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Humoral Immune Response of Rabbits to Acholeplasmal Lipoglycans

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Lipoglycans extracted from *Acholeplasma* species with hot aqueous phenol were immunogenic for rabbits when introduced by an appropriate method. All lipoglycans examined elicited antibody associated with a heavy, 2-mercaptoethanol-sensitive immunoglobulin fraction when inoculated intravenously adsorbed to autologous rabbit erythrocytes. This antibody was specific for the *Acholeplasma* species from which the lipoglycan was extracted. Extensive immunization of these animals with acholeplasmal lipoglycans produced significant increases in sheep erythrocyte hemolysin. Some, but not all, *Acholeplasma* species yielded lipoglycans that were immunogenic when emulsified with Freund complete adjuvant and introduced via the footpad into rabbits. Such animals produced antibodies corresponding to the M and G immunoglobulin classes that reacted with both homologous and heterologous acholeplasmal lipoglycans by precipitation in immunodiffusion as well as passive hemagglutination. None of the animals inoculated demonstrated a significant anamnestic response after booster injections either intravenously or via the footpads.

Several members of *Mollicutes*, in particular the genus *Acholeplasma*, contain membrane-associated lipoglycans, previously referred to as lipopolysaccharides (9, 12, 13). These lipoglycans react serologically with specific antisera raised in rabbits against membrane preparations of the appropriate organism (14). In contrast to gram-negative bacterial lipopolysaccharides, the mycoplasmal lipoglycans contain no phosphate, ethanolamine, or 2-keto-3-deoxyoctonic acid but consist of a long oligosaccharide chain attached covalently to a diglyceride (13). Each species of *Acholeplasma* contains one structurally homogeneous polymer (12, 13). The sugar composition varies both qualitatively and quantitatively between lipoglycans from different species and consists of hexoses, hexosamines, and deoxyhexosamines (13). The fatty acids in the lipoglycans from all species, except *A. axanthum*, occur esterified, presumably to the glycerol moiety. The predominating fatty acids of *A. axanthum* lipoglycan are 3-hydroxymyristic and 3-hydroxypalmitic acids (12, 13). Ninety percent of the total fatty acids in this lipoglycan are linked in *N*-acyl bonds to the amino sugars (12). However, in all the acholeplasmal lipoglycans, the amino groups are *N*-acylated, probably with acetyl groups. This present study was undertaken to determine the immunogenicity of the purified lipoglycans and to characterize their antigenic specificity.

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MATERIALS AND METHODS

The lipoglycans from five species of *Acholeplasma* (*A. axanthum* strain S743, *A. granularum* strain BTS39, *A. laidlawii* strain B, *A. modicum* strain PG49, and *A. oculi* strain 19L) and *Mycoplasma neurolyticum* strain PG39 were used in this study. The organisms were grown as previously described (13). The lipoglycans were extracted from lipid-depleted cells with hot aqueous phenol (13) and purified by digestion with nucleases followed by chromatography on controlled-pore-size glass beads (12, 13). These purified preparations were homogenous and contained less than 0.01% protein (12, 13).

Immunization of rabbits. Adult New Zealand white or red rabbits weighing 2 to 3 kg were inoculated either via the footpads or marginal ear vein. The initial course of injection for the footpad route consisted of a primary injection of 0.5 ml containing 1 mg of lipoglycan emulsified with an equal volume of Freund complete adjuvant. On days 14 and 28, a similar dose of lipoglycan emulsified with an equal volume of Freund incomplete adjuvant was injected into the footpads. Sera obtained from these animals 7 days after the second booster injection are referred to as early immune sera. Subsequently, these animals were given bimonthly footpad inoculations of 100 μg of lipoglycan dissolved in phosphate-buffered saline (0.01 M sodium phosphate buffered with 0.15 M NaCl, pH 7.0) for a period of 10 months. Sera collected 10 days after the last injection are referred to as late immune sera.

Intravenous inoculations employed lipoglycans adsorbed onto autologous rabbit erythrocytes. Suspensions (10%) of autologous rabbit erythrocytes in phosphate-buffered saline were mixed with an equal volume of lipoglycan (200 μg) and incubated for 2 h at room temperature followed by overnight incubation at 4°C. The adsorbed erythrocytes were washed twice and resuspended to 10% in phosphate-buffered saline. The initial course of inoculation for the intravenous introduction of antigen consisted of four daily injections (0.5 ml of 10% adsorbed erythrocyte suspension each) followed by 3 days of rest over a 3-week period. Sera collected 3 days after the last injection are referred to as early immune sera. After four bimonthly injections via the footpads with 100 μg of lipoglycan dissolved in phosphate-buffered saline, a second series of three daily intravenous injections with lipoglycan adsorbed to autologous erythrocytes was made. Sera collected 5 days after the last injection are referred to as late immune sera.

Sera were collected at selected intervals and stored at −56°C. Antisera were prepared against mycoplasmal membranes as described previously (14).

Serological tests. Immunodiffusion was carried out in petri dishes or microscope slides with 0.5% Ionagar (Oxoid) in a modified barbital buffer (2) and patterned after Ouchterlony (11). Precipitin bands were developed at 4°C in a moist chamber. Passive hemagglutination and passive immune hemolysis were performed in microtiter plates (Cooke Engineering Corp., Alexandria, Va.). Twofold serial dilutions of 50 μl of heat-inactivated (60°C, 30 min) antiserum were mixed with 50 μl of a 2% suspension of sheep or rabbit erythrocytes coated with the appropriate lipoglycan. Guinea pig complement (25 μl of a 1:80 dilution) was added for the hemolysis reaction. Control experiments indicated that the lipoglycan-coated erythrocytes were not lysed by complement more dilute than 1:30 unless antiserum to specific lipoglycan or to erythrocytes was present. The lipoglycan-coated erythrocytes for serological tests were prepared by incubating at room temperature for 60 min a 5-ml volume of lipoglycan (100 μg/ml) with erythrocytes obtained by centrifugation (800 × *g*) of 10 ml of a photometrically prepared 2% suspension. Incubation at 37 or 4°C for periods of 1 to 15 h did not alter significantly the adsorption of lipoglycan to the erythrocytes. Lipoglycan concentrations of less than 50 μg/ml or greater than 500 μg/ml were less satisfactory. At low concentrations too little lipoglycan was adsorbed per erythrocyte, whereas at high concentrations considerable lysis of erythrocytes occurred with some lipoglycans, in particular those from *A. oculi* and *A. axanthum*. Mild deacylation (N KOH, 37°C, 10 min) or heating at 100°C for 2 h, procedures used to facilitate adsorption of bacterial lipopolysaccharides (3, 5, 6, 10), did not alter the efficiency of adsorption of acholeplasmal lipoglycans. After adsorption, the lipoglycan-coated erythrocytes were washed three times with modified barbital buffer and resuspended in the same buffer to a final concentration of 2%. Serum hemolysis titers to sheep erythrocytes were estimated for 50% endpoints by the method of Campbell et al. (2).

Fractionation of antisera. Antisera (5 to 10 ml) were passed through a column of either Sephadex G-200 or Ultragel AcA-22 (Pharmacia, Uppsala, Sweden; 2.5 by 90 cm) at a flow rate of 11 to 15 ml/h with 0.1 M tris(hydroxymethyl)aminomethane containing 0.2 M NaCl, pH 8.0, as eluting solvent (4). The protein content of each 2-ml fraction was estimated by measuring adsorbancy at 280 nm. Fractions forming separate protein peaks were pooled, dialyzed against 10 volumes of NaCl (0.12 M) at 4°C for 48 h, lyophilized, and restored to the original volume of serum applied to the column.

Density gradient centrifugation was performed in a SW50 rotor with a preformed sucrose gradient (10 to 40%). After centrifugation at 149,000 × *g* for 12 h, 15-drop fractions were collected from the bottom of the centrifuge tube and assayed for serological activity.

Mercaptoethanol treatment of the serum was accomplished by reacting 4 ml of the serum (diluted 1:2 with saline) with 4 ml of 2-mercaptoethanol (0.2 M) for 30 min at room temperature. The reaction was terminated by dialysis against iodoacetamide (0.2 M) for 24 h followed by dialysis against phosphate-buffered saline for 48 h.

RESULTS

Typical passive hemagglutination titers of rabbit sera after inoculation with lipoglycans from mycoplasmas are shown in Tables 1 and 2. Although the titers are not high, they are significant and reproducible in that no more than a twofold difference was noted among the four to eight animals used for each immunogen. Generally, the emulsion of lipoglycan in Freund adjuvant enhanced the antibody level. An exception was noted with the lipoglycan from *A. granularum*, which exhibited the greatest enhancement of antibody production when the lipoglycan was adsorbed onto autologous rabbit erythrocytes before intravenous inoculation. The lack of immunogenicity in adjuvant may be due to its smaller molecular size (12). Similar titers were noted with most of the sera when assayed by the passive hemolysin technique with sheep erythrocytes. Although rabbit erythrocytes could be used interchangeably in the passive hemagglutination assays, they produced inconsistent results when used with guinea pig complement in the passive hemolysis test. No attempts were made to standardize this test further by alterations of the source or concentration of complement. A potential problem with the use of sheep erythrocytes in these tests was noted with sera collected after repeated inoculations of the lipoglycans into rabbits. Many of these sera showed significant increases in sheep erythrocyte hemolysin (Table 3). Hence, it was necessary to heat inactivate and repeatedly adsorb these sera with normal sheep erythrocytes before assaying with lipoglycan-coated sheep erythrocytes. This adsorption was not necessary when rabbit erythrocytes were used, since no

TABLE 1. *Passive hemagglutination titers of rabbit sera produced in response to footpad inoculations*[a]

In vitro antigen (lipoglycan-sheep erythrocyte)	Antiserum titer to acholeplasmal lipoglycan							
	A. axanthum		*A. granularum*		*A. laidlawii B*		*A. modicum*	
	Early[b]	Late[c]	Early	Late	Early	Late	Early	Late
A. axanthum	256	64	16	16	32	32	<4	32
A. granularum	<4	32	16	16	512	128	<4	128
A. laidlawii	<4	<4	<4	4	128	16	<4	<4
A. modicum	<4	<4	<4	<4	<4	<4	256	256
A. oculi	16	32	32	4	4	4	512	256
M. neurolyticum	32	32	<4	<4	32	128	8	16
E. coli O111-B4 lipopolysaccharide	<4	<4	<4	<4	<4	<4	<4	<4

[a] Reciprocal of the highest dilution of serum that produces visible agglutination with 2% sheep erythrocytes coated with lipoglycan. Pooled sera of four animals per immunogen, except eight animals inoculated with *A. modicum*, lipoglycan.

[b] Serum collected 7 days after the initial immunization protocol (see text).

[c] Serum collected 10 days after the last booster (lipoglycan in phosphate-buffered saline). All sera were adsorbed with washed sheep erythrocytes before assay with antigen. All preimmune titers < 4.

TABLE 2. *Hemagglutination titers of rabbit sera produced in response to intravenous inoculations*[a]

In vitro antigen (lipoglycan-sheep erythrocyte)	Antiserum titer to acholeplasmal lipoglycan							
	A. axanthum		*A. granularum*		*A. laidlawii*		*A. modicum*	
	Early[b]	Late[c]	Early	Late	Early	Late	Early	Late
A. axanthum	64	32	>4	>4	>4	16	>4	>4
A. granularum	>4	>4	64	128	>4	>4	>4	>4
A. laidlawii	>4	>4	>4	>4	128	64	>4	>4
A. modicum	>4	>4	>4	>4	>4	>4	32	128

[a] Reciprocal of the highest dilution of serum that produces visible agglutination with 2% sheep erythrocytes coated with lipoglycan. Pooled sera of eight animals inoculated with lipoglycan, except four animals inoculated with the lipoglycan of *A. laidlawii* B.

[b] Serum collected 3 days after the initial course of immunization (see text).

[c] Serum collected 5 days after the secondary course of immunization (see text). All sera were adsorbed with washed sheep erythrocytes before assay with antigen. All preimmune titers < 4.

hemolysin to rabbit erythrocytes was produced on immunization with lipoglycans. As can be noted in Table 3, no significant enhancement of hemolysin activity occurred when membranes were used as immunogens. Usually the greatest activity was noted with sera collected after several booster injections of lipoglycans in phosphate-buffered saline.

Analyses of the antisera by immunodiffusion indicated that the method of immunization had a significant effect on the precipitin patterns. None of the sera obtained after intravenous injection of the lipoglycan-coated autologous erythrocytes, regardless of the passive hemagglutination titer, yielded precipitin bands with any of the lipoglycans. These same lipoglycans produced precipitin bands with antisera produced by footpad inoculation (Fig. 1). Sera collected during the initial immunization period produced two diffuse bands. However, sera collected after extensive booster injections with lipoglycans in phosphate-buffered saline usually gave rise to only one distinct band. The addition of 2-mercaptoethanol to the wells did not alter the number of precipitin bands. Attempts to recover the second band by reintroducing the lipoglycan in adjuvant into extensively immunized rabbits were unsuccessful.

Sera obtained after footpad immunization with the lipoglycans in adjuvant revealed cross-reactivity among certain lipoglycans when examined by passive hemagglutination (Table 1) and by immunodiffusion (Fig. 2). These data suggest the presence of shared antigenic determinants among the lipoglycans from *A. granularum, A. modicum, A. oculi,* and *A. axanthum.* The lipoglycan from *A. laidlawii* did not react visibly with the antiserum to the lipoglycans from *A. modicum* (Fig. 2A) or *A. axanthum* (Fig. 2B). None of the acholeplasmal lipoglycans reacted with antiserum prepared against the lipoglycan from *Thermoplasma* (9, 13). This antiserum precipitates with its homologous lipoglycan.

The serological titers shown in Tables 1 and 2 suggested that an anamnestic response was

TABLE 3. *Sheep erythrocyte hemolysin activity of rabbit serum after immunization with Acholeplasma*

Immunogen	Route[a]	H_{50}/ml of serum[b]		
		Preimmune	Early serum	Late serum
A. axanthum lipoglycan	i.v.	24	129	140
A. axanthum lipoglycan	fp	6	12	25
A. axanthum membrane	fp	32	28	18
A. granularum lipoglycan	i.v.	17	81	68
A. granularum lipoglycan	fp	17	41	21
A. granularum membrane	fp	16	12	30
A. laidlawii lipoglycan	i.v.	24	136	105
A. laidlawii lipoglycan	fp	72	48	63
A. laidlawii membrane	fp	ND	24	10
A. modicum lipoglycan	i.v.	5	562	933
A. modicum lipoglycan	fp	11	45	147
A. modicum membrane	fp	ND	10	7

[a] i.v., Intravenous; fp, footpad.

[b] Mean H_{50} (50% hemolytic complement units) of 20 nonimmunized rabbits, 19 ± 17.

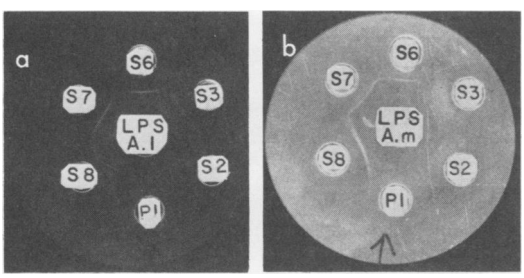

FIG. 1. *Immunodiffusion patterns obtained with sera after immunization or rabbits via the footpads with acholeplasmal lipoglycans. (a) Center well: 100 μg of A. laidlawii lipoglycan; (b) center well: 100 μg of A. modicum strain PG49 lipoglycan. Outer wells: antiserum to the homologous lipoglycan selected intervals; PI, preimmune; S2, 7 days after initial immunization; S3, 20 days after initial immunization; S6, 5 days after third booster (ca. 6 months); S7, 5 days after fourth booster (8 months); S8, 5 days after fifth booster (10 months).*

absent in rabbits immunized with lipoglycans. Even after extensive booster injections, titers were seldom greater than those obtained after an initial course of immunization, regardless of the route of injection. Sera, selected as representative of each immunization procedure, were subjected to gel filtration, sucrose density gradient centrifugation, and degradation with 2-mercaptoethanol. The antibody obtained after intravenous inoculation of the lipoglycan of *A. axanthum* adsorbed to autologous rabbit erythrocytes is characterized in Fig. 3 and Table 4. All

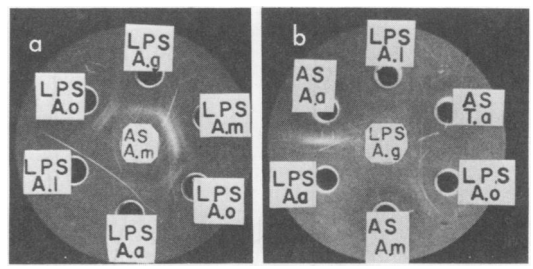

FIG. 2. *Cross-reactivity among acholeplasmal lipoglycan as evidenced by immunodiffusion. (a and b) AS Am, early antiserum after footpad inoculation with lipoglycan from A. modicum; AS A.a., early antiserum after footpad inoculation with lipoglycan from A. axanthum; AS T.a., early antiserum after footpad inoculation with membrane preparation of T. acidophilum; LPS A.a., 100 μg of A. axanthum lipoglycan; LPS A.o., 100 μg of A. oculi lipoglycan; LPS A.m., 100 μg of A. modicum lipoglycan; LPS A.g., 100 μg of A. granularum lipoglycan; LPS A.l., 100 μg of A. laidlawii lipoglycan.*

of the antibody activity was found in fractions corresponding to the heavy class of immunoglobulins. This antibody activity was destroyed by reacting the whole serum or its fractions with 2-mercaptoethanol (Table 4). On the other hand, similar experiments with antiserum to the lipoglycan of *A. modicum* emulsified with adjuvant and injected via the footpad showed antibody activity in fractions corresponding to a heavy, 2-mercaptoethanol-sensitive immunoglobulin as well as a light, 2-mercaptoethanol-resistant immunoglobulin (Fig. 4 and 5; Table 5). Both early and late sera contained a light, 2-mercaptoethanol resistant immunoglobulin with antibody activity against the lipoglycan. Immunodiffusion of this immunoglobulin against anti-rabbit immunoglobulin G (IgG; Miles Laboratories, Elkhart, Ind.) produced a precipitin band indicative of its identity as IgG.

The Jerne plaque technique (8) was utilized to verify the character of the antibody produced in response to extensive immunization with these immunogens. The spleens were removed from rabbits after immunization via the footpad and intravenous routes with the lipoglycan of *A. modicum*. The individual antibody-producing cells were visualized with sheep erythrocytes coated with the lipoglycan. Background plaques with uncoated sheep erythrocytes served as controls. These data (Table 6) suggest that the predominant antibody produced against this lipoglycan following the footpad route of immunization is IgM. The number of plaques are not increased by the use of facilitating antisera. The plaques were eliminated with 2-mercaptoethanol treatment (8). Extensive immunization by

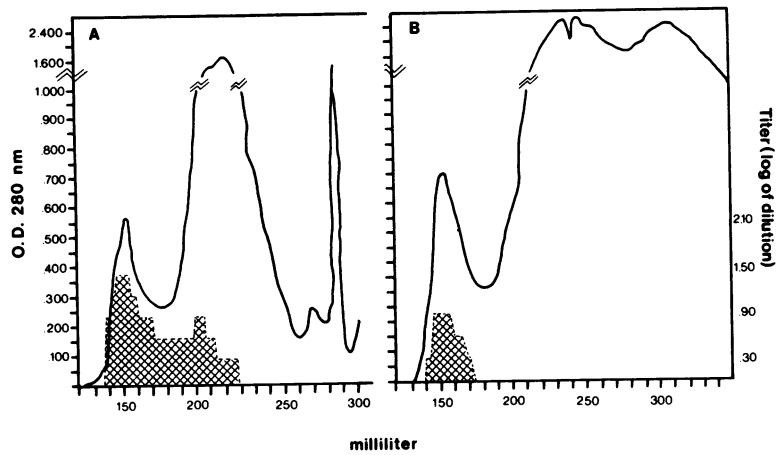

FIG. 3. *Fractionation of rabbit antiserum to lipoglycan of A. axanthum on Sephadex G-200. (A) 5 ml of serum collected 3 days after initial course of intravenous inoculation with lipoglycan-autologous rabbit erythrocyte immunogen. (B) 5 ml of serum collected 5 days after secondary course of immunization. Aliquots (5 ml) eluted with 0.1 M tris(hydroxymethyl)aminomethane–0.2 M NaCl, pH 8.0. Solid line represents absorbancy at 280 nm. Cross-hatched area represents indirect hemagglutination titers.*

TABLE 4. *Passive hemagglutination titers and mercaptoethanol sensitivity of sucrose density gradient fractions of anti-A. axanthum lipoglycan*

Fraction no.[a]	Early Serum[b]				Late serum[c] (pool 1)	
	Pool 1[d]		Pool 2[e]			
	IHA titer[f]	2-ME sensitivity[g]	IHA titer	2-ME sensitivity	IHA titer	2-ME sensitivity
1	2	+	2	+	2	+
2	2	+	2	+	8	+
3	4	+	8	+	32	+
4	8	+	16	+	32	+
5	32	+	16	+	8	+
6	64	+	2	+	4	+
7	128	+	2	+	4	+
8	16	+		NA	2	+
9	4	+		NA	2	+
10	2	+	2	NA		NA
11	2	+		NA		NA
12–23		NA		NA		NA

[a] Fifteen-drop fractions were collected from the bottom of the gradient.
[b] Serum collected 3 days after the initial course of immunization (see text).
[c] Serum collected 5 days after the secondary course of immunization (see text).
[d] Pool 1: 10 to 170 ml of column eluate (Fig. 3).
[e] Pool 2: 171 to 230 ml of column eluate (Fig. 3).
[f] Reciprocal of the highest dilution of serum fraction that produced detectable agglutination of 2% lipoglycan-coated sheep erythrocytes. IHA, Indirect hemagglutination.
[g] 2-Mercaptoethanol (2-ME, 0.1 M) was added to the wells of a microtiter plate containing serum fraction serially diluted with phosphate-buffered saline. Plates were read after a 1-h incubation at room temperature with lipoglycan-coated sheep erythrocytes. + denotes mercaptoethanol sensitivity. NA, Not applicable.

the intravenous route of administration leads to the production of antibodies, detected by both the direct and indirect techniques, that reacted with untreated sheep erythrocytes as well as sheep erythrocytes with adsorbed lipoglycan. This antibody activity is probably associated with the significant increase in hemolysin activity noted in Table 3. The high levels of hemolysin after extensive intravenous introduction of these immunogens limits the usefulness of this technique.

DISCUSSION

The humoral immune response elicited by rabbits to the lipoglycans isolated from several species of *Acholeplasma* was dependent upon

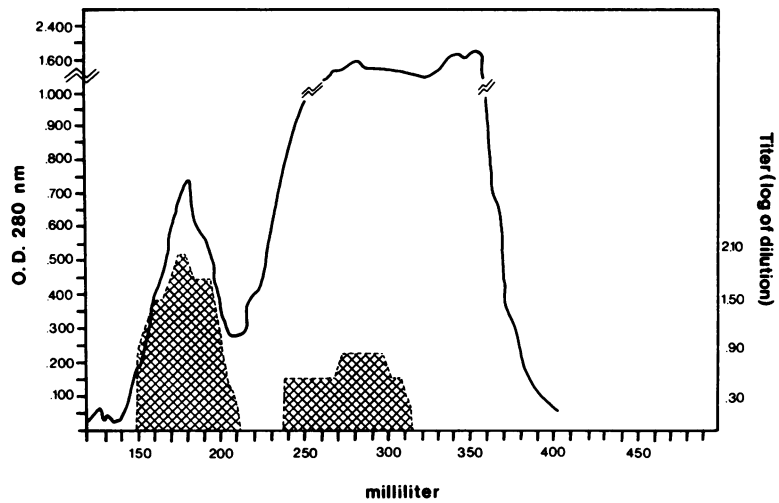

FIG. 4. *Fractionation of 10 ml of early immune serum to lipoglycan of A. modicum on Sephadex G-200. Aliquots (5 ml) eluted with 0.1 M tris(hydroxymethyl)aminomethane–0.2 M NaCl, pH 8.0. Solid line represents absorbancy at 280 nm in a Beckman DU-2 spectrophotometer. Cross-hatched area represents indirect hemagglutination titers. Early sera: serum collected 3 days after the initial course of immunization via the footpad with lipoglycan emulsified with Freund adjuvant (complete and incomplete).*

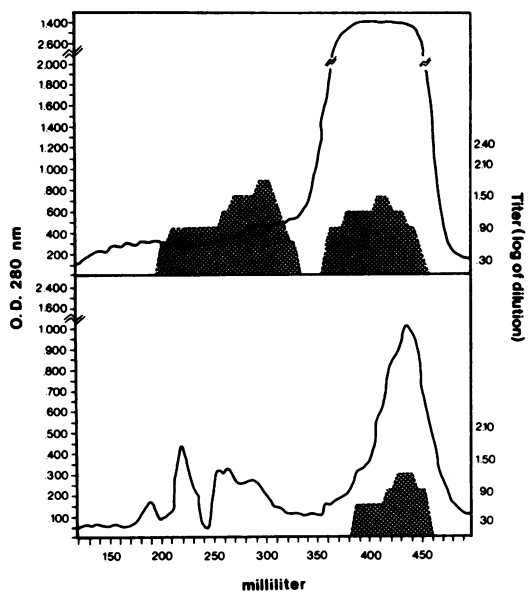

FIG. 5. *Fractionation of late antiserum against A. modicum lipoglycan on Ultragel AcA-22. Eight milliliters of serum placed on a 90- by 2.5-cm column and eluted in 5-ml aliquots with 0.1 M tris(hydroxymethyl)aminomethane–0.2 M NaCl, pH 8.0. Solid line indicates absorbancy at 280 nm measured in Beckman Du-2. Cross-hatched area indicates indirect hemagglutination titer. Upper curve: serum collected 10 days after secondary course of immunization; dialyzed against 0.1 M tris(hydroxymethyl)-aminomethane buffer, pH 8.0; diluted with phosphate-buffered saline 1:2. Lower curve: serum collected 10 days after secondary course of immunization; reacted with an equal volume of 0.2 M 2-mercaptoethanol for 30 min at 20°C, dialyzed against 0.2 M iodoacetamide for 24 h, and dialyzed against phosphate-buffered saline for 48 h.*

the route of immunization as well as the character of the immunogen preparation. When the lipoglycan was presented adsorbed onto autologous rabbit erythrocytes, the only antibody activity noted was associated with a heavy immunoglobulin that was sensitive to 2-mercaptoethanol. This antibody activity was measurable by the passive hemagglutination and hemolysis techniques, but did not form precipitin bands in Ouchterlony immunodiffusion. In addition, no significant secondary response as exemplified by a significant rise in titer or a switch to IgG antibody was noted in these animals after extensive inoculation with any of these lipoglycans. These results are reminiscent of those reported in the literature (7, 10, 15), where antibody predominantly belonging to the IgM class is obtained after intravenous inoculation of rabbits and mice with the lipopolysaccharides of gram-negative bacteria.

The literature also contains reports where antibody predominantly of the IgG type may be obtained with such polysaccharides (1) and lipid A of these polysaccharides (5) if an appropriate immunogen preparation and method of immunization is employed. In the present study introduction of the acholeplasmal lipoglycans emulsified with adjuvant and injected via the footpads yielded antibody activity associated with a light, 2-mercaptoethanol-resistant as well as a heavy, 2-mercaptoethanol-sensitive immuno-

TABLE 5. *Passive hemagglutination titers and mercaptoethanol sensitivity of sucrose gradient fractions of early antiserum to A. modicum lipoglycan*[a]

Fraction no.[b]	Pool I[c]		Pool II[d]	
	IHA titer[e]	2-ME sensitivity[f]	IHA titer	2-ME sensitivity
1	2	+		NA
2	2	+		NA
3	2	+	2	+
4	2	+	4	+
5	4	+	8	+
6	16	+	8	+
7	32	+	4	+
8	8	+		NA
9	2	+		NA
10		NA		NA
11		NA		NA
12		NA	2	−
13		NA	4	−
14		NA	8	−
15		NA	16	−
16		NA	16	−
17		NA	32	−
18		NA	8	−
19		NA	2	−
20–23		NA		NA

[a] Serum collected 7 days after the initial course of immunization (via the footpad with lipoglycan and Freund adjuvant; see text).

[b] Each fraction was a 15-day aliquot collected from the bottom of gradient.

[c] Combined eluate from Sephadex G-200 column fractionation (145 to 215 ml).

[d] Combined eluate from Sephadex G-200 column fractionation (235 to 315 ml).

[e] Reciprocal of the highest dilution of serum fraction that produced detectable agglutination of 2% lipoglycan-coated sheep erythrocytes. IHA, Indirect hemagglutination.

[f] 2-Mercaptoethanol (2-ME, 0.1 M) was added to the wells of microtiter plate containing aliquot of fraction diluted with phosphate-buffered saline. Plates were scored after 1 h of incubation at room temperature; + indicates loss of agglutination (sensitivity to mercaptoethanol); − indicates no reduction in IHA titer; NA = not applicable.

globulin fraction. However, analyses of individual antibody-producing spleen cells by the Jerne plaque assay technique failed to demonstrate a significant increase in facilitated plaques in these animals even after extensive immunization. In all sera examined during the year of immunization, the predominant antibody was found to be of the heavy, 2-mercaptoethanol-sensitive class.

The sera obtained after the footpad route of inoculation with adjuvant yielded greater heterogeneity of antibody activity as determined by cross-reactivity in the serological tests as well as immunoglobulin class. These data suggest that

TABLE 6. *Individual antibody-producing cells in spleen of rabbits after extensive inoculation with the lipoglycan of A. modicum*

Route of immunization	Plaque-forming cells/10^6 spleen cells			
	Sheep erythrocytes		Sheep erythrocytes + *A. modicum* lipoglycan	
	Direct	Indirect	Direct	Indirect
Footpad[a]	24 ± 7	12 ± 8	2,520 ± 120	914 ± 259
Intravenous[b]	878 ± 114	1462 ± 163	768 ± 154	1,600 ± 272

[a] Two rabbits were boosted via the footpad with 200 μg of lipoglycan in phosphate-buffered saline 3 weeks after the late immune sera were collected (see text); 5 days later they were exsanguinated, and the spleens were aseptically removed and assayed for individual antibody-producing cells.

[b] Two rabbits were boosted with 0.5 ml of 10% autologous erythrocytes with adsorbed lipoglycan via the marginal ear vein 3 weeks after collection of the late immune sera (see text). Five days later they were exsanguinated, and the spleens were aseptically removed and assayed for individual antibody-producing cells.

emulsions of the lipoglycans with light mineral oils and mycobacterium may lead to the formation of micelles in which various orientations of the lipoglycans may be presented to the host as antigenic determinants. Adsorption of the lipoglycan molecules onto erythrocytes appears to confine the orientation so that fewer antigenic determinants are presented. Thus, such antisera elicit fewer cross-reactions among the acholeplasmal lipoglycans. Antisera produced against intact membranes of the *Acholeplasma* also yielded less cross-reactivity among antibodies with specificity against the lipoglycans (14). Attempts will be made to fragment the lipoglycans, characterize chemically the fragments, and determine the nature of the determinant groups associated with the differing antibody responses obtained.

Although no significant anamnestic response was noted among these animals over a 10-month period, as evidenced by a fourfold or greater rise in antibody titer, some animals yielded an antibody activity associated with a light immunoglobulin that was resistant to mild treatment with 2-mercaptoethanol. Immunodiffusion studies with anti-rabbit IgG indicated that this immunoglobulin was IgG. Additional studies are required to determine the portion of the lipoglycan responsible for the observed serological cross-reactivity.

The lipoglycan of *A. granularum* and *A. oculi* (data not presented here) did not induce a significant antibody response when introduced with adjuvant via the footpad. These lipoglycans appear to be of a smaller molecular size than other acholeplasmal lipoglycans as determined by glass bead column filtration (12). Relatively high-titer serum could be obtained by adsorbing

these particular lipoglycans onto autologous rabbit erythrocytes before inoculation via the intravenous route. This suggests that there is a minimal size for these lipoglycans below which rabbits will not elicit antibody after introduction of antigen emulsified in adjuvant. All of the acholeplasmal lipoglycans isolated to date (9, 12, 13, 14) were capable of eliciting antibodies in rabbits when administered intravenously adsorbed to autologous rabbit erythrocytes.

Another phenomenon that requires further study relates to the increased sheep erythrocyte hemolysin activity associated with repeated injections of these lipoglycans. The data suggest that some determinant exposed on the isolated lipoglycan is responsible for the observed increase in hemolysin activity. The observation that such increases in hemolysin do not occur after immunization with acholeplasmal membrane preparations suggests that the responsible determinant group must be sequestered in the membrane or its immunogenicity suppressed by other antigens present in the membrane. The nature of this determinant in the isolated lipoglycan and its possible relationship to the Forsmann type antigen are under investigation.

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