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Identification of the Major Antigenic Determinants on Lipoglycans from *Acholeplasma granularum* and *Acholeplasma axanthum*

T. H. AL-SAMARRAI, P. F. SMITH,* AND R. J. LYNN

Department of Microbiology, University of South Dakota, Vermillion, South Dakota 57069

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The major antigenic determinants of the lipoglycans from *Acholeplasma granularum* and *Acholeplasma axanthum* were found to be the oligosaccharide sequences Glc*p*(β1→2)-Glc*p*(α1→4)-Glc*p* and Glc*p*(β1→2)-Glc*p*(β1→2)-Glc*p*, respectively. The disaccharides sophorose and maltose inhibited hemagglutination by specific antiserum of sheep erythrocytes coated with lipoglycan from *A. granularum*. Only sophorose possessed this capacity with the lipoglycan from *A. axanthum*. Periodate oxidation destroyed the ability of both lipoglycans to interact with specific antibody, a result compatible with structural data on the lipoglycans.

Structural features of oligosaccharide chains are known to dictate the antigenic specificity of many biological molecules, including streptococcal, klebsiella, and pneumococcal polysaccharides (5, 8, 9, 15), gram-negative bacterial lipopolysaccharides (3), blood groups (4), and glycolipids (14). Lipoglycans, which have been isolated from the cytoplasmic membranes of species of *Acholeplasma*, have been shown to elicit the production of specific antibodies in rabbits when introduced via the footpad together with Freund complete adjuvant, by the intravenous route when adsorbed onto autologous erythrocytes, or by either route as integral parts of the acholeplasmal membrane (7). The class of antibody produced is principally immunoglobulin M. Since adsorption onto erythrocytes does not mask the major antigenic sites of these lipoglycans, the antigenic determinants must reside in a part of the molecule distinct from the adsorbing site (1, 7).

Most acholeplasmal lipoglycans consist of long oligosaccharide chains attached to a diacyl glycerol (13). Deacylation does not interfere with antigenic specificity, indicating that the oligosaccharide portions contain the antigenic determinants. The complete structure of the lipoglycan from *Acholeplasma granularum* has been determined (12). It is a linear oligosaccharide of 12 repeating units of 9 sugars [Glc*p*(β1→2)-Glc*p* (α1→4)-Glc*p*(α1→3,4)-FcNA c(β1→3)-Gal*p* (α1→3)-Gal*p*(α1→3)-Gal*p* (α1→3,4)-GlcNAc(β1→3,4)-GlcNAc(β1→4), where Glc is glucose, FcNAc is *N*-acetylfucosamine, Gal is galactose, and GlcNac is *N*-acetylglucosamine] covalently attached to diacyl glycerol. Only a partial structural characterization has been accomplished thus far for the lipoglycan from *Acholeplasma axanthum*. It differs from the lipoglycan of *A. granularum* in several important features: (i) the oligosaccharide chain contains periodic galactose 1-phosphate and glycerol phosphate residues; (ii) one of every four amino groups of its amino sugars contains N-acyl long-chain fatty acids, some of which are α-hydroxy fatty acids; and (iii) its molecular weight is five times greater, i.e., 100,000. The terminal residues of this lipoglycan from *A. axanthum* have been identified as Glc*p*(β1→2)Glc*p*(β1→2)-Glc*p*(β1→6)Gal*p*- (P. F. Smith, unpublished data).

The purpose of this investigation was to define which sugar residues serve as antigenic determinants on these two distinctive lipoglycans.

MATERIALS AND METHODS

Isolation of lipoglycans. The lipoglycans were isolated from lipid-depleted cells of *A. granularum* BTS39 and *A. axanthum* S743 as previously described (13). The lipoglycan from *A. granularum* was further purified by passage through an anion-exchange resin (11), rendering it totally free from contaminating nucleic acids. Such treatment of the lipoglycan from *A. axanthum* was not possible because of its phosphate content. However, nucleic acid contamination of this lipoglycan was less than 1%. Protein contamination of both lipoglycans was not detectable, i.e., less than 0.01%. Both preparations were homogeneous by criteria previously established (11).

Immunization of rabbits. Adult New Zealand white rabbits were used. A 10% suspension of autologous rabbit erythrocytes in phosphate-buffered saline (0.01 M sodium phosphate [pH 7.0] made 0.15 M with NaCl) was mixed with an equal volume of lipoglycan (100

μg/ml) and incubated for 2 h at room temperature and then overnight at 4°C. After the coated erythrocytes were washed three times in phosphate-buffered saline, the cells were resuspended to a final concentration of 10% in the same medium. Immunizations were carried out by four daily intravenous injections of 1 ml of the 10% suspension of lipoglycan-coated erythrocytes followed by a 3-day rest period over a period of 4 weeks. Three days after the last intravenous injection, 200 μg of lipoglycan suspended in phosphate-buffered saline was injected into the footpad. Sera were collected by cardiac puncture 5 days after this footpad injection. Rabbits were given additional monthly injections of 100 μg of lipoglycan into the footpad.

Serological tests. Passive hemagglutination tests were performed in microtiter plates. Twofold serial dilutions of 50 μl of heat inactivated sera (60°C, 30 min) were mixed with 50 μl of a 2% suspension of sheep erythrocytes coated with the appropriate lipoglycan. After incubation for 30 min at room temperature, the plates were examined for hemagglutination. Lipoglycan-coated erythrocytes were prepared by mixing a 5-ml volume of lipoglycan (50 μg/ml in phosphate-buffered saline) with sheep erythrocytes obtained by centrifugation (800 × *g*) of 10 ml of a photometrically prepared 2% suspension (2). After 45 min at room temperature, the cells were washed three times with phosphate-buffered saline and resuspended in modified barbital buffer (2) to a photometrically determined concentration of 2%.

The passive hemagglutination test served to measure the effects of a variety of oligosaccharides on the lipoglycan-antibody interaction. Oligosaccharides were selected to represent all of the possible anomeric and numerical carbon atom linkages of glucose disaccharides as well as a sampling of unrelated sugars (see Table 2).

Various concentrations of oligosaccharides dissolved in 0.15 M NaCl (0.5 ml) were mixed with the highest dilution (1:16 for *A. granularum*; 1:64 for *A. axanthum*) of the appropriate antiserum (0.5 ml) exhibiting maximum hemagglutination. After incubation for 45 min at 37°C, 50 μl of a 2% suspension of sheep erythrocytes coated with 1 U of lipoglycan was added to 50 μl of antiserum-oligosaccharide mixture in microtiter plates. After mixing, the plates were held for 30 min at room temperature and examined for hemagglutination. Controls containing no oligosaccharide were included through all steps. One unit of lipoglycan is defined as the smallest amount of lipoglycan giving detectable agglutination of erythrocytes by homologous antiserum. One unit of antiserum is defined as the highest dilution of antiserum giving detectable agglutination of erythrocytes coated with one unit of lipoglycan.

Periodate oxidation of lipoglycans. Both lipoglycans were subjected to oxidation with sodium metaperiodate as described previously (1). Briefly, 0.1 μmol of lipoglycan was reacted at room temperature with 0.04 mM periodate for 17 h. The reaction was terminated by the addition of glucose, and the generated aldehyde groups were reduced with sodium borohydride. After dialysis against deionized water, the preparations were lyophilized.

TABLE 1. Passive hemagglutination titers of pooled rabbit antisera against lipoglycans

Antigen[a]	Antiserum titer[b] to acholeplasmal lipoglycan	
	A. axanthum	*A. granularum*
A. axanthum	64	8
A. granularum	<4	16

[a] Sheep erythrocytes coated with lipoglycan.
[b] Reciprocal of antiserum dilution exhibiting detectable agglutination.

RESULTS

The titers of pooled antisera prepared against *A. granularum* and *A. axanthum*, as well as the cross-reactivities, are shown in Table 1. Although the antiserum against *A. axanthum* lipoglycan did not exhibit detectable cross-reactivity with *A. granularum* lipoglycan, cross-reactivity was observed with antiserum against *A. granularum* and the lipoglycan of *A. axanthum*. No anemestic response by rabbits was noted. These results are compatible with previous observations (7). Preimmune rabbit serum exhibited no titer even when used undiluted.

An initial determination of the nature of the antigenic determinants of both lipoglycans and the possible shared determinants was made by examination of the inhibitory effects of selected oligosaccharides on passive hemagglutination of lipoglycan-coated erythrocytes. Since glucose residues are terminal in both lipoglycans, all of the different structural arrangements of glucose saccharides were tested. Since periodic α-galactose 1-phosphate residues are found on the lipoglycan from *A. axanthum*, galactose 1-phosphate also was used. In addition, unrelated oligosaccharides served as controls. The results are shown in Table 2. Sophorose [Glc*p*(β1→2)-Glc*p*] inhibited hemagglutination of both *A. axanthum* and *A. granularum* lipoglycans. Maltose [Glc*p*(α1→4)-Glc*p*] inhibited hemagglutination of only *A. granularum* lipoglycan. None of the other oligosaccharides exhibited any inhibitory effect at the maximum concentration tested, i.e., 6 mg/ml. A slight inhibitory effect by isomaltose was noted with *A. granularum* but appeared to be due to contamination (2%) with maltose, as assessed by gas-liquid chromatography. The structures of the inhibitory disaccharides matched the structures of the terminal three sugars of each repeating sequence of the particular lipoglycans. Cross-reactivity between the two lipoglycans is the result of identical shared structures of the terminal disaccharides, i.e., Glc*p*(β1→2)-Glc*p*. Monosaccharides exhibited no inhibitory activity, nor did galactose 1-phosphate.

Confirmation of these results was obtained by examination of the effects of periodate oxidation

of the lipoglycans. Previous structural analyses of the lipoglycan from *A. granularum* have demonstrated that all of the glucose but none of the galactose or amino sugar residues are destroyed by periodate oxidation (12). In the case of the lipoglycan from *A. axanthum*, periodate treatment destroys all of the galactose and about half of the glucose residues (P. F. Smith, unpublished data). Exposure of the lipoglycans to $NaIO_4$ followed by dialysis and borohydride reduction resulted in the destruction of greater than 99% of the hemagglutination activity of *A. granularum* and greater than 95% of that of *A. axanthum* (Table 3).

TABLE 3. Minimum concentrations of intact and periodate-treated lipoglycans causing total inhibition of hemagglutination of lipoglycan-coated erythrocytes by specific antisera

Antiserum[a]	Lipoglycan concn (μg/0.1 ml)	
	Intact	Periodate treated
A. axanthum	1.6	30
A. granularum	0.8	>200

[a] Antiserum (0.1 ml, 1:4 dilution) was mixed with 0.1 ml of various concentrations (0.2 to 200 μg/0.1 ml) of lipoglycans in phosphate-buffered saline.

DISCUSSION

Hemagglutination inhibition and susceptibility of lipoglycans to periodate oxidation support the identification of the antigenic determinant as the terminal three sugar residues of each repeating sequence of lipoglycans. Hence, the antigenic determinant of the lipoglycan from *A. granularum* is Glc*p*(β1→2)-Glc*p*(α1→4)-Glc*p*-, whereas that from *A. axanthum* is Glc*p*(β1→2)-Glc*p*(βl→2)-Glc*p*. The identity of the first sugar linkage in both lipoglycans explains the cross-reactivity of their respective antisera. The reduced inhibitory activity toward hemagglutination by maltose relative to that of sophorose suggests that with the lipoglycan from *A. granularum*, the terminally linked sugar residue exerts a stronger influence on antigenic specificity than the second sugar of the sequence. Furthermore, the non-inhibitory activity of gentiobiose on antibody against the lipoglycan from *A. axanthum* suggests that the linkage between the third and fourth sugars from the terminus exerts little or no antigenic activity. This same conclusion also applies to the branched galactose 1-phosphate residues. The possibility exists that more than one antibody is involved in the case of *A. granularum*. Such a situation would not alter the conclusion regarding the nature of the specific antigenic determinant. However, only one precipitin line is seen with purified lipoglycan as antigen and anti-lipoglycan antibody (11). The validity for the use of the tests used here has precedence in the work of Morgan and Watkin (10) and Kabat and Lestowitz (6), who used hemagglutination inhibition to identify oligosaccharide units involved in hemagglutination.

TABLE 2. Minimum concentration of oligosaccharides causing total inhibition of passive hemagglutination of lipoglycan-coated erythrocytes by specific antisera

Oligosaccharide	Major glucoside linkage	Oligosaccharide concn (mg/ml)[a]	
		A. axanthum	*A. granularum*
Kojibiose	α (1 → 2)	>6	>6
Sophorose	β (1 → 2)	2	2
Nigeran	α (1 → 3)	>6	>6
Laminaran	β (1 → 3)	>6	>6
Maltose	α (1 → 4)	>6	4
Cellobiose	β (1 → 4)	>6	>6
Isomaltose	α (1 → 6)	>6	>6
Gentiobiose	β (1 → 6)	>6	>6
Galactose 1-PO_4	NA[b]	>6	ND[c]
Lactose	NA	>6	>6
Melibiose	NA	>6	>6
Stachyose	NA	>6	>6

[a] The concentration of oligosaccharides tested ranged from 0.5 to 6 mg/ml.
[b] NA, Not applicable.
[c] ND, Not done.

The location of the major antigenic determinants on the terminal three sugar units of these acholeplasmal lipoglycans indicates that this portion of the molecules is unobstructed and not involved with their attachment to eucaryotic cells. Whether these portions of the lipoglycans also are exposed at the surface of the acholeplasmal cells has not been established. However, they would appear to contribute to the overall antigenic specificity of each species. Since periodate oxidation does not interfere with binding of these lipoglycans to eucaryotic cells, the antigenic determinants must be distinct from the binding sites of the molecules.

LITERATURE CITED

1. **Al-Samarrai, T. H., P. F. Smith, and R. J. Lynn.** 1982. Isolation and characterization of the sheep erythrocytes receptor for acholeplasmal lipoglycans. Infect. Immun. **38**:1078–1087.
2. **Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf.** 1970. Methods in immunology. W. A. Benjamin, Inc., New York.
3. **Dmitriev, B. A., L. Backinowsky, V. L. Lvov, N. K. Kochetkov, and I. L. Hofman.** 1973. Immunochemical studies on *Shigella dysenteriae* lipopolysaccharides. Eur. J. Biochem. **40**:533–539.
4. **Furukawa, K., N. Kochibe, H. Takizawa, and S. Iseki.** 1972. Immunochemical studies on bacterial blood group substances. VI. Structures of A active oligosaccharides isolated from *Salmonella riogrande* lipopolysaccharide. Jpn. J. Microbiol. **16**:199–204.
5. **Heidelberger, M., W. F. Dudman, and W. Nimmich.** 1970.

Immunochemical relationships of certain capsular polysaccharides of *Klebsiella pneumococci* and *Rhizobia*. J. Immunol. **104**:1321–1328.

6. **Kabat, E. A., and S. Leskowitz.** 1955. Immunochemical studies on blood groups. XVII. Structural units involved in blood group A and B specificity. J. Am. Chem. Soc. **77**:5159–5164.
7. **Lynn, R. J., P. A. Guidici, E. J. Hollemweguer, and P. F. Smith.** 1980. Humoral immune response of rabbits to acholeplasmal lipoglycans. Infect. Immun. **29**:926–933.
8. **McCarty, M.** 1956. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates. J. Exp. Med. **104**:629–643.
9. **McCarty, M.** 1958. Further studies on the chemical basis for the serological specificity of group A streptococcal carbohydrate. J. Exp. Med. **108**:311–323.
10. **Morgan, W. T. J., and W. M. Watkins.** 1953. The inhibition of the haemagglutinins in plant seeds by human blood group substances and simple sugars. Br. J. Exp. Pathol. **34**:94–103.
11. **Smith, P. F.** 1977. Homogeneity of lipopolysaccharides from *Acholeplasma*. J. Bacteriol. **130**:393–398.
12. **Smith, P. F.** 1981. Structure of oligosaccharide chain of lipoglycan from *Acholeplasma granularum*. Biochim. Biophys. Acta **665**:92–99.
13. **Smith, P. F., T. A. Langworthy, and W. R. Mayberry.** 1976. Distribution and composition of lipopolysaccharides from mycoplasmas. J. Bacteriol. **125**:916–922.
14. **Sugiyama, T., P. F. Smith, T. A. Langworthy, and W. R. Mayberry.** 1974. Immunological analysis of glycolipids and lipopolysaccharides derived from various mycoplasmas. Infect. Immun. **10**:1273–1279.
15. **Wheat, R. W., C. Dorsch, and G. Godoy.** 1965. Occurrence of pyruvic acid in the capsular polysaccharide of *Klebsiella rhinoschleromatis*. J. Bacteriol. **89**:539.