The partial characterization of the structure of the lipoglycan (LG) from *Acholeplasma axanthum* is added to the previous complete structural analysis of the lipoglycan from *A. granularum*. The terminal sequence of *A. axanthum* LG is Glcp(β1→2)-Glc(β1→2)-Glc(β1→6); of *A. granularum* Glcp(β1→2)-Glc(α1→4)-Glc(β1→4). These specific residues define the major antigenic determinants of the LG as determined by blockage of hemagglutination of LG coated erythrocytes by specific oligosaccharides and binding of radiolabeled LG to specific immunoglobulins. The binding of LG to mammalian cells occurs by an interaction between specific eucaryotic cell receptors and the internal sequence of the oligosaccharide chain of LG. Size and sugar chains of LG rather than fatty acid residues appears to define the binding site on the LG.

Acholeplasmal lipoglycans are heteropolysaccharides covalently attached to a diacyl glycerol [1]. They are membrane-associated and appear to be homogeneous as to size and structure for each species. However, both size and structure differ between species. Molecular size differences range from 20,000 to 150,000. These lipoglycans exhibit antigenic specificity [2,3], are immunogenic [2], adsorb specifically to mammalian cells [4], induce pyrogenicity in rabbits and clot *Limulus* lysate [5], and, in the case of *Acholeplasma laidlawii*, serve as a specific receptor for the enveloped mycoplasma virus, MVL2 [6].

Lipoglycans from two species, *A. granularum* and *A. axanthum*, were selected for elucidation of the structure of the oligosaccharide chains and subsequent definition of the role of specific chemical features involved in various biological interactions. *A. granularum* was chosen as a representative of a non-pathogen and *A. axanthum* as an example of a possible pathogen. The complete structure for the lipoglycan from *A. granularum* has been determined [7]. It has a monomeric weight of 20,000 and is composed of glucose, galactose, N-acetylglucosamine, N-acetylfucosamine, glycerol, and fatty acid esters. It consists of a linear oligosaccharide chain with 12 repeating units of nine sugars:

\[
\text{Glc}(\beta 1\rightarrow 2)-\text{Glc}(\alpha 1\rightarrow 4)-\text{Glc}(\alpha 1\rightarrow 3)+4)-\text{FcNAc}(\beta 1\rightarrow 3)-\text{Galp}(\alpha 1\rightarrow 3)-
\]

\[
\text{Galp}(\alpha 1\rightarrow 3)-\text{Galp}(\alpha 1\rightarrow 3)+4)-\text{FcNAc}(\beta 1\rightarrow 3)+4)-\text{GlcNAc}(\beta 1\rightarrow 4)-
\]

attached to diacylglycerol. Although the position of the linkages (three or four) on the amino sugars has not been resolved, it is presumed they are on carbon 4 from indirect evidence.
The lipoglycan from A. axanthum has been only partially characterized. Its monomeric weight is about 100,000 and is composed of glucose, galactose, N-acetylfucosamine, N-acetylquinovosamine, glycerol, fatty acids, and phosphorus. A trace of galactosamine may be present. The existence of phosphorus as an integral component of the molecule was detected upon attempts to remove traces of contaminating nucleic acids by passage through an anion exchange resin. In contrast to the lipoglycan from A. granularum, the lipoglycan from A. axanthum could not be eluted with water. Treatment with cold 70 percent HF, which releases inorganic phosphorus without hydrolysis of glycosidic bonds, liberated half of the galactose residues of the lipoglycan. Assay of a galactose in the intact molecule with galactose oxidase, which oxidizes the -CH₂OH at the sixth position, likewise detected half of the galactose. These findings together with the ratio of galactose:P of two indicate that half of the galactose exists as galactose phosphate side chains. Treatment of the intact lipoglycan with glycosidases, which should remove appropriate terminal sugar residues, indicated that the terminal three residues are β-linked glucose, followed by one β-linked galactose and then two α-linked glucose residues. Chromium trioxide oxidation, which selectively destroys β-linked neutral and amino sugars, resulted in the loss of six of every eight glucose, one of two galactose, and all of the amino sugars. Analysis of the products of Smith degradation suggest that all of the galactose residues are linked 1→6, two of eight glucose residues are linked 1→2, three are 1→4, and three are 1→3. All of the amino sugars survive periodate oxidation and are probably linked 1→4. One large fragment remains after Smith degradation which, after de-N-acylation, appears to contain 12 sugar residues of which three are glucose, six are fucosamine, and three are quinovasamine. Controlled acid hydrolysis of this fragment yields a trisaccharide of glucose and a pentasaccharide of fucosamine. All of the phosphorus of the lipoglycan is associated with this 12 sugar fragment, suggesting that the galactose phosphate residues are distributed evenly along the molecule on one of the three glucose residues unoxidized by periodate. All of the amino sugars are N-acylated. Three of every four N-acyl groups are acetyl while one of four is a long chain fatty acid. Some of these fatty acids are hydroxy fatty acids. These data and other evidence not mentioned here are suggestive of an oligosaccharide chain consisting of 24 repeating units of 19 sugar residues each:

GlcP(β1→2)-GlcP(β1→2)-GlcP(β1→6)-GalP(β1→4)-GlcP(α1→4)-
GlcP(α1→4)-FucNAc(β1→4)-FucNAc(β1→4)-FucNAc(β1→4)-FucNAc(β1→4)-
FucNAc(β1→4)-FucNAc(β1→3)-GlcP(β1→3)-
GalP-P-(α1→6)

GlcP(β1→3)-GlcP(β1→4)-QvNAc(β1→4)-QvNAc(β1→4)-QvNAc(β1→2)-

The placement of the fucosamine and quinovosamine segments is arbitrary and could be reversed. More data are required to confirm this tentative structural arrangement.

Knowledge of the structural features of these two lipoglycans has facilitated an examination of the specific nature of their antigenic determinants [8]. Specific antisera were raised against each lipoglycan in rabbits by injection of autologous erythrocytes coated with appropriate lipoglycan. Two types of experiments were performed. One type examined the blocking of hemagglutination by specific antiserum of sheep erythrocytes coated with homologous lipoglycan as well as the interference of antibody binding of radiolabeled lipoglycans by unlabeled disaccharides; the second type examined binding of radiolabeled disaccharide by antibody against lipoglycan.
Varying concentrations of disaccharides and oligosaccharides (0.5 to 6 mg/ml) were mixed with a predetermined dilution of antiserum. After incubation for 45 minutes at 37°C, lipoglycan-coated sheep erythrocytes were added. Following 30 minutes at 22°C, microtiter plates were examined for hemagglutination. Among all of the carbohydrates tested, including all of the possible anomer and carbon number linkages of glucose as well as α-galactose-1-phosphate and oligosaccharides unrelated to lipoglycan structures, hemagglutination of *A. granularum* lipoglycan was inhibited completely by sophorose (Glcps(β1–2)-Glcps, 2 mg/ml) and maltose (Glcps(α1–4)-Glcps, 4 mg/ml), while for *A. axanthum*, only sophorose (2 mg/ml) was effective. These results are compatible with the terminal sugar sequences of both lipoglycans and indicates that the terminal three sugar units of each oligosaccharide chain define the antigenic specificity. The first two sugar radicals exert a greater influence than the second and third since maltose was less effective in the case of *A. granularum* lipoglycan than sophorose. Furthermore, the identical nature of the terminal two sugar radicals in both lipoglycans explains the cross-reactivity of their antisera. Pretreatment of antisera against both lipoglycans with unlabeled sophorose was followed by addition of an excess amount of lipoglycans, labeled with ¹⁴C-palmitic acid. Immunoglobulins were then precipitated with cold ammonium sulfate [9], washed, and counted. In both cases increasing amounts of sophorose caused decreasing adsorption of radiolabeled lipoglycans to immune globulins (Table 1). No such effect was noted with normal serum.

The availability of ¹⁴C-maltose permitted examination of its binding to immune globulins against both lipoglycans (Table 2). The amount of maltose bound was proportional to the dilution of antiserum against *A. granularum* lipoglycan. No significant binding occurred with either normal serum or antiserum against *A. axanthum* lipoglycan, as would be predicted from the oligosaccharide structures.

It has been reported previously that acholeplasmal lipoglycans bind to sheep and rabbit erythrocytes and to cultured mouse fibroblasts and rabbit epidermal cells [4]. The receptor sites on these eucaryotic cells are distinct for the lipoglycans since gram-negative bacterial lipopolysaccharides do not compete for binding of lipoglycans. The lipoglycan receptor has been isolated and characterized as a lipoglycoprotein from a variety of eucaryotic cell types [10]. However, this aspect of the study is not the subject of this report. Rather, attempts to define the portion of the lipoglycan molecule which is involved in attachment to erythrocytes will be

|  | *A. granularum* |  | *A. axanthum* |
|---|---|---|---|
|  | Lipoglycan Binding (Counts per Minute) |  | Lipoglycan Binding (Counts per Minute) |
| Sophorose Added (mg) | Specific Antiserum | Normal Serum | Sophorose Added (mg) | Specific Antiserum | Normal Serum |
| 0 | 41,621 | 801 | 0 | 3,886 | – |
| 0.5 | 37,831 | 689 | 0.4 | 2,841 | – |
| 1.0 | 32,531 | 628 | 0.8 | 2,503 | – |
| 1.25 | 2,392 | 490 | 5.0 | 1,296 | 137 |
| 2.0 | 27,120 | 823 |  |  |  |
TABLE 2
Binding of $^{14}$C-maltose to Immune Globulins in Antiserum
Against Lipoglycan from A. granularum

| Serum Dilution | Specific Antiserum A. granularum (Counts per minute) | Normal Serum |
|----------------|---------------------------------------------------|--------------|
| 1:2            | 19,150                                            | 1,120        |
| 1:4            | 6,823                                             | 580          |
| 1:8            | 4,362                                             | 253          |
| 1:16           | 1,250                                             | 123          |
| 1:32           | 126                                               | 68           |

presented. Obviously, the terminal sugar sequences of the oligosaccharide chains are not involved since lipoglycans bound to erythrocytes react with specific antisera. Lipoglycans from A. granularum and A. axanthum were preincubated with a variety of compounds, including mono-, di-, and oligosaccharides, proteins (bovine serum albumin, hemoglobin, glycophorin), and amino acids to determine their effect on attachment or competition for binding sites on erythrocytes. In no case was any inhibition observed that could not be considered non-specific, i.e., >200 μg/ml. Periodate-oxidized, borohydride-reduced lipoglycans were capable of binding to erythrocyte receptors although these coated erythrocytes no longer could agglutinate in the presence of specific antisera against lipoglycans. Exposure of erythrocytes, pretreated with oxidized lipoglycans, to untreated lipoglycans also were no longer capable of hemagglutination. Apparently the receptor sites on the erythrocytes were occupied with lipoglycans on which the antigenic determinants had been destroyed.

Deacylation of the lipoglycan from A. granularum destroyed its capacity to bind to erythrocytes. However, deacylation of the lipoglycan from A. axanthum had no effect. Since A. axanthum lipoglycan contains N-acyl fatty acid residues, these would not be removed by mild alkali. Hence, a deacylated lipoglycan from another species, A. modicum, was examined. This deacylated lipoglycan exists as a monomer, a dimer, and a tetramer [3]. The dimer and tetramer retained the ability to attach to erythrocytes. Therefore, it would appear that size rather than fatty acid residues is important for binding. The monomer weight of the lipoglycan from A. granularum is 20,000, from A. modicum 36,000, and from A. axanthum 100,000. The smallest molecule found to bind to erythrocytes was the dimer from A. modicum, which possesses a size of 72,000 daltons. Intact lipoglycans exist in polymeric form and this fact probably explains why all bind to mammalian cells. Presumably, internal segments of oligosaccharide chain are involved. Further evidence of the non-involvement of fatty acyl groups was the finding that simultaneous or pre-exposure of erythrocyte receptors to glycolipids, acylated glucose, and acylated polysaccharides had no effect on lipoglycan binding.

The biological significance of these lipoglycans from Acholeplasma, which are considered non-pathogenic, is not clear. Likely possibilities include the expression of a distinctive surface property for each species, stabilization of the cell membrane, and, in the case of biological interactions, modulation of the immune response.
ACHOLEPLASMAL LIPOGLYCANS

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