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Serological Reactions of Glycolipids from Streptococcal L-Forms

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Parent bacteria and L-forms of some streptococci of group A, group D, and *Streptococcus* MG share common antigens as shown by cross-reactivity in growth inhibition, complement fixation, and Ouchterlony tests. Cross-reactivity between these streptococci or their L-forms with a staphylococcal L-form, or *Mycoplasma pneumoniae*, was found only rarely and only in low-titer complement fixation tests. Removal of all three serological reactivities from positive antisera by addition of chloroform-methanol (7:3) extracts of the streptococci or their L-forms, and failure to remove activities by protein extracts, indicated the glycolipid nature of the cross-reacting antigenic determinants.

Although streptococcal L-forms are immunogenic and the resultant antibodies have been detected by complement fixation, agglutination, precipitation, immunofluorescence, or growth inhibition tests (2, 5-10, 13, 14), the natures of the responsible antigens or antigenic determinants have not been defined. Furthermore, the specificity of antibody response to streptococcal L-forms of differing parentage is unclear. Because some serological reactions of similar membrane-bounded organisms (mycoplasma) are determined by glycolipids (18, 20-22), we investigated the role of lipids in the serology of some streptococcal L-forms.

MATERIALS AND METHODS

Microorganisms. All L-forms and parental bacteria used were taken from lyophilized cultures. Group A strains ADAL, and its M-type 14 parent, AEDL, and its type-12 parent, and GL8L, and its type-19 parent were obtained originally from Louis Dienes (Massachusetts General Hospital) and have been employed in many studies (2, 3, 7, 13, 14, 17). *Streptococcus* MGL and MGP were obtained from Sarabelle Madoff (15) of the same laboratory. Group A strains 64X392 (a type-1 clinical isolate) and 60X298 (type 13, received from the Center for Disease Control, Atlanta, Ga.) were induced to the respective L-forms in our laboratory by the use of group C streptococcal phage-associated lysin (12): they were passed weekly 150 times in liquid media without penicillin before lyophilization.

Group D streptococcus F24 and its L-form were supplied by James R. King (11). Group D streptococci 54X474 (a clinical isolate) and H69D5 (originally received as ATCC 9854 from the American Type Culture Collection, Rockville, Md.) were taken from our laboratory collection. *Staphylococcus aureus* L (ATCC 23832) was received from Marvin Boris (Pediatric Research Laboratory, North Shore Hospital, Manhasset, N.Y.).

GL8L (on solid media without serum or extra salt) and MGL (on removal of penicillin) are revertable. We have never been able to revert any of the other L-forms to the parental bacteria.

Media. Parental streptococci were grown in Trypticase soy broth (TSB: Baltimore Biological Laboratories [BBL]). Strains AEDL, GL8L, *S. aureus* L, and 60X298L were grown in TSB containing 3% NaCl and 10% horse serum. L-forms were grown for 24 to 48 h and parents for 18 h. Strains ADAL, 64X392L, and F24L were grown for 18 h in brain heart infusion (BHI) (BBL) containing 2.3% NH_4Cl and 0.5% glucose without horse serum. MGL was grown in BHI containing 0.5% yeast extract (Difco), 10% sucrose, 10% horse serum, and 1,000 U (1 ml) of penicillin G. All incubations were carried out aerobically at 37 C.

Purification of membranes. For several experiments, purified membranes were prepared from L-forms according to the procedure of Panos et al. (17).

Lipid fractionation. Lipid fractions of bacteria, L-forms, or L-form membranes were prepared according to the procedure of Plackett (18).

Protein preparation. L-forms (ADAL) were defatted with chloroform-methanol (CM; 2:1), suspended in deionized water, and sonically treated at 10 kc in a Raytheon sonic oscillator for 30 min at 4 C. After centrifugation at 30,000 $\times$ *g* for 1 h, the soluble supernatant fluid was passed through a column of Sephadex G25. As determined by absorption at 280 nm, all of the protein appeared in a single peak. This material was used for control (i.e., nonlipid) absorption of antisera.

Thin-layer chromatography. Lipid fractions from bacteria, L-forms, or membranes were separated by thin-layer chromatography by using a solvent system of chloroform-methanol-water (65:25:4). Plates and

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detecting systems for total lipids, glycolipids, and phospholipids were as described in Razin et al. (21).

Preparation of antigens. Before use as antigens, L-forms were washed in 3% saline and then lysed in distilled water. To prepare parent bacteria, a 180- to 430-mg (wet weight) sample of washed cells (optical density [OD_{660}] no greater than 25) in 36 ml of water was shaken with 44 g of 0.17-mm diameter glass beads in a Braun disintegrator at 4 C until there was a 10-fold reduction in OD_{660} (5 min). Both L-forms and parent streptococci were heated at 56 C for 45 min and suspended in saline, at a concentration of between 2.0 and 4.0 mg of protein per ml, for injection.

Preparation of antisera. Antisera were prepared in adult New Zealand white rabbits. A 1-ml portion of the saline suspension of L-forms or bacteria was injected intravenously three times a week on consecutive days for 4 weeks. The rabbits were bled from the ear vein 6 days after the last injection. After 6 days of rest, the animals were reinjected on 3 more consecutive days and bled again 6 days later. Antisera made to group D streptococci (H69D5) and to group C streptococci (26RP66) were previously prepared in a similar fashion in this laboratory. All antisera were heated at 56 C for 1 h prior to use in serological tests.

Absorption of antisera. Antisera were treated by adding 1 mg of dry lipid to 0.1 ml of antiserum, mixing by pipette, incubating 2 h at 37 C, and refrigerating overnight. The lipid was a CM (7:3) extract of L-form or *Streptococcus* (B. Prescott et al., Bacteriol Proc., p. 74, 1971). Any insoluble material was removed by centrifugation.

Growth inhibition tests. Growth inhibition tests were carried out with sterile, filter paper disks of 6.35-mm diameter (#470E Schleicher & Schuell Co.) saturated with 0.02 ml of antisera. Plates were made with Trypticase soy agar (BBL) plus 10% horse serum and penicillin or with BHI plus NH_4Cl and 10% horse serum. Plates were spread with 1:10 and 1:100 dilutions in the respective broth of an overnight growth of bacterial L-forms and allowed to dry at 37 C for 2 h. The disks were placed on the plate with sterile forceps, and the plates were incubated in a moist chamber at 37 C and examined at 24 h and 3 days. Growth inhibition was considered positive if growth did not occur within 2 mm of the disk; however, nearly all zones of inhibition measured between 3 and 10 mm in width and no control sera (preimmunization or normal rabbits) showed any zones of inhibition exceeding an ill-defined 1 to 1.5 mm.

Complement fixation test. Dried lipid fractions were dissolved in propylene glycol and prepared as 0.1% solutions in phosphate-buffered saline, or 100 μg of lipid (with 2 mg of egg lecithin as adjuvant) was dissolved in 1 ml of Veronal-buffered saline. The various streptococcal antisera were diluted 1:40. All reactions were tested by the micro complement fixation technique (23).

Gel precipitin tests. For double immunodiffusions, a microversion of the Ouchterlony (16) test was used. Six 1-mm holes were located peripherally 4 mm from a central well on 1 by 3 inch (2.54 by 7.62 cm) slides coated with 1% Noble agar (Difco) with 0.10% Merthiolate. Lipid fractions, prepared as described above, were tested against undiluted antisera.

RESULTS

Table 1 shows that rabbit antisera against two group A L-forms, a group A parent, a group D L-form, and a group D *Streptococcus* all inhibited growth of both group A and group D L-forms. In addition, when tested against CM (7:3) extracts of these L-forms or of parent group A or D streptococci, the same antisera fixed complement and produced positive Ouchterlony tests. Antiserum against a staphylococcal L-form reacted homologously in all three serological tests, but was negative against streptococci, streptococcal L-forms, and their CM (7:3) extracts. Conversely, antisera to group A or group D L-forms, or to a group D parent, occasionally gave minimal titers on complement fixation with CM (7:3) extracts of staphylococcal L-forms: these are of dubious significance and are not shown in Table 1.

Antiserum against *Streptococcus* MG-L inhibited growth of L-forms of groups A and D and gave positive Ouchterlony and complement fixation tests against CM (7:3) extracts of both L-forms and parent streptococci. Although not shown in Table 1, antisera against a number of other streptococcal L-forms (AED-L, GL8-L, and 298-L group A) also produced positive Ouchterlony tests with CM (7:3) extracts of both group A and group D L-forms and parent bacteria. In growth inhibition tests, these antisera inhibited both group A and group D L-forms, with the exception of 298-L antiserum which inhibited growth of 298/L only.

In the positive Ouchterlony tests, single precipitin lines of identity were produced between CM (7:3) extracts and antisera from group A and group D L-forms or streptococci and by MG-L antiserum. An example is shown in Fig. 1.

As also shown in Table 1, absorption of positive antisera with CM (7:3) glycolipid extracts of either L-forms or parent bacteria of groups A and D resulted in simultaneous removal of growth-inhibiting, precipitating activity, and (where tested) complement-fixing activity. In limited tests, absorption of groups A and D L-form antisera with CM (7:3) extracts of the staphylococcal L-form failed to remove growth-inhibiting or precipitating activity: complement fixation by such absorbed antisera was not tested.

Absorptions with a protein fraction from a delipidated group A L-form (ADAL) also failed to remove any serological reactivity from any positive antisera (data not shown).

Analyses of the CM (7:3) extracts from both L-forms and parent streptococci of groups A and D and of the staphylococcal L-form are reported

TABLE 1. *Serological reactions of streptococci, their L-forms, or glycolipid extracts thereof*

Antiserum versus	Streptococcal antigen and test used															
	Group A								Group D					Other		
	392 L			392 P		ADAL			F24L			F24P		MGL		
	G[a]	C[b]	O[c]	C	O	G	C	O	G	C	O	C	O	G	C	O
Group A streptococci																
392L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
392L (absorbed with streptococcal extract)[d]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	N	−
392L (absorbed with staphylococcal extract)[e]	+	N	+	N	+	N	N	+	N	N	+	N	+	N	N	N
392P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N
392P (absorbed with streptococcal extract)[d]	−	N	N	N	N	N	N	N	−	N	−	N	N	N	N	+
ADAL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+
ADAL (absorbed with streptococcal extract)[d]	−	N	−	N	−	N	−	−	−	N	−	N	−	N	N	N
Group D streptococci																
F24L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F24L (absorbed with streptococcal extract)[d]	−	−	−	−	−	N	N	−	−	−	−	N	−	N	N	N
F24L (absorbed with staphylococcal extract)[e]	+	N	+	N	+	N	N	+	N	N	+	N	+	N	N	N
H69D5P	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+
H69D5P (absorbed with group D, 474P extract)	−	N	N	N	−	N	N	N	−	N	−	N	N	N	N	N
Streptococcus MGL	+	+	+	N	+	+	N	+	+	+	+	N	+	+	+	+
Staphylococcus L[f]	−	−	−	N	−	−	−	−	−	−	−	N	−	N	N	−

[a] Growth inhibition vs. living organism.
[b] Complement fixation using chloroform-methanol 7:3 (CM7:3) as antigen.
[c] Ouchterlony tests (using CM7:3 extract as antigen).
[d] Identical results with CM7:3 extracts of 392L, 392P, F24P, and 474P.
[e] CM7:3 extract of *Staphylococcus* L, ATCC 23832.
[f] Positive in G, C, and O tests vs. *Staphylococcus* L, ATCC 23832, or CM7:3 extracts thereof.
N = Not done.

elsewhere (B. Prescott et al., Bacteriol. Proc., p. 74, 1971). In brief, they show that the extracted material, which is derived entirely from membrane, contains approximately 0.6% nitrogen but is predominantly glycolipid with glucose as the only carbohydrate. Silica gel thin-layer chromatography shows that the glycolipid patterns from whole L-forms, whole streptococci, and L-form membranes of both groups A and D are similar. In contrast, the pattern from the staphylococcal L-form is slightly different.

DISCUSSION

Results indicate that some L-forms and streptococci of groups A and D share a common membrane antigen(s) which produces growth-inhibiting, complement-fixing, and precipitating antibodies in rabbits. *Streptococcus* MG appears to have similar antigens, whereas a staphylococcal L-form possesses an antigen with low complement fixation activity. Some group A L-forms (i.e., 298-L) may lack such antigens or possess others. Dannis et al. (3) also showed membrane antigen(s) common to a group A streptococcus and its L-form by lines of identity in agar gel diffusion and by cross-absorptions. They were also able to remove growth-inhibiting antibody from L-form antisera by absorption with parental (protoplast) membrane. However, contrary to our results, they did not find growth inhibition of the L-form by parental antiserum.

The evidence obtained by our use of CM 7:3 lipid extracts as antigens and as immunoabsorbents, together with knowledge of the composition of these extracts (B. Prescott et al., Bacteriol. Proc., p. 74, 1971) and the failure of protein preparations to absorb serological activity, suggests strongly that the determination of antigenic reactivity in all three serological tests resides in a glycolipid. The situation thus re-

sembles that reported among *Mycoplasma* (18, 20–22).

The Ouchterlony tests reported were performed only with glycolipid, i.e., CM (7:3) fractions. Therefore, the multiple precipitin bands (presumably due chiefly to membrane proteins) reported by other (7, 13) were not seen. However, multiple bands (not all of which produced lines of identity between groups A and D) were seen when trypsinized, heated, ammonium-sulfate precipitated, or crudely sonicated preparations of L-forms or membranes were used as antigen. By use of trypsinized protoplast membrane supernatant fluids, Freimer (4) was unable to show a relation of group D membrane antigens to those of group A. This disparity in his results and ours is unexplained, but may depend on the undefined nature of antigens or haptens present in a trypsinized extract or on the possibility that all group A or group D membranes may not contain all the same antigens. We have indicated above that one group A streptococcal L-form (298-L) and its antisera reacted differently from other L-forms and antisera tested. Furthermore, Lynn and Haller (13) showed that antisera to two different group A streptococcal L-forms did not both inhibit the growth of all group A L-forms tested and that antisera to the parent streptococci frequently showed patterns of inhibition different than those shown by the corresponding L-form antisera.

Cross-reacting glycolipids have been isolated from *Acholeplasma* (formerly *Mycloplasma*) *laidlawii* and *Streptococcus* MG (19), and a cross-reaction between "polysaccharides" of *Mycoplasma mycoides* and a group K streptococcus has been reported (24). Recent studies utilizing hybrid reaggregated membranes of mycoplasma (1, 22) have confirmed the importance of the glycolipid component in specificity of complement fixation and metabolic inhibition tests. In our limited experience, antisera against CM 7:3 extracts of *Mycoplasma pneumoniae* and streptococci (MG, group A, group D, or their L-forms) usually failed to cross-react in all three serological tests, although in one isolated instance, an extract of *M. pneumoniae* fixed complement when added to antiserum made against a group A L-form. Because the *Streptococcus* MG (19) and the other streptococcal (B. Prescott et al., Bacteriol. Proc., p. 74, 1971) glycolipids appear to be entirely glucosyl compounds, whereas the glycolipid of *M. pneumoniae* contains both glucose and galactose (18), the general lack of cross-reactivity may be explainable. We have not yet explored relationships between the composition

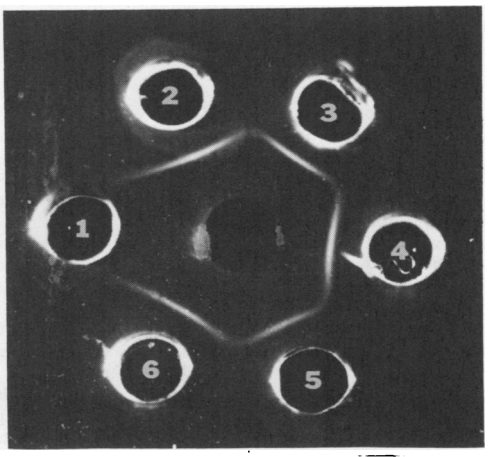

FIG. 1 *Example of lines of identity in Ouchterlony test with antiserum to Streptococcus MG-L form in center well. Similar patterns were produced by antisera to either L-forms or parents of groups A and D streptococci. The outer wells contain CM (7:3) extracts of (1) Staphylococcus aureus ATCC-L (ATCC 23832); (2) group A parent streptococcus 64 × 392; (3) group A L-form 64 X 392L; (4) group A L-form ADAL; (5) group D L-form F24L; and (6) group D parent streptococcus 54 X 474.*

of streptococcal and *A. laidlawii* or other mycoplasma glycolipids.

It therefore appears that growth-inhibiting, and some complement-fixing and precipitating, activities of antisera to streptococci or their L-forms may be determined by membrane glycolipids shared by some representatives of groups A and D, as well as by *Streptococcus* MG. The variations in reported results suggest, however, that the antigenic determinants (i.e., glycolipids) may also be different in L-forms derived from different strains of even a single serological group (e.g., group A). Variation could also originate in the parent bacterium or result from differences in the manner of induction, the media used, or the passage history and stability of the L-form tested, but no data are available concerning these possibilities. More extensive serological testing and characterization of glycolipids from a wider selection of other bacteria and L-forms is required and is in progress.

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