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Immunological Properties of Glycolipids from Membranes of *Acholeplasma laidlawii*

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Glycolipids, the predominant class of lipids in the membranes of *Acholeplasma laidlawii*, are the haptenic determinants that react with anti-*A. laidlawii* serum to fix complement. The predominant complement-fixing activity of the membrane glycolipids was associated with the monoglucosyl diglyceride, diglucosyl diglyceride, glycerylphosphoryl diglucosyl diglyceride (GPDD), and an unknown lipid B, which did not react with ninhydrin but released glucose and glycerol and traces of phosphorus upon hydrolysis. The glycolipids monoglucosyl diglyceride and diglucosyl diglyceride or GPDD and unknown lipid B were paired as a result of their cross-reactions with selective antisera prepared with the aid of reconstituted membrane complexes containing membrane lipids. Reconstituted membrane complexes assembled from [^{14}C]monoglucosyl diglyceride and delipidated membrane proteins gave optimal complement fixation titers before saturation of the complexes with the [^{14}C]monoglucosyl diglyceride. The phosphoglycolipid of the membrane, GPDD, was anticomplementary as a pure lipid, a cholesterol liposome, and a reconstituted membrane complex. This anticomplementary activity, which was caused by 3 μg of pure GPDD, affected both human and guinea pig complement. Although human C1, C4, C3, and C5 were not inhibited by GPDD, C2 was inhibited 10-fold by reconstituted membrane complexes containing 150 μg of GPDD. A role for this phosphoglycolipid is discussed in the hypothetical mechanism of inhibition of C2 attachment to SAC1, 4 sites.

The glycolipids of mycoplasmal membranes, the haptenic determinants that react with specific antibody to fix complement (21), are intimately involved in the mechanism of immune lysis (4) and are easily complexed with heterologous or homologous delipidated membrane proteins for immunogenic purposes (29). The immunological properties of the membrane glycolipids from *Acholeplasma laidlawii* (21, 38; M. D. Ryan and L. L. Matz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M28, p. 71) are of particular interest because the chemical structures of the lipids are well characterized (34, 35) and because of the recent advances in understanding the ultrastructure of the membrane of *A. laidlawii* (40). Studies of the antigenic properties of glycolipids from *A. laidlawii* have been complicated by the anticomplementary (AC) activity of both cells (5) and extracted lipids (3). The anticomplementarity of individual immunologically active lipids (26), particularly the phosphatidyl glycerol from *Mycoplasma pneumoniae*, suggested the association of the AC activity of *A. laidlawii* with a lipid, perhaps a glycolipid, of the membrane. Although some immunochemically active lipids reportedly interfere with complement in a nonspecific manner (26), studies using purified complement components and lipid-sensitized liposomes (12) suggested a more specific role for these lipids in complement inactivation. The object of this study was to determine the complement-fixing haptenicity and the AC activity of specific glycolipids from the membrane of *A. laidlawii*.

MATERIALS AND METHODS

Preparation of membrane suspensions. Cells of *A. laidlawii* (ATCC 14192) were harvested after growth on tryptose media, pH 7.5, for 16 h at 37 C (23). Membranes were isolated from sonicated cell suspensions (27) by centrifugation at 100,000 $\times g$ for 1 h in a model L2-65B Beckman ultracentrifuge. Membranes were either resuspended in a 1:20 dilution of 0.5 M tris(hydroxymethyl)aminomethane buffer containing 0.01 M $MgCl_2$ and 0.01 M β-mercaptoethanol (1/20 β buffer) (27) or lyophilized and stored at 4 C.

Isolation of membrane glycolipids. Lipids were extracted from lyophilized membranes with chloroform-methanol (2:1) at room temperature. The extracted membranes were removed from the supernatant by centrifugation after each of three extrac-

tions. Although chloroform-methanol (2:1) was most effective as a solvent, releasing 32.5% of the membrane dry weight as lipid, membrane suspensions were also extracted with 90% cold acetone, 1/20 β buffer, or 0.01% saponin in 1/20 β buffer. The relative amount of each lipid extracted varied with the extraction procedure. Dry weight was determined by deposition of the lipid on constant-weight planchets and drying under vacuum over silica gel until a constant weight was obtained. The sugar content of lipids was estimated by the phenol-sulfuric acid assay (9), and the total carbon content was determined by the dichromate-sulfuric acid test (2). Silica gel H chromatoplates of 0.25-mm thickness were routinely prepared by using a thin-layer spreader (Quickfit Instruments, Walton Stone, Staffordshire, England). The lipids were separated on silica gel H plates with either chloroform-methanol-water (65:25:4), chloroform-methanol (60:30), or propanol-ammonium hydroxide-water (60:30:10). The criteria for identification of glycolipids are based on characterization detailed in the literature; monoglucosyl diglyceride, diglucosyl diglyceride, and glyceryl-phosphoryl diglucosyl diglyceride (GPDD) were identified by R_f values (34, 35), incorporation of D-[^{14}C]glucose (36), and staining chromatoplates with periodate-Schiff reagents (32). Other polar lipids were identified by co-chromatography with known compounds. Lipids separated on chromatoplates were extracted from the dislodged gel with chloroform-methanol (2:1) at least three times. Lipids extracted from the sedimented gel were estimated by the dichromate-sulfuric acid and/or the phenol-sulfuric acid assays. Lipids were isolated in larger amounts for use in immunological tests by extraction of lyophilized membrane preparations with chloroform-methanol (2:1) and column chromatography on silicic acid (34) and by concomitant thin-layer chromatography (TLC) on silica gel H. Extensively rechromatographed lipid antigens were prepared by silicic acid chromatography, TLC with chloroform-methanol-water at least twice, and finally two-dimensional TLC at least twice with chloroform-methanol-water and propanol-ammonium hydroxide-water. [^{14}C]monoglucosyl diglyceride, used to determine optimum protein/lipid ratios for complement fixation, was biosynthesized by incubation of a 1.0-ml membrane suspension (2.0 mg of protein/ml) with 0.025 ml of [U-^{14}C]uridine diphosphate glucose (New England Nuclear Corp.) at 37 C for 1 h. It was isolated after extraction of the incubation mixture with chloroform-methanol (2:1) by TLC on silica gel H with chloroform-methanol-water (65:25:4) as solvent.

Preparation of reconstituted membrane complexes. Reconstituted membrane complexes were formed by addition of Mg^{2+} to a mixture of sodium dodecyl sulfate (SDS) solutions of membrane lipids (16). This method was later modified as follows: lipid and protein were dissolved in SDS (0.5 mg of membrane protein per ml), incubated for 20 min at 60, 28, and 4 C, diluted fivefold with 100 mM Mg^{2+} in 1/20 β buffer, and reincubated for 20 min at 60, 28, and 4 C.

Preparation of cholesterol-lecithin liposomes. Cholesterol-lecithin liposomes were prepared by evaporating chloroform-methanol (2:1)-solubilized membrane lipids to dryness followed by resolubilization in a chloroform-methanol (2:1) solution of lecithin (1.0 mg/ml). The lipid-lecithin complex was evaporated to dryness and resuspended in a cholesterol suspension in 10% ethanol in distilled water (20 mg/ml) to a final molar ratio of 10:1 cholesterol to lecithin in the final liposome (21).

Antisera. Anti-*A. laidlawii* serum was prepared in rabbits by subcutaneous injection of 1.0 ml (15–20 mg of cellular protein) of *A. laidlawii* (grown in tryptose media [23] supplemented with 1% rabbit sera) with complete Freund adjuvant (1:1) three to six times at 10- to 14-day intervals. Selective antisera were prepared by absorbing anti-*A. laidlawii* serum with reconstituted membrane complexes containing membrane protein and all glycolipids except one for 30 min at 37 C and sedimenting at 100,000 × *g* for 1 h at 4 C to remove antigen-antibody complexes. The supernatant from the third antigen-antibody absorption was considered selective antiserum.

Complement. Guinea pig complement was used throughout, except in studies on complement components in which human complement components and stable cellular intermediates (Cordis Laboratories, Miami, Fla.) were substituted. Human complement components were used in hemolytic assays (20) to determine inhibition of specific complement components.

Complement fixation tests. Membrane or lipid antigens that had been grown in tryptose media (23) supplemented with porcine sera were assayed using standard complement fixation procedures (15, 25) by reaction with anti-*A. laidlawii* serum. After elution from chromatoplates, the lipid antigens were stabilized and enhanced by sensitizing cholesterol-lecithin liposomes (21) with the lipids. Veronal buffer (0.147 M) (VB^{2+}), pH 7.2, containing 0.0003 M Mg^{2+} and 0.00015 M Ca^{2+} was used throughout. Complement fixation microtiter tests were conducted by using 0.05 ml of VB^{2+}, 0.025 ml of lipid antigen, 0.025 ml of inactivated antiserum, and 0.025 ml (2 U) of complement added to wells of V microtiter plates. After incubation at 37 C for 1 h, 0.025 ml of a standardized 2.0% suspension of sensitized sheep erythrocytes was added, and samples were reincubated at 37 C for 1 h and allowed to settle for 1 to 2 h. The complement fixation titer was the reciprocal of the highest dilution of antiserum that resulted in 50% hemolysis as visually approximated. Isofixation curves (25) were determined by complement fixation tube tests using 0.9 ml of G1-GVB^{2+}, which consists of equal volumes of VB^{2+}, 5% glucose in distilled water, and this mixture made up to 0.1% gelatin with a distilled water solution of 1.0% gelatin, 0.2 ml of lipid antigen, 0.2 ml of rabbit anti-*A. laidlawii* serum, and 0.2 ml (2 to 6 U) of complement. After incubating for 1 h in a 37 C shaking water bath, 0.2 ml of a standardized indicator system was added. Reincubation in a 37 C shaking water bath for 1 h was followed by a 5-min incubation at 4 C and a 5-min centrifugation at 1,500 × *g*. An end point of 50% hemolysis was determined by reading optical density of the reaction supernatant at 545 nm in a Zeiss

M4 QIII spectrophotometer. Anticomplementarity was assayed in a complement fixation microtiter or tube test by replacing the anti-*A. laidlawii* serum with the appropriate buffer.

RESULTS

Complement fixation by the polar lipids extracted from membranes of A. laidlawii. Membranes (antigen 1) of *A. laidlawii* extracted with 90% cold acetone yielded lipid extracts (antigen 2) that consistently gave higher titers than native membranes (Table 1). Delipidated membrane protein (Table 1, antigen 3) possessed no complement-fixing activity, and when reconstituted with membrane-lipid mixture (Table 1, antigen 4), it gave higher titers than when reconstituted with isolated glycolipids (Table 1, antigens 5 and 6). Minimally delipidated membrane proteins or slightly altered membrane vesicles extracted with 0.01% saponin (Table 1, antigens 7 and 8) also consistently reacted differently than native membrane vesicles.

Lipid extracts were separated into seven easily recognizable and rather homogeneous (28) lipids on silica gel H chromatoplates. The lipids were tested serologically after binding to cholesterol-lecithin liposomes, which gave much better experimental reproducibility than individual lipids (22). Monoglucosyl diglyceride, diglucosyl diglyceride, GPDD, unknown lipid B, and phosphatidyl glycerol, whose immunological properties have been debated in the literature (7, 28) and which may be contaminated with trace amounts of GPDD, all reacted with anti-*A. laidlawii* serum (Table 2, column C). The complement-fixing reactivity of the lipid aliquots was not directly related to lipid content of the membrane (percent dry weight), but the lipids that contained glucose ([^{14}C]glucose incorporation) were the predominant complement-fixing determinants of the membrane. This became increasingly evident when the glucose content of unknown B was determined upon hydrolysis and when the AC activity of GPPD was diluted out or was not effective, as against antiserum absorbed with acetone-extracted membranes (Table 2, column E). Membrane suspensions from *A. laidlawii* that contained all of the membrane lipids absorbed nearly all, whereas acetone-extracted membrane suspensions that contained only trace amounts of membrane lipids absorbed only trace amounts, of the antibody-to-membrane lipids in anti-*A. laidlawii* serum (Table 2, columns D and E).

Membrane lipids were tested for antigenicity by combination with delipidated membrane proteins after 90% cold-acetone extraction of membrane (Table 2, column F). These reconstituted membrane complexes containing individual lipids were immunologically specific for the reconstituted lipid (Table 2, column G). The highest complement-fixing titers without AC activity were observed in preparations E, H, and I (Table 3). Because preparation E contained five times more antigen and preparation H took so long to reconstitute, the reconstituted membrane complexes of preparation I (Table 3) that were formed by melting together SDS solutions of delipidated membrane proteins and extracted membrane lipids after dilution with 100 mM Mg^{2+} were used throughout the study. The relationship between the complement-fixing titers, the amount of lipid, and the amount of protein in these complexes (Fig. 1A) was of interest as an assembly process. The titer of the complex increased with the amount of [^{14}C]monoglucosyl diglyceride reaggregated with 200 μg of membrane protein until it reached a limit of 75 μg of [^{14}C]monoglucosyl diglyceride. In contrast, the amount of lipid absorbed by the 200 μg of membrane protein increased up to 150 μg of [^{14}C]monoglucosyl diglyceride. The labeled lipid found in the supernatant remained at a low level up to 150 μg of [^{14}C]monoglucosyl diglyceride and then greatly increased at 200 μg of lipid. When the [^{14}C]monoglucosyl diglyceride concentration was held constant at 150 μg and the protein concentrations were varied, the complement fixation titer and the [^{14}C]monoglucosyl diglyceride incorporation were optimal at 2 mg of protein per ml or 200 μg of protein per 150 μg of [^{14}C]monoglucosyl diglyceride (Fig. 1B).

The complement-fixing titers against the reconstituted membrane complexes containing

TABLE 1. *Complement fixation titers of membranes, reconstituted membranes, and membrane extracts from A. laidlawii*

Antigen	Source	Complement fixation titer of antiserum
1	Membranes	128
2	90% Cold-acetone extract of membranes	256
3	Membranes delipidated with 90% cold acetone	8
4	Reconstituted membrane complex (2 + 3)	256
5	Delipidated membrane proteins plus diglucosyl diglyceride	64
6	Delipidated membrane proteins plus GPDD (after dilution)	64
7	Saponin-extracted membrane	64
8	Saponin extract	256

TABLE 2. *Complement fixation reactivity with anti-A. laidlawii serum of the polar lipids from membranes of A. laidlawii*

A	B	C	D	E	F	G	H	I
Polar lipids of the membranes[a]	% Membranes[b] (% dry wt)	[^{14}C]glucose incorporation[c] (counts/min)	CF[d] of sensitized liposomes or reconstituted membrane complexes to antiserum	CF of sensitized liposomes to antiserum absorbed with membranes	CF of sensitized liposomes to antiserum absorbed with acetone-extracted membranes	CF of sensitized liposomes to antiserum absorbed with reconstituted membrane complexes containing membrane lipids	CF of sensitized liposomes to antiserum absorbed with reconstituted membrane complexes containing all lipids except diglucosyl diglyceride	Lipids
	0.52	123						Carotenoids
	0.63	437	32	16	32	16	16	Cardiolipin
	0.58	205, 309	128	16	128	16	16	Monoglucosyl diglyceride
	1.37	6, 450	512–256	16	256	16	64	Diglucosyl diglyceride
	0.20	484	128	16	128	16	16	Phosphatidyl glycerol
	0.64	559	AC	16	128	16	16	GPDD
	0.38	0	128–256	16	128	16	16	Unknown B

[a] Separation of polar lipid components from the 90% cold-acetone extract of membranes on silica gel H chromatoplates with chloroform-methanol-water (65:25:4). The carotenoids moved with the solvent front.

[b] Percent dry weight = [micrograms of lipid (phenol sulfuric acid positive)]/[microgram of membrane (dry weight)].

[c] Corrected counts per minute of [^{14}C]glucose incorporated from [^{14}C]uridine diphosphate glucose. The lipids were extracted from the TLC gel, dried onto chromatography paper, and counted in a Nuclear Chicago scintillation system in a toluene-base scintillation mixture containing 4% 2,5-diphenyloxazole and 0.12% *p*-bis-(*o*-methylstyryl)-benzene.

[d] Complement fixation titer. These titers are not a comparison of absolute antigenicity of the lipids, but the relative reactivity of the isolated lipids as components of the membrane of *A. laidlawii*.

individual lipids were very similar to those against cholesterol-lecithin liposomes prepared with the same individual lipid (Table 2). The complement-fixing antibodies of the absorbed anti-*A. laidlawii* serum selective for individual lipids could be completely absorbed with membrane vesicles or reaggregated membrane complexes containing a mixture of membrane lipids, but could not be absorbed with acetone-extracted membranes. Furthermore, when a reconstituted membrane complex containing all the lipids except diglucosyl diglyceride was used to absorb the anti-*A. laidlawii* serum, the activity against diglucosyl diglyceride was the only remaining complement-fixing activity of the antiserum (Table 2).

Selectivity of the anti-A. laidlawii serum after absorption with reconstituted membrane complexes. Extensively rechromatographed monoglucosyl diglyceride, diglucosyl diglyceride, GPDD, and unknown lipid B (see Materials and Methods) were differentiated and identified by using selective antisera (Table 4). Except in the reactions that were complicated by the AC activity of GPDD, the observed complement fixation titers using sensitized cholesterol-lecithin liposomes were at least four times the titer found for nonreacting purified lipids (Table 4). The lowered concentration of monoglucosyl diglyceride and diglucosyl diglyceride in the chloroform-methanol column effluent (Table 4), substantiated by the decreased immunological reactivity of these lipids in the chloroform-methanol effluent, suggested a cross-reactivity between monoglucosyl diglyceride and anti-diglucosyl diglyceride antibody, which indicated similar antigenic structures of the two lipids. Cross-reactivity between un-

known lipid B and anti-GPDD suggested a similarity of the antigenic structures of GPDD and unknown B. The same rationale indicated that the antigenic structures of monoglucosyl and diglucosyl diglycerides are different from those of GPDD and unknown lipid B. Hydrolysis of unknown lipid B released glucose, glycerol, and traces of phosphorus, strongly suggesting a glycolipid structure for the unknown lipid.

AC activity of GPDD, a phosphoglycolipid from membranes of A. laidlawii. Although our results suggested that several of the lipids were antigenic, only GPDD, as identified by the criteria described in Materials and Methods, was AC (Table 2, column C). This lipid was also antigenic as exhibited by the diluted lipid (Table 2, column D) and the isofixation curve of the protein-GPDD complex (Fig. 2). The AC activity of GPDD was evident with 3 μg of the pure lipid (Fig. 3A). In contrast, the GPDD-reconstituted membrane complex required 60 μg to reach optimal anticomplementarity (Fig. 3B), but the level of anticomplementarity was greater. The AC action of GPDD was effective against both human and guinea pig complement. Ten micrograms of GPDD inhibited 13 50% hemolytic complement (CH_{50}) units of guinea pig complement (Fig. 3B) in the linear portion of the curve. Although complement components C1, C4, C3, and C5 were not inhibited by GPDD, C2 was inhibited 10-fold by 150 μg of GPDD in the reconstituted membrane complex (Table 5). The observed decrease in CH_{50} units fixed in the C3 assay when the GPDD complex was present was not considered significant within the dilution range of the assay system (Table 5).

DISCUSSION

Complement fixation by the polar lipids from membranes of A. laidlawii. The predominant complement-fixing activity of the membrane lipids of *A. laidlawii* was associated with the glycolipids monoglucosyl diglyceride, diglucosyl diglyceride, glycerylphosphoryl diglucosyl diglyceride, and an unknown lipid B, which did not react with ninhydrin but released glycerol, glucose, and traces of phosphorus upon hydrolysis. The glycolipids of *M. pneumoniae* that are di- and trihexosyldiglycerides containing galactose and/or glucose and the glycolipids of T-strain mycoplasmas that have not been characterized have also been associated with the complement-fixing activity of their respective cells (21, 30). Although glycolipids such as those from T-strain mycoplasmas are also immunologically active in passive hemagglutination tests, their serological activity is most reliably estimated by the complement fixation test because small amounts of insoluble antigens are reac-

TABLE 3. *Immunological reactivity of reconstituted membrane complexes in the complement fixation test*

Prepn	Components incubated with protein[a]		Incubation		CF reactivity[b]		
	Lipid	Mg^{2+} (mM)	Time (h)	Temp (C)	Dilution[c]	Titer of antiserum	AC
A	0.1 ml of acetone extract and lecithin	20	1.0	37	1:8	32	+
B	0.1 ml of acetone extract and SDS	20	1.0	37	1:8	8	−
C	0.1 ml of acetone extract and 10% ethanol	20	1.0	37	1:8	32	−
D	0.1 ml of acetone extract and 10% ethanol	20	0.33	60	1:8	32	+
			0.33	37			
			0.33	28			
E	0.5 ml of acetone extract and 10% ethanol	20	0.5	37	1:8	256	±
					1:64	256	−
F	0.5 ml of acetone extract and 10% ethanol	20	16.0	4	1:8	128	++
					1:64	8	−
G	0.1 ml of acetone extract and SDS	20	16.0	4	1:8	256	++
					1:64	8	−
H	0.1 ml of acetone extract and SDS	20	16.0 (Dialysis)	4	1:8	64	−
					1:64	128	−
I	0.1 ml of acetone extract and SDS	100	0.33	60	1:8	128	−
			0.33	28			
			0.33	4			

[a] 0.1 ml of membrane protein extracted with 90% cold acetone.
[b] Reactivity in complement fixation (CF) microtiter test.
[c] Dilution of antigen (reconstituted membrane complex).

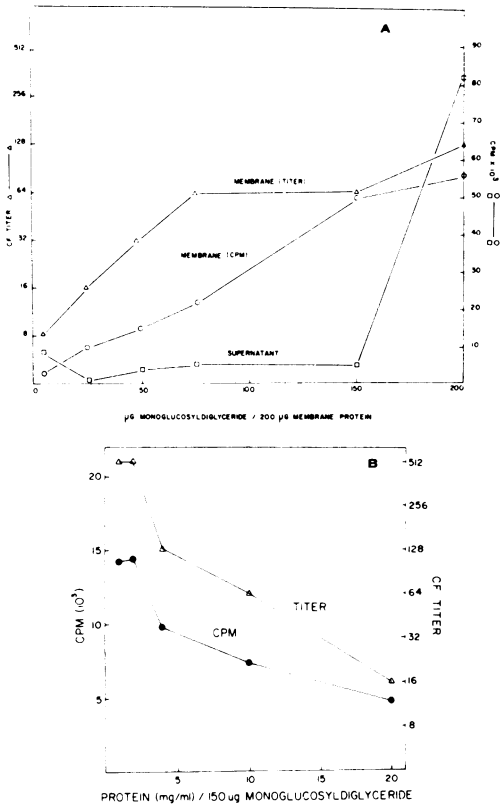

FIG. 1. *Delipidated membrane protein with increasing amounts of* [^{14}C]*monoglucosyl diglyceride assayed for counts per minute (CPM) of lipid incorporated* (○) *and complement fixation titer* (△). *The incorporated* [^{14}C]*monoglucosyl diglyceride in the supernatant was also determined as counts per minute of lipid* (□). *Sedimentable reconstituted membrane complexes contained 200 (Fig. 1A) or 150 (Fig. 1B) µg of delipidated membrane protein.*

tive, isofixation curves can be prepared, and small quantities of antibodies are sensitively assayed. Reliable complement fixation assays of these glycolipids from membranes of *A. laidlawii* required the formation of cholesterol-lecithin liposomes, substantiating the requirement for auxiliary lipids by these and other immunologically reactive lipids (26).

The serological activity of each of these glycolipids was confirmed by absorption and cross-absorption experiments using selective antisera to reconstituted membrane complexes formed by Mg^{2+} flocculation of SDS-solubilized membrane lipids and proteins, which were melted together to increase the rate of reconstitution. Although these reconstituted membrane complexes of *A. laidlawii* and native membrane of *A. laidlawii* reacted similarly in immunological tests, were both triple-layered structures (6), and were both dependent on the same kinds of intimate bonds, the resemblance of the complexes to native membrane at a molecular level is uncertain because of the absence of a template (35). Thus, the degree of binding of membrane proteins and lipids in our reconstituted complexes is not erroneous, but may not be similar to that in native membrane structures.

AC activity of the GPDD isolated from membranes of A. laidlawii. Only one of the

TABLE 4. *Complement fixation titers of selective antisera and purified lipids*

Silicic acid column	Lipid	Complement fixation titer of antisera				
		Unabsorbed antiserum	Antisera absorbed with all lipids[a] except:			
			Monoglucosyl diglyceride	Diglucosyl diglyceride	GPDD	Unknown B
Acetone effluent	Monoglucosyl diglyceride	256	64	128	32	32
	Diglucosyl diglyceride	512	16	256	32	32
	GPDD	512	16	32	128	32
Chloroform-methanol (2:1) effluent	Monoglucosyl diglyceride	128	16	32	16	16
	Diglucosyl diglyceride	512	16	128	16	16
	GPDD	AC[b]	AC	AC	AC	AC
	Unknown B	512	16	64	128	128

[a] Anti-*A. laidlawii* serum absorbed with reconstituted membrane complexes containing all lipids reacted with the purified lipids at a titer of 16.

[b] Anticomplementary

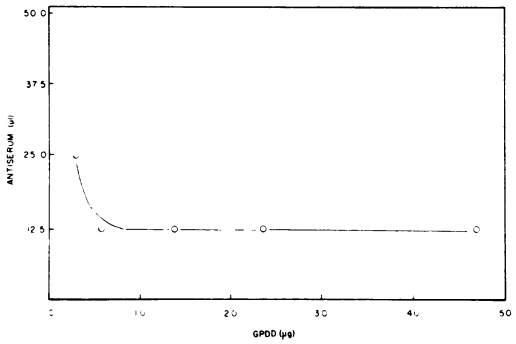

FIG. 2. *Isofixation curve demonstrating the antigenic character of a reconstituted membrane complex prepared from 150 µg of GPDD and 200 µg of delipidated membrane protein. Dilutions of the complex were used as lipid antigen in the complement fixation tube test using 6 U of guinea pig complement. An end point of 50% hemolysis was determined spectrophotometrically by reading the optical density of the reaction supernatant at 545 nm.*

membrane lipids, the phosphoglycolipid GPDD, was AC as a pure lipid, a cholesterol-lecithin liposome, and a reconstituted membrane complex. The AC activity of membranes and cells of *A. laidlawii* is probably caused by GPDD, since the proteins of the membranes remaining after mild delipidation are not AC. The AC activity of GPDD is unlike that of phosphatidyl glycerol in *M. pneumoniae* in that it is not diminished by lecithin (27). It is, however, enhanced by Mg^{2+} as in other AC systems (25).

Our results, according to the following rationale, strongly suggest that some GPDD is on the surface of cells of *A. laidlawii*, even though lectin binding studies suggest that no carbohydrate-binding sites are found on the cell membrane surface of *A. laidlawii* (31). Localization of other glycolipids on the surface of cells of *M. pneumoniae* (4) has been suggested because of the role of lipid hapten in immune lysis. Immune lysis of *M. pneumoniae* is apparently caused by interaction of antibody with lipid hapten and concomitant complement binding, which causes discontinuity and weakening of the bimolecular lipid membrane and finally osmotic bursting (4, 18). Liposomes sensitized with lipid haptens also undergo immune lysis, and studies on sensitized liposomes have revealed that the enzymatic degradation of lipids is not involved in immune lysis (12, 13, 17). The C2 and C8 components of complement are essential for complement action in immune lysis of liposomes, although the efficiency of the reaction is increased when all the components are present (11). As in immune lysis, which requires surface availability of glycolipids to spe-

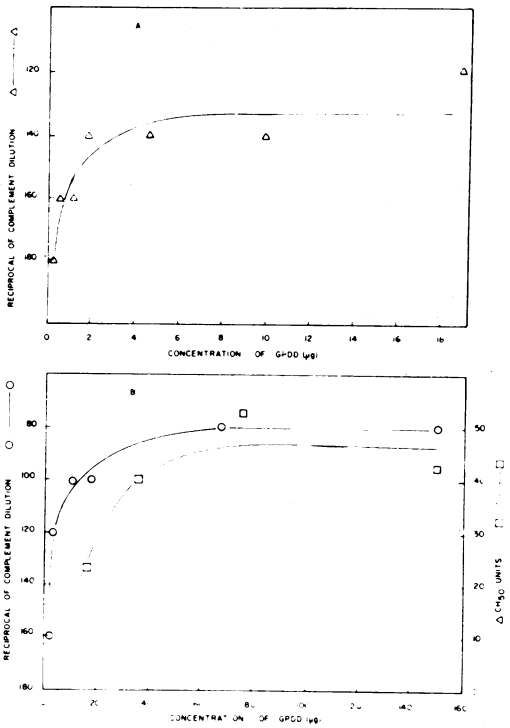

FIG. 3. *Anticomplementarity as a function of concentration of GPDD. (A) Anticomplementarity microtiter assay. Data are given as the reciprocal of the complement dilution resulting in a 50% hemolytic end point as determined by visual approximation. (B) Anticomplementarity of a reconstituted membrane complex containing GPDD. The 50% end point was approximated visually from the anticomplementarity microtiter test and determined spectrophotometrically using the anticomplementarity tube test with 2 U of guinea pig complement. A CH_{50} unit of complement is that amount of complement necessary to achieve 50% hemolysis of the indicator system. ΔCH_{50} units represent a loss of hemolytic activity of guinea pig complement after incubation with GPDD-complex.*

TABLE 5. *Inhibition of human complement components by reconstituted membrane complexes containing GPDD*

Cellular intermediate used for assay	Hemolytic assay for individual component	Donor pool and buffer (CH_{50})	Donor pool and GPDD complex (CH_{50})
EAC4	C1	8,000	7,692
EAC1	C4	65,306	71,910
EAC1, 4	C2	2,963	280[a]
EAC1, 4	C3	10,667	5,479
EAC1, 4	C5	39,024	39,024

[a] Concentration of GPDD complex/2 = 1,290; concentration of GPDD complex/8 = 1,690; concentration of GPDD complex/16 = 2,273.

cific antibodies and subsequent binding of complement, the AC action of GPDD also requires surface localization for its interaction with complement components or complexes that are impermeable to the membrane of *A. laidlawii*.

GPDD probably inhibits C2, one of the complement components essential for hemolysis (19, 24). Although C1, C4, C3, and C5 are not inhibited by GPDD, our data do not exclude additional inhibition of C6 through C9. The mechanism of C2 inhibition relies upon the decreased generation of SAC4, 2 sites (19). The generation of fewer numbers of C4, 2 sites could be caused by (i) repression of the cleavage of C2 by C1 serine esterase of the SAC1, 4 complex (18), thus limiting the availability of C2a, the cleaved fragment, for completion of the SAC1, 4, 2a complex; (ii) blockage of the attachment of C2a to the membrane-bound SAC1, 4; or (iii) binding and/or inactivation of C2a by GPDD instead of SAC1, 4. Our results do not favor any of these mechanisms at this time.

Other phosphoglycolipids with structures similar to GPDD (33), recently isolated and characterized, differ from the phosphoglycolipid from *A. laidlawii*, which is unique in having the glycerophosphate residue on the terminal glucose. The occurrence of these phosphoglycolipids in streptococcal cells (8, 10, 14) suggests a general role for them in AC action, in addition to their role as intermediates in the biosynthesis of teichoic acids (1, 39, 41).

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