracted by the phenol-chloroform-petroleum ether method as described before (11). The extracted material was purified further as described previously (34), using digestions with nucleases and pronase, ultracentrifugation, and chromatography over Sepharose CL4B. The resultant material contained 3 to 5% 2-keto-3-deoxyoctolusonic acid and had <1% contaminating nucleic acids and proteins. In addition, the LPS from the serogroup 06 strains were serologically analyzed by Y. Knirel, Moscow, Russia, using subtype-specific sera, and each LPS was found to have been extracted from the correct strain.

Preparation, screening, and evaluation of MAbs. MAbs were obtained as described previously (39), using spleen cells of mice immunized with a single strain of whole, heat-killed bacteria as the source of the antibodies. Fusions were performed with cells from animals immunized with the same agent. Hybridomas were selected by enzyme-linked immunosorbent assay (ELISA) as described previously (39), using the various LPS from the different strains as coating antigens. Antibodies were produced as ascites fluid in pristane-primed BALB/c mice. ELISA titers were determined by assaying 10-fold dilutions of MAb-containing mouse ascites fluid. Three or more consecutive points on the linear portion of the resulting dilution curve (optical density at 405 nm [OD_{405}] versus dilution) were selected, and the straight line defined by these points was determined by linear regression analysis, employing a Casio X-3800P scientific calculator. The resulting equation was solved for y (MAb dilution) at an x (OD_{405}) value of 0.5.

Immunoblots. Whole bacteria (6×10^6 CFU/10 μl) or purified LPS (2.5 mg per lane) were boiled in sample buffer for 5 min and run at 30 mA per gel at 4°C for 3 h in a Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system, using a dual slab apparatus (Bio-Rad, Richmond, Calif.) with a 4% stacking gel and a 15% separating gel (37). The resultant gels were blotted to 0.45-μm-pore-size nitrocellulose membranes (Bio-Rad) and reacted with 1:16 dilutions of MAb-containing tissue culture supernatant, and MAb binding was demonstrated by further incubation with rabbit anti-mouse immunoglobulin G (IgG) plus IgM plus IgA (Zymed, South San Francisco, Calif.) and goat anti-rabbit IgG-peroxidase conjugate, followed by incubation in the presence of 4-chloro-1-naphthol (Bio-Rad) as previously described (37).

Human serum. Pre- and postimmunization sera from humans given 100 μg subcutaneously of a vaccine composed of the Fisher IT-1 high-molecular-weight polysaccharide were obtained as described previously (27). Sera were obtained prior to immunization and 28 to 42 days postimmunization and were stored at −20°C.

ELISA employing human sera. Human sera were diluted in phosphate-buffered saline (PBS) containing 5% skim milk and 0.05% Tween 20 and added to microtiter wells of ELISA plates previously sensitized for 2 h at 37°C with *P. aeruginosa* LPS suspended at 10 μg of 0.04 M phosphate buffer (pH 7.2) per ml, and then the plates were washed and blocked at 4°C for at least 12 h with 5% skim milk. After the sera were incubated for 2 h at 37°C, plates were washed and a mixture of appropriate dilutions of alkaline-phosphatase-conjugated goat anti-human IgG, IgM, and IgA was added for 2 h at 37°C; plates were washed, and the *para*-nitro-*O*-phenylphosphate substrate was added. The OD_{405} was read at 60 min, and the titer was determined as described above for the MAbs, except that regression calculations were performed with the Statview SE + Graphics (Abacus Concepts, Inc., Berkeley, Calif.) program on a MacIntosh II computer.

ELISA inhibition. ELISA plates were sensitized with LPS from the various serogroup 06 strains as described above and blocked with 5% skim milk. Dilutions (1:100) of postimmunization human sera were added in duplicate to these wells, which were incubated for 1 h at 37°C; plates were washed, and then a 1:1,000 dilution of MAb LC3-2H2 (described in Results) was added. Control wells receiving human sera and no MAb and neither sera nor MAb were included. The MAb was left for 1 h at 37°C, and the wells were washed. Next we added alkaline phosphatase-conjugated goat anti-mouse gamma-chain-specific antibody diluted in PBS–5% skim milk–0.05% Tween 20 containing 2% normal human serum. This reagent was incubated for 1 h at 37°C. Plates were washed, substrate was added, and the OD of the wells was read at 30 to 60 min.

The inhibition of binding of the MAb by human sera was calculated as follows: % inhibition = 100 − {[(mean OD of wells with human serum and MAb − mean OD of wells with human serum only)/(mean OD of wells with MAb − mean OD of wells with no antibody)] × 100}.

Opsonophagocytic assay. Bacteria were grown for 4 h in tryptic soy broth, washed twice in RPMI, and suspended to a concentration of 2×10^7 CFU/ml in RPMI with 10% fetal bovine serum. Components of the assay included 0.1 ml of the bacterial suspension; 0.1 ml of dilutions of heat-inactivated (56°C, 30 min) pre- or postimmunization human serum or ascites fluid containing the MAb; 0.1 ml of 10% fresh, nonimmune human serum as a complement source, adsorbed previously with 1 mg of lyophilized serogroup 06 bacteria per ml of 10% complement at 4°C for 30 min; and 0.1 ml of RPMI containing 2×10^6 human peripheral blood leukocytes obtained from whole blood by dextran sedimentation. A sample was removed for dilution and bacterial enumeration at the start of the assay, the tubes were incubated for 90 min with end-over-end rotation, and then a sample was removed for dilution and enumeration of the surviving bacteria. Percent kills were calculated as described before (1). The titer was determined to be the highest serum dilution giving ≥50% kill of bacteria compared with the bacterial count of the control after 90 min of incubation without serum.

In vivo protection assays. The dose of MAb needed to protect 50% (i.e., the protective dose, or PD_{50}) of outbred Swiss-Webster mice (Hilltop Farms, Scottsdale, Pa.) against lethality associated with *P. aeruginosa* intraperitoneal (i.p.) infection was determined by giving groups of five mice graded doses of each MAb (range, 0.01 to 100 μg per mouse in 10-fold dilutions) intravenously 30 to 45 min prior to challenge. The challenge inoculum was calculated to be 25 to 50 times the 50% lethal dose. The PD_{50} was determined by the Spearman-Karber method (10).

Statistical analyses. Kendall rank correlation coefficients and *t* tests were performed with the Statview SE + Graphics software program (Abacus Concepts) on a MacIntosh computer.

RESULTS

ELISA and immunoblot analyses of MAb reactivity with serogroup 06 LPS subtypes. MAbs were prepared by immunizing donor mice with intact bacteria and screening hybridomas with purified LPS from the homologous subtype strain. A total of 34 cell lines were selected from 121 hybridomas whose MAb products demonstrated reactivity during initial screening against LPS from the homologous subtype. There were five fusions in all, each employing spleen cells from at least one mouse immunized with a single subtype strain; all five subtypes were represented among these five fusions. Five of the cloned cell lines, emanating from four separate fusions, were selected for further analysis on the basis of distinctive subtype specificities. These five hybridomas were produced with immunizing and LPS screening antigens from the following strains: Fisher IT-1 (P6-2A2 and P6-1D2); 06a,6b (LB1-1C3); 06a,6c (LC3-2H2); and 06a,6d (LD3-4D6). Mouse ascites fluid containing MAbs produced by these five hybridomas were assayed by ELISA against all five purified serogroup 06 subtype LPS (Table 1). MAb P6-2A2 (IgG2a) reacted with all *P. aeruginosa* LPS subtype antigens except that from strain 06a,6c. LC3-2H2 (IgG1) reacted with LPS from all five subtypes. (Seven of the 34 originally selected hybridomas produced MAb reactive with all five subtype antigens: four were obtained from mice immunized with the 06a,6d subtype strain, and three were from the mice immunized with the 06a,6c strain.) LB1-1C3 (IgG2a) reacted strongly with 06a,6b, weakly with 06a,6c, Fisher IT-1, and Habs 06, and not at all with 06a,6d. LD3-4D6 (IgM) was specific for 06a,6d. P6-1D2 (IgM) reacted strongly with Fisher IT-1 and Habs 06, weakly with 06a,6b, and not at all with 06a,6c and 06a,6d. None of the five MAbs exhibited ELISA binding activity (data not shown) against purified LPS from Fisher immunotypes 2 to 7 or from *P. aeruginosa* AK1401 and PAC557, rough mutant strains which express one of two structurally characterized *P. aeruginosa* core types and the D-rhamnan common antigen (2, 18, 41).

TABLE 1. ELISA reactivity of MAbs with *P. aeruginosa* serogroup 06 LPS subtypes

MAb	Reciprocal titer[a] with subtype strain:				
	06a,6b	06a,6c	06a,6d	Fisher 1	Habs 06
P6-2A2	33,113	<100	33,113	11,220	50,118
LC3-2H2	25,118	33,113	19,952	25,118	33,113
LB1-1C3	25,118	165	<100	251	251
LD3-4D6	<100	<100	1,659	<100	<100
P6-1D2	112	<100	<100	19,952	5,011

[a] Reciprocal titer was determined by regression analysis of the linear portion of the dilution curve obtained when OD (x) was plotted against ascitic fluid dilution (y); the resultant equation was solved for y (titer) when $x = 0.5$.

Immunoblot analysis of MAbs against whole-cell extracts and purified LPS from various serogroup 06 subtypes (Fig. 2) revealed patterns of MAb specificity generally in accordance with those demonstrated by ELISA. In particular, the immunoblot analysis appeared to corroborate the predominant reactivity of MAbs LB1-1C3, LD3-4D6, and P6-1D2 with subtypes 06a,6b, 06a,6d, and Fisher IT-1, respectively. The analysis also confirmed the reactivity of MAb P6-2A2 with all subtypes except 06a,6c and the reactivity of MAb LC3-2H2 with all subtypes. Minor discrepancies included the following. (i) By ELISA, MAb LC3-2H2 bound to LPS from all five subtypes; by immunoblot analysis, however, although the MAb recognized at least one antigen representing each of the five subtypes, it did not bind to purified LPS from strain 06a,6d or a whole-cell extract from the Habs 06 subtype. (ii) By ELISA, MAb P6-1D2 bound to Fisher IT-1 and Habs 06 LPS; by immunoblotting, in contrast, this MAb bound to IT-1 cell extract but not purified IT-1 LPS and only weakly to purified LPS from the HAbs 06 strain.

The banding patterns observed on immunoblots were also of interest. A typical ladder pattern commonly associated with O-side chain-containing LPS was produced by all of the MAbs except P6-2A2 (Fig. 2). This MAb reacted predominantly with faster-migrating (lower-molecular-weight) material compared with that recognized by the other four MAbs; although some multiple banding was observed, it was associated primarily with lower-molecular-weight material and was not in a characteristic ladder pattern. The distinctive immunoblot patterns produced by various subtype-reactive MAbs are illustrated by the different staining configurations observed when IT-1 whole-cell extract (lane 1 in each blot shown in Fig. 2) was reacted with MAbs P6-2A2, LC3-2H2, and P6-1D2, all of which reacted strongly with the IT-1 subtype in ELISA and immunoblotting assays. As indicated, P6-2A2 reacted primarily with fast-migrating material, while LC3-2H2 recognized more slowly migrating bands and P6-1D2 developed only the most slowly migrating (highest-molecular-weight) bands.

Opsonophagocytic activity of MAbs against serogroup 06 LPS subtype strains. All five MAbs mediated opsonophago-

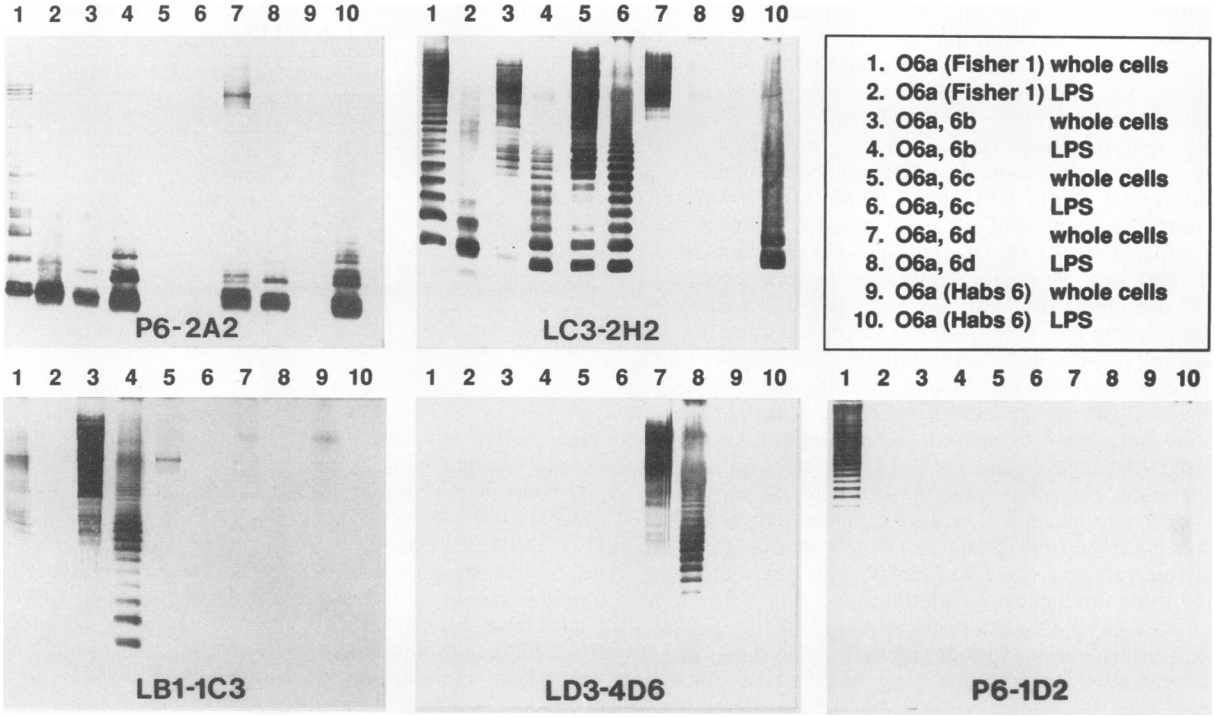

FIG. 2. Binding of the various MAbs indicated in the label on each blot to boiled whole cells and purified LPS extracted from the five subtype strains of *P. aeruginosa* serogroup 06. Preparation in each lane corresponds to number in legend.

cytic killing of 06 subtype strains in the presence of human peripheral blood leukocytes and fresh human serum; subtype specificity coincided almost exactly with that demonstrated by ELISA (Table 2). The correlation between opsonic (Table 2) and ELISA (Table 1) titers was high ($P = 0.001$, Kendall rank correlation corrected for ties). There was no apparent relation between MAb isotype and opsonic potential; good opsonic activity was seen as long as the MAb exhibited binding activity by ELISA against the particular subtype strain.

Protective activity of MAbs against i.p. challenge with serogroup 06 LPS subtype strains in mice. Protection was conferred by four of five MAbs against live i.p. challenge with at least one subtype strain (Table 3); the fifth MAb, LD3-4D6, afforded no in vivo protection despite binding activity against subtype 06a,6d in ELISA and immunoblot assays and documented opsonic activity against this subtype. The most broadly protective MAb was LC3-2H2, which prevented mortality by all subtypes except Habs 06. The failure of protection by LC3-2H2 against Habs 06 was consistent with the inability of this MAb to recognize a whole-cell extract of Habs 06 on immunoblots but was inconsistent with the MAb's demonstrated binding activity against Habs 06 LPS in ELISA and immunoblot assays and opsonic activity against this subtype. Although at least one subtype-associated discrepancy was noted between opsonic and protective activities in the cases of all five MAbs (two in the case of P6-2A2), there was, nevertheless, overall agreement between these in vitro and in vivo MAb functions. For example, a high opsonic titer (>1:500) corresponded to a low PD_{50} (<20 μg per mouse), or a low opsonic titer (<1:100) corresponded to a high PD_{50} (>126 μg per mouse) in 19 of 25 MAb-subtype pairings ($P = 0.02$ by Kendall rank correlation corrected for ties; Tables 2 and 3). There was also good agreement between ELISA titers and PD_{50} values ($P = 0.04$ by Kendall rank correlation corrected for ties; Tables 1 and 3).

Serum antibody responses to serogroup 06 LPS subtypes in normal human subjects immunized with Fisher IT-1 high-

TABLE 2. Opsonophagocytic killing of *P. aeruginosa* serogroup 06 LPS subtype strains by various subtype-reactive MAbs[a]

MAb	Titer[b] against subtype strain:				
	06a,6b	06a,6c	06a,6d	Fisher IT-1	Habs 06
P6-2A2	500	<100	1,000	2,000	5,000
LC3-2H2	500	10,000	1,000	500	1,000
LB1-1C3	10,000	100	<100	<100	<100
LD3-4D6	<100	<100	1,000	<100	<100
P6-1D2	100	100	<100	10,000	5,000

[a] See Table 1 for ELISA reactivities of MAbs with various serogroup 06 subtype antigens.
[b] The titer represents the reciprocal of the highest dilution of ascites fluid yielding ≥50% bacterial kill.

TABLE 3. Protective activity of LPS-reactive MAbs against i.p. challenge with *P. aeruginosa* serogroup 06 LPS subtypes in mice[a]

MAb	PD_{50} (μg per mouse) against subtype strain:				
	06a,6b	06a,6c	06a,6d	Fisher IT-1	Habs 06
P6-2A2	>200	>200	12.6	2	>200
LC3-2H2	5	0.8	20	2	>316
LB1-1C3	0.2	12.6	>316	>126	>200
LD3-4D6	>200	>316	>316	>126	>126
P6-1D2	>200	>200	>200	0.8	>316

[a] See Table 1 for ELISA reactivities of MAbs with various serogroup 06 subtype antigens.

TABLE 4. ELISA titers against various *P. aeruginosa* serogroup 06 subtype strains in human sera obtained 28 to 42 days after immunization with 100 μg of Fisher IT-1 subtype high-molecular-weight polysaccharide vaccine

Vaccinee	Preimmune titer range	Postimmunization serum titer[a] against LPS from subtype strain:				
		06a,6b	06a,6c	06a,6d	Fisher IT-1	Habs 06
1	75–219	7,831	4,333	4,447	8,788	5,043
2	94–150	4,172	4,870	2,641	4,645	1,077
3	75–169	10,766	5,333	5,835	8,747	6,360
4	All ≤50	1,018	843	1,462	908	823
5	All ≤50	2,100	1,389	1,372	1,949	1,160
6	56–188	3,256	4,164	1,477	2,595	2,823
7	≤50–98	2,112	1,831	3,007	3,578	2,520
8	94–263	9,706	11,000	12,740	8,567	13,200

[a] See footnote *a*, Table 1.

molecular-weight polysaccharide vaccines. Serum was obtained from eight normal adult volunteers 28 to 42 days after subcutaneous injection with 100 μg of a high-molecular-weight polysaccharide vaccine closely related to the O-polysaccharide of Fisher IT-1 LPS (27, 36). Immune sera produced in response to immunization with LPS-derived material from this single subtype were evaluated for binding activity by ELISA (Table 4) and opsonic activity (Table 5) against all five serogroup 06 subtypes, including IT-1.

All eight subjects demonstrated impressive increases in serum ELISA reactivity against LPS from all five serogroup 06 subtypes (Table 4). The ELISA titers of individual subjects against various subtypes were similar; those individuals with high titers to one subtype antigen also tended to have high titers against the other subtypes (e.g., see subjects 3 and 8 in Table 4). Moreover, titers against the homologous IT-1 subtype were not consistently higher than those demonstrated against heterologous subtypes.

There was a strong correlation between ELISA reactivity (Table 4) and opsonic titers (Table 5) of postimmunization sera ($P < 0.001$ by Kendall rank correlation). While seven of eight subjects had no detectable preimmunization opsonic activity against any of the subtype strains, all subjects had readily detectable opsonic activity against all five subtype strains after immunization. As in the case of ELISA reactivity, most subjects exhibited similar titers against the various serogroup 06 subtypes.

TABLE 5. Opsonic titers of human sera 28 to 42 days after immunization with 100 μg of *P. aeruginosa* serogroup 06 (Fisher IT-1 subtype) high-molecular-weight polysaccharide vaccine

Vaccinee	Opsonic titer[a] against strain:				
	06a,6b	06a,6c	06a,6d	Fisher IT-1	Habs 06
1	8	256	128	256	32
2	8	8	32	128	32
3	256	256	256	256	256
4	64	64	64	64	64
5	64	64	32	128	64
6	64	64	64	64	64
7	64	64	64	64	64
8	512	512	512	512	512

[a] Reciprocal of highest twofold serum dilution yielding ≥50% killing of the starting inoculum after 90 min. All preimmunization sera had reciprocal titers of <4, except that of vaccinee 8, for which there was a titer of 8 against all strains.

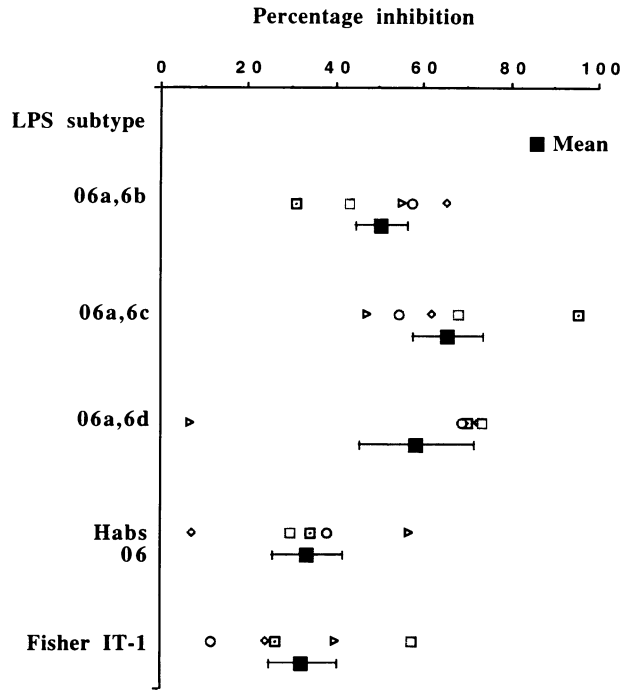

FIG. 3. Inhibition of the binding of MAb LC3-2H2, reactive with LPS from all representative structures of serogroup 06, by 1:100 dilutions of antisera obtained from humans immunized with 100 μg of the high-molecular-weight polysaccharide antigen from the Fisher IT-1 strain of *P. aeruginosa*. Each symbol represents the values for one individual, and the larger solid box indicates the mean for the group. Bars indicate the standard errors of the means.

Inhibition by postimmunization sera of binding by MAb LC3-2H2 to serogroup 06 subtype LPS. MAb LC3-2H2 reacts with LPS from all five serogroup 06 subtypes and thus may recognize a common epitope shared by all five subtypes. We investigated whether this putative common epitope was recognized by sera from five subjects immunized with IT-1 high-molecular-weight polysaccharide vaccine by evaluating the ability of each serum to inhibit the ELISA reactivity of MAb LC3-2H2 with each of the 06 subtype antigens. All of the immune sera inhibited MAb binding to multiple LPS subtypes (range, 31 to 65% inhibition) (Fig. 3). Two of the serum samples inhibited MAb binding to all five subtypes, and the remaining three serum samples appeared to inhibit MAb binding to four of five subtype antigens. In each of the three instances in which MAb reactivity did not appear to be inhibited by immune serum, a different antigen and a different serum were involved (Fig. 3). These data suggest that the basis for the observed cross-reactivity of immune sera produced through immunization of human subjects with IT-1 high-molecular-weight polysaccharide may be the induction of antibody that recognized the common epitope recognized by MAb LC3-2H2.

DISCUSSION

Our results document that it is possible to produce O-side chain-specific MAbs capable of distinguishing among structurally related *P. aeruginosa* serogroup 06 LPS subtypes. These findings reinforce the concept that there are essentially two levels of structural and antigenic variation among LPS from different *P. aeruginosa* strains. These are expressed either as a

serogroup-defining difference in the monosaccharide composition or as a subtype-determining variation in the anomeric configuration or substitution pattern of a basic O-side chain repeat unit. Our data thus confirm, in the case of the most common *P. aeruginosa* serogroup isolated from patients with *P. aeruginosa* infections (35), a finding previously reported for other *P. aeruginosa* serogroups as well (19, 45).

ELISA and immunoblot analyses of five different MAbs employing purified LPS or whole bacteria representing the five known IATS serogroup 06 subtypes revealed five distinct patterns of subtype specificity, ranging from recognition of a single subtype to reactivity with all five. Failure of the MAbs to react with LPS from Fisher IT-2 through IT-7 strains or with LPS from rough strain AK 1401 or PAC1R suggested that the MAbs recognized O-side chain-related structures not shared by other serogroups.

Striking qualitative differences were observed in the migration characteristics of LPS species recognized by different subtype-specific MAbs on immunoblots produced from SDS-PAGE gels run under identical conditions. The typical ladder pattern manifested by more slowly migrating LPS species that stained with MAb LC3-2H2, for example, contrasted with the faster-migrating LPS molecules recognized by P6-2A2. It is tempting to speculate on the basis of these staining patterns that the greater protective activity of MAb LC3-2H2 against live challenge with 06 subtype strains was somehow related to the ability of this antibody to recognize larger LPS molecules containing longer O-side chains. Similarly, the greater protective capacity of MAb P6-2A2 against subtypes IT-1 and 06a,6d compared with 06a,6b may have been related to recognition by this MAb of more slowly migrating material produced by the IT-1 and 06a,6d subtypes compared with that produced by 06a,6b.

Opsonophagocytic activity of MAbs exhibited a pattern of subtype specificity that corresponded closely with binding activity. Moreover, although some discrepancies existed between opsonic activity and protection against live i.p. challenge in mice, most MAbs that were opsonic for a particular subtype strain also protected against that strain in vivo. Among the exceptions to this general pattern was LD3-4D6, which demonstrated binding activity against subtype 06a,6d LPS in ELISAs and immunoblot assays and opsonic activity against this strain but no in vivo protection. The basis for this and other documented discrepancies between MAb opsonophagocytic and protective activity is unknown. However, the finding emphasizes the potential dichotomy between in vitro and in vivo MAb function and the resulting need to carry out in vivo as well as in vitro testing of vaccine-induced immune sera or MAbs intended for clinical use.

A potential concern stemming from demonstrated subtype-related differences in O-side chain structure and antigenicity, and corresponding variations in the subtype specificity of O-side chain-reactive MAbs, is the immunoprophylactic or therapeutic coverage provided by O-side chain-based vaccines (5, 6, 7, 28, 43) or MAbs directed to these structures (12, 24, 25). For example, an octavalent *P. aeruginosa* O-polysaccharide–toxin A conjugate vaccine was recently evaluated for immunogenicity in humans solely on the basis of antibodies induced against prototype strains used in making the vaccine (6, 44). The possible subtype specificity of immune responses to this vaccine was thus ignored. Likewise, animal and human immune responses to *P. aeruginosa* high-molecular-weight polysaccharide vaccines have been evaluated primarily against those strains employed in vaccine preparation (27, 29). Similarly, a cocktail of human MAbs directed against LPS from five different *P. aeruginosa* serogroups (42) appears to have been screened against a single strain from each of the represented serogroups, raising the possibility that one or more of the component MAbs is directed against subtype- rather than serogroup-specific determinants.

It was encouraging that despite the restrictive subtype specificity of some MAbs in this study (e.g., LB1-1C3, LC3-4D6, and P6-1D2), others (e.g., P6-2A2 and LC3-2H2) appeared to cross-react more broadly among different serogroup 06 subtypes. For practical purposes, in fact, since LC3-2H2 reacted with all 06 LPS subtypes, it should be considered a serogroup- rather than a subtype-specific antibody, while the epitope recognized by LC3-2H2 is properly seen as a serogroup rather than a subtype determinant.

The demonstration of a murine MAb capable of recognizing an LPS determinant apparently common to all subtypes of IATS serogroup 06 paralleled the finding that human subjects immunized with a high-molecular-weight polysaccharide vaccine derived from Fisher IT-1 *P. aeruginosa* (one of five 06 subtypes) exhibited similar serum antibody responses to all 06 subtypes in terms of both binding activity and opsonic function. These parallel observations were linked by the demonstration that postimmunization sera from human subjects vaccinated with the Fisher IT-1 high-molecular-weight polysaccharide were capable of inhibiting binding by MAb LC3-2H2 to all serogroup 06 subtype antigens. Together, these observations suggest the feasibility of producing serogroup-specific MAbs and polyclonal antibodies to epitopes shared by various LPS subtypes. Further suggested is the likelihood that the cross-reactive serum antibody response induced by immunization with the Fisher IT-1 high-molecular-weight polysaccharide vaccine was based at least in part on antibodies similar or identical in epitope specificity to that of MAb LC3-2H2.

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