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Isolation and Characterization of the Sheep Erythrocyte Receptor for Acholeplasmal Lipoglycans

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A receptor specific for lipoglycans from *Acholeplasma axanthum* and *Acholeplasma granularum* was isolated from sheep erythrocyte stroma by extraction with *n*-pentanol and permeation chromatography. The purified receptor appeared as one band on sodium dodecyl sulfate-polyacrylamide gels and stained with Coomassie blue, periodate-Schiff reagent, and Sudan black. It was distinct from the erythrocyte receptor for gram-negative lipopolysaccharides and the glycophorin receptor for certain species of *Mycoplasma*. Periodate oxidation and trypsin did not affect the receptor activity in intact erythrocytes, but the purified receptor was susceptible to proteolytic digestion. Specific receptors, sensitive to trypsin digestion, could be isolated from rabbit kidney and cultured rabbit epidermal cell membranes. These could be distinguished from the receptor from erythrocytes by their solubility in *n*-pentanol. The segment of the lipoglycan molecule which binds to these receptors was not lipoidal in nature and was distinct from the specific antigenic determinants of the lipoglycans.

Acholeplasmal lipoglycans have been demonstrated to attach to sheep and rabbit erythrocytes, cultured rabbit epidermis, and mouse fibroblasts in vitro as assessed by passive agglutination of lipoglycan-coated cells in the presence of specific antibody (9). The structural analogy between these lipoglycans and bacterial lipopolysaccharides, which are known to bind to receptors on eucaryotic cells (13, 22), suggested that a similar receptor might exist for acholeplasmal lipoglycans. Furthermore, the endotoxin-like activity, e.g., pyrogenicity and *Limulus* lysate clotting (17), of these lipoglycans should require close interaction with eucaryotic cells. This report describes the isolation and characterization of a receptor, distinct from that involved in the binding of gram-negative lipopolysaccharide, from sheep erythrocyte membranes to which lipoglycans from *Acholeplasma axanthum* and *Acholeplasma granularum* become attached.

MATERIALS AND METHODS

Lipoglycans were isolated and purified from lipid-depleted cells of *A. axanthum* S743 and *A. granularum* BTS39 as previously described (20). The lipoglycan from *A. granularum* was purified further by passage through an anion-exchange resin (19) rendering it totally free from contaminating nucleic acids. Since the lipoglycan from *A. axanthum* contains phosphorus (P. F. Smith, unpublished data), further purification by treatment with an anion-exchange resin was not possible. However, nucleic acid contamination of this preparation was less than 1% (20). Since lipoglycans give opalescent solutions, some of the absorption at 260 mm may not be due to nucleic acids. Protein contamination of both lipoglycans was not detectable, i.e., less than 0.01%. Both preparations were homogenous by criteria previously described (18, 20). Radiolabeled lipoglycans were prepared by growth of *A. axanthum* and *A. granularum* in 1-[^{14}C]palmitic acid. Typically, 1 liter of culture contained 50 μCi of 1-[^{14}C]palmitic acid (58.6 mCi/mmol). The lipoglycans were isolated and purified as described previously (20), except the dialysis step was substituted with diethyl ether extraction for removal of phenol from the aqueous phenol extract. The specific activity of the ^{14}C-labeled lipoglycan from *A. granularum* was estimated to be 2,000 cpm/μg; that of lipoglycan from *A. axanthum* was 960 cpm/μg.

Treatment of erythrocytes with various reagents. A variety of treatments were applied to intact erythrocytes to obtain some initial characterization of the receptor. Periodate oxidation was conducted by suspending 2 ml of packed sheep erythrocytes in 100 ml of phosphate-buffered saline made 0.1 to 0.5 mM with $NaIO_4$. After incubation at either 4 or 37°C in the dark for 45 min, excess periodate was destroyed by the addition of glucose to a final concentration of 0.5%. Treated erythrocytes were washed three times in phosphate-buffered saline containing 0.5% glucose and 0.5% gelatin and finally suspended in 100 ml of phosphate-buffered saline containing 0.5% gelatin.

Treatment of erythrocytes with formaldehyde was carried out by suspending 2 ml of packed cells in 100 ml of phosphate-buffered saline containing 2% formaldehyde, incubating overnight at 4°C with gentle stirring followed by washing four times, and resuspending in 100 ml of phosphate-buffered saline.

Exposure of erythrocytes to Na_2SO_3 followed a similar procedure, except for the use of 0.01 M Na_2SO_3, a temperature of 37°C, and a time of 60 min.

Erythrocytes also were treated with various concentrations of the following enzymes: trypsin, pronase, chymotrypsin, and papain (100 to 500 μg/ml in Tris-hydrochloride, pH 6.5); wheat germ lipase and phospholipases A, C, and D (50 μg/ml in Tris-hydrochloride, pH 6.5); *N*-acetylneuraminidase (10 U/ml in Tris-hydrochloride, pH 6); and α- and β-glucosidases and galactosidases (50 to 500 μg/ml). Conditions were as previously described (19). After incubation for 60 min at 37°C, the cells were washed four times and suspended in phosphate-buffered saline to the original volume.

Blockage of binding of lipoglycan to sheep erythrocytes with putative inhibitor. By using procedures previously described to bind the lipoglycan to sheep erythrocytes (9), we determined that 15 μg of acholeplasmal lipoglycan was the minimal amount needed to incubate with 0.1 ml of packed sheep erythrocytes to optimally sensitize them for agglutination with rabbit antilipoglycan serum. One coating unit refers to this quantity.

Attempts were made to block the receptors on erythrocytes or the portion of the lipoglycan molecule which attached to the receptor by exposure of either erythrocytes or lipoglycans with putative inhibitor. Various amounts of carbohydrates, proteins, and lipids were incubated with erythrocytes (2% suspension in phosphate-buffered saline) for 1 h at 37°C. After washing four times, the cells were exposed to 5 μg of lipoglycan per ml by the coating procedure described previously (9).

Conversely, various amounts of potential inhibitor were added to 15-μg amounts of lipoglycan in phosphate-buffered saline and incubated for 1 h at 37°C. Then 0.1 ml of packed cells was added, incubation was continued for 45 min, and erythrocytes were washed three times and suspended in phosphate-buffered saline.

Enzymatic treatment of purified receptor. Purified receptor from erythrocyte stroma (50 μg for each treatment) was incubated with each of the following enzymes: trypsin, pronase, papain (30 μg/ml); α- and β-glucosidases and galactosidases and fucosidase (20 μg/ml); wheat germ lipase and phospholipases A, C, and D (10 μg/ml). The preparations were incubated in the appropriate buffers for 72 h at 37°C. A drop of toluene was added to each to prevent bacterial growth. Receptors from kidney and epidermal cell membranes were treated with trypsin and wheat germ lipase only. Reaction mixtures were dialyzed against deionized water, centrifuged, and lyophilized. Enzyme-treated receptor, receptor alone, enzyme alone, and boiled enzymes were assayed for receptor activity by hemagglutination inhibition.

Preparation of erythrocyte stroma. Sheep erythrocytes (20% suspension in modified Alsevers solution; Colorado Serum Co., Denver, Colo.) were washed three times in 0.15 M NaCl by centrifugation at 4°C and 1,000 × *g*. Two methods for preparation of stroma were used. One was the method of Marchesi and Palade (11) in which erythrocytes were lysed in 10 volumes of 5 mM Tris-hydrochloride buffer (pH 7.0) containing 1 mM EDTA, stirred for 15 min, and sedimented at 4°C and 25,000 × *g* for 30 min. The pellet was suspended in the same buffer and centrifuged at 25,000 × *g* for 10 min. Hemoglobin was removed by suspension of the pellet in 0.05 M Tris-hydrochloride (pH 7.0) containing 0.5 M NaCl and 1 mM EDTA followed by centrifugation as above. Repeated washing in this medium yielded a milky white pellet of erythrocyte stroma. The pellet was suspended in a small volume of deionized water and lyophilized. The second method was that described by Schroffel et al. (15). Washed erythrocytes were lysed in 9 volumes of 15 mM sodium barbital buffer (pH 7.3) containing 3 mM $MgCl_2$. The erythrocyte stroma were sedimented at 20,000 × *g* and 4°C for 40 min and then washed four times in the same buffer, suspended in deionized water, and lyophilized. Both preparations were stored at −20°C until further use.

Isolation of lipoglycan receptor from erythrocyte stroma. The procedure used for isolation of lipoglycan receptor was based upon the method of Kirchhof et al. (6) for isolation of J blood group active lipoprotein from bovine erythrocytes. Sheep erythrocyte stroma (40 mg) were suspended in 0.5 ml of boiled cold deionized water, frozen at −20°C, held overnight, thawed, and washed with deionized water at 4°C. The supernatant fluids from the washings, obtained by centrifugation at 45,000 × *g* were dialyzed against water, lyophilized, and stored at −20°C. The pellet was suspended in 80 ml of cold deionized water and homogenized. To this suspension, kept in an ice bath, an equal volume of cold (4°C) *n*-pentanol was added, and the mixture was vigorously stirred for 30 min. After centrifugation at 30,000 × *g* and 4°C for 15 min, the mixture was separated into an insoluble interfacial film, an upper organic phase, and a lower aqueous phase. The aqueous phase and the interfacial material were extracted with *n*-pentanol, dialyzed against five changes of deionized water, lyophilized, and stored at −20°C. The material from the aqueous phase is referred to as crude lipoglycan receptor. The *n*-pentanol phases were dried under vacuum at 45°C.

A portion of crude receptor was fractionated on a glass bead column as detailed below, and another portion was subjected to dextran sulfate precipitation (7). Lyophilized crude receptor (10 mg) was dissolved in 0.04 ml of 5% dextran sulfate plus 0.1 ml of M $CaCl_2$ or with $CaCl_2$ alone, held at 4°C for 24 h, and centrifuged. The supernatant fluid was collected and dialyzed against deionized water, lyophilized, and stored at −20°C. This fraction is referred to as α-lipoprotein (7). The precipitate was dissolved in 0.15 M NaCl to its original volume and treated with 0.1 ml of M potassium oxalate to remove calcium. After 24 h at 4°C, the precipitate was sedimented, and the supernatant fluid, termed β-lipoprotein, was dialyzed and lyophilized.

Further purification of crude receptor as well as both α- and β-lipoprotein fractions was accomplished on a column (0.5 by 25 cm) of controlled-pore-size glass beads (CPG-10-75; Electro-Nucleonics, Inc., Fairfield, N.J.), which have an operating range of molecular weight 3×10^3 to 3×10^4. Each of the three different fractions was applied separately, and each was eluted with 1 M NaCl. Fractions (2 ml) were collected, dialyzed against deionized water, and lyophilized. The columns were then eluted with absolute methanol. Since receptor activity appeared solely in the methanol eluate, this fraction was reapplied to the column and eluted with methanol, collecting 2-ml fractions. Each methanol fraction was extracted twice

with chloroform-methanol (1:2, [vol/vol]), and both phases were dried under a stream of nitrogen.

Preparation of kidney cell membrane. Four New Zealand White rabbits were sacrificed, and the kidneys were removed and chilled in an ice bath for 30 min. After removal of the capsule and adipose tissue, the kidneys were minced into small pieces, washed with phosphate-buffered saline, and filtered through nylon cloth. After five washings, the tissue was ground in a tissue grinder and washed four times with 0.1 M Tris-hydrochloride buffer (pH 7.2) by centrifugation. The ground tissue was frozen and thawed three times and subjected to three additional washings. Connective tissue was removed by centrifugation at 60 × *g* for 20 min. The supernatant fluid containing the membranes was centrifuged at 4°C and 25,000 × *g* for 15 min. Viscous material was removed by resuspension of the pellet in 0.05 M Tris-hydrochloride (pH 7.2) containing 0.5 M NaCl followed by centrifugation as above. After an additional three washings, the greyish white pellet was suspended in deionized water and lyophilized.

Preparation of rabbit epithelial cell membranes. Rabbit epidermal cells, SF 1 Ep (ATCC No. CCL 68), were grown as monolayers in 32-oz. (ca. 960-ml) bottles on Eagle minimal essential medium containing 7% calf serum. Cells from 100 bottles were removed by scraping into phosphate-buffered saline (pH 7.2). The harvested cells were washed three times by centrifugation at 4°C and 12,000 × *g* for 10 min. Membranes were obtained by three cycles of alternate freezing and thawing. The resultant membranes were washed five times with phosphate-buffered saline (pH 7.2) by centrifugation at 4°C and 16,000 × *g* for 15 min. The resulting pellet was lyophilized.

Isolation of receptor from tissues other than erythrocytes. Extraction with *n*-pentanol, as used with erythrocyte stroma, was employed for both rabbit kidney cell membranes and the rabbit epidermal cell membranes. Since the activity resided in the organic phase, rather than in the aqueous phase as with erythrocytes, dried pentanol extracts were extracted twice with petroleum ether (boiling point, 30 to 60°C). The petroleum ether-insoluble residue was taken up in methanol, centrifuged to remove insoluble material, and dried under a stream of nitrogen. Further purification of the methanol-soluble material was accomplished by passage through the glass bead column as described above for erythrocytes. The petroleum ether-soluble material was dried under a stream of nitrogen.

Gel electrophoresis of receptor. The method of Davis (3) was employed for sodium dodecyl sulfate-polyacrylamide electrophoresis. Gels with various concentrations (7 and 10%) of polyacrylamide in 0.19 M Tris-glycine buffer (pH 8.5) containing sodium dodecyl sulfate (1%) were prepared. Most of the electrophoresis was performed with 7% gels. Samples derived during fractionation of erythrocyte stroma were treated with 1% sodium dodecyl sulfate for 1 h at 37°C. After samples were mixed with equal volumes of 40% sucrose, they were applied to the gel and subjected to current of 3 mA/gel in an analytical gel electrophoresis apparatus (Canalco, Bethesda, Md.) in 0.19 M Tris-glycine buffer (pH 8.5) at room temperature. Bromophenol blue served as the tracking dye. Electrophoresis was terminated when the tracking dye reached a distance 1 cm from the bottom of the gel. Gels were removed and stained with Coomassie blue, periodate-Schiff reagent, or Sudan black (3).

Treatment of lipoglycan with various reagents. Attempts were made to define what characteristics of lipoglycan are required for its binding to erythrocyte receptor. Deacylation was performed only with lipoglycan from *A. granularum* since *N*-acyl fatty acids from *A. axanthum* are resistant to this procedure (18). Lipoglycan was incubated with 1 N NaOH for 15 min at 37°C, dialyzed, and lyophilized.

Both lipoglycans were subjected to oxidation with periodate. Approximately 0.1 μmol of lipoglycan in 0.1 M acetate buffer (pH 5.0) was incubated with 0.04 mM $NaIO_4$ for 17 h at 37°C. Reaction mixtures were neutralized with 0.1 N NaOH, and reduction was carried out with $NaBH_4$ (0.1 mg/ml) for 2 h. Excess borohydride was inactivated by acidification to pH 5.0 with 1 N acetic acid. Reaction mixtures were dialyzed against deionized water and lyophilized. Erythrocytes were coated by exposure to treated lipoglycans (100 μg/ml) and tested for hemagglutination activity with antiserum against the appropriate lipoglycan.

To evaluate these preparations for binding without retention of antigenic specificity, the treated erythrocytes were exposed to 1 coating unit of untreated lipoglycan and tested for hemagglutinating activity.

Binding of radiolabeled lipoglycans to erythrocyte stroma. Binding experiments were conducted at 37°C in 1.5-ml Eppendorf microtubes. Radiolabeled lipoglycans (20 μg representing 20,000 to 40,000 cpm in 50 μl of 0.15 M NaCl) were added to different concentrations of erythrocyte stroma, incubated for 1 h, and centrifuged for 5 min in a microfuge at 13,000 rpm, and pellets were washed twice with phosphate-buffered saline. Washed pellets, suspended in deionized water, were transferred to scintillation vials, solubilized with BioSolv (Beckman Instruments, Inc., Fullerton, Calif.), and counted in a Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.).

Competitive binding experiments were conducted by the addition of 50 μl of labeled lipoglycan to different concentrations of purified lipoglycan receptor. After 1 h, a constant amount of erythrocyte stroma was added to each mixture and reincubated for 1 h. The mixtures were counted as described above.

Analytical procedures. Antiserum against the lipoglycans was produced in rabbits as previously described (9). 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 appropriately treated sheep erythrocytes. After incubation for 30 min at room temperature, the plates were examined for hemagglutination.

Inhibition of passive hemagglutination. Various amounts of the potential inhibitors were added to either the sheep erythrocytes or the lipoglycan and preincubated for various periods of time up to 1 h before assay for sensitized erythrocytes by the passive hemagglutination technique. Inhibition was considered positive when no agglutination occurred at dilutions of 1:2 or greater with homologous antiserum.

Thin-layer chromatography of lipids extractable from receptors isolated from all three tissues employed silica gel G and the solvent system, $CHCl_3$–CH_3OH–H_2O (65:25:4 [vol/vol]) (19).

The method of Marchesi and Andrews (10) was

followed for the isolation of glycoproteins from erythrocyte stroma. Glycophorin was extracted from stroma with 0.3 M lithium diiodosalicylate and purified by phosphocellulose chromatography.

Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as a standard; carbohydrate was determined by the phenol-sulfuric acid method (1). All chemicals and enzymes were obtained from commercial sources.

RESULTS

Characterization of lipoglycan-receptor on intact erythrocytes. Neither ionic strength nor pH appeared to have an effect on the attachment of lipoglycans to erythrocytes. Incubation of 1 coating unit of lypoglycan with erythrocytes in the presence of various inorganic salts (NaCl, 0.7 M; $CaCl_2$, 0.3 M; $MgCl_2$, 0.3 M) or in the presence of 5 mM EDTA had no significant effect on hemagglutination. Likewise, variation of pH between 6.0 and 8.0 did not influence the agglutination.

Pretreatment of erythrocytes with $NaIO_4$ at either 4 or 37°C did not abolish the ability of erythrocytes to be coated with lipoglycans. On the contrary, treated cells exhibited a twofold increase in hemagglutination capacity. With periodate-treated erythrocytes, a relationship was seen between the concentration of lipoglycans used for coating, the serum titer, and hemolysis of agglutinated erythrocytes during incubation at 4°C. Lysis occurred increasingly earlier as the concentration of coating lipoglycan was increased while maintaining a constant antiserum titer. Furthermore, lysis occurred earlier in higher than in lower antiserum concentrations.

Formaldehyde treatment of erythrocytes before coating with either lipoglycan resulted in a twofold decrease in hemagglutination capacity. Hence some alteration, either chemical or physical, of the binding sites resulted from modification of free amino groups. Among all of the enzymatic treatments of erythrocytes, only trypsin treatment exhibited an effect. Pretreatment with trypsin actually increased twofold their capacity to hemagglutinate. Thus, such treatment appeared to unmask the receptor, and the results also suggested that glycophorin plays no role in binding of lipoglycans. Glycosidases were used with both untreated and trypsin pretreated erythrocytes. In neither instance was there any effect on hemagglutination capacity. None of the lipolytic enzymes had any effect.

Preexposure of erythrocytes with different carbohydrates (D-glucose, D-fucose, D-galactose, D-ribose, D-mannose, kojibiose, sophorose, nigeran, laminaran, maltose, cellobiose, *N*-acetylglucosamine, isomaltose, gentibiose, lactose, melibiose, and stachyose, 5 to 10 mg/ml), various proteins (bovine serum albumin and human hemoglobin, 2 to 10 mg/ml), phospholipids (phosphatidyl ethanolamine and phosphatidyl choline, 100 to 500 μg/ml; sphingosine and sphingomyelin, 1 mg/ml; acylated sugars, 100 to 500 μg/ml), and *Escherichia coli* lipopolysaccharide failed to inhibit coating of cells with lipoglycans as assessed by hemagglutination.

Isolation of receptor from erythrocyte stroma. Table 1 presents data on erythrocyte coating inhibition as measured by hemagglutination inhibition and the chemical composition of fractions derived during isolation of lipoglycan receptor from sheep erythrocytes. Freezing and thawing of erythrocyte stroma prepared in the presence of either Mg^{2+} or EDTA resulted in partial release of receptor activity into a soluble form with concomitant loss from the stroma. Better recovery of receptor was obtained by extraction of stroma with *n*-pentanol by which receptor activity was recovered in the aqueous phase. This crude lipoglycan receptor exhibited from 4 to 8 major bands on polyacrylamide gels, a significant reduction from the 18 observed with erythrocyte stroma. Fewer protein bands, i.e., four, were observed when stroma were prepared in the presence of EDTA but in the absence of Mg^{2+}.

This crude lipoglycan receptor could be separated into two fractions by treatment with $CaCl_2$ or with $CaCl_2$ plus dextran sulfate. The precipitable portion, termed β-lipoprotein or low-density lipoprotein, was low in protein and high in carbohydrate. The opposite was true for the supernatant fraction, termed α-lipoprotein or high-density lipoprotein. Gels on which these two fractions were electrophoresed exhibited two major bands for the β-lipoprotein (not shown) and four major bands for α-lipoprotein (Fig. 1). The specific activity for lipoglycan coating was fivefold greater in the α-lipoprotein than in the β-lipoprotein fraction. These two fractionation steps resulted in 13-fold purification from stroma prepared in the presence of EDTA.

Further purification was accomplished employing a column of controlled-pore-size glass beads. The eluate of the column to which was applied the α-lipoprotein fraction with 0.05 M Tris-hydrochloride (pH 7.0) containing 1 M NaCl at 4°C contained no coating inhibitory activity. All of the activity was found in the methanol eluate, which exhibited two bands on the gels, both of which stained with Coomassie blue and periodate-Schiff reagent. Repassage of the total methanol eluate through the column, but collecting fractions rather than the total eluate, resulted in the recovery of essentially all of the activity in the 20 to 26 ml (51% [dry weight] of the total eluate) fraction and 36 to 40 ml (14% [dry weight]) fraction. The first fraction

TABLE 1. Isolation of lipoglycan receptor from sheep erythrocyte stroma[a]

Fraction	Mg^{2+} stroma					EDTA stroma				
	Protein (μg/mg)	Carbohydrate[c] (μg/mg)	Total lipid P[d] (μg/mg)	Inhibitory concn[e] (μg)	Sp act[f]	Protein (μg/mg)	Carbohydrate (μg/mg)	Total lipid P (μg/mg)	Inhibitory Concn (μg)	Sp act
Stroma	566 ± 38	0.03 ± 0	ND[g]	400 ± 28	1	450 ± 14	0.08 ± 0.01	ND	250 ± 7	1
Crude receptor	606 ± 23	0.17 ± 0.01	1.6	70 ± 7	5.7	133 ± 5	0.07 ± 0.01	1.8	30 ± 3	8.3
β-Lipoprotein	250 ± 7	0.23 ± 0.01	ND	150 ± 7	2.7	67 ± 4	0.17 ± 0.03	ND	50 ± 5	5
α-Lipoprotein	667 ± 18	<0.01	ND	30 ± 3	13.3	268 ± 11	<0.01	ND	10 ± 1	25
CPG-10-75 column										
Total methanol eluate	78 ± 6	0.20 ± 0.03	3.5	12 ± 2	33.3	75 ± 5	0.18 ± 0.03	3.9	16 ± 2	15.6
Pooled fraction (20 to 26 ml)	35 ± 1	0.10 ± 0.01	0.4	100 ± 7	4	35 ± 3	0.11 ± 0.01	0.5	100 ± 7	2.5
Pooled fraction (36 to 40 ml)	266 ± 9	<0.01	14.1	2.5 ± 0.3	160	259 ± 13	<0.01	14.2	2 ± 0.3	125

[a] Starting materials and fractions are described fully in the text. For starting material, 500 to 1,200 mg of stoma was utilized.
[b] Protein concentration was determined by the method of Lowry et al. (8).
[c] Carbohydrate concentration was determined by the method of Ashwell (1).
[d] Total phosphorus was determined by the method of Smith et al. (20).
[e] The inhibitory concentration is the smallest quantity of material which, when incubated with 3 binding units of lipoglycan, prevented subsequent sensitization of sheep erythrocytes as determined by lack of hemagglutination with rabbit antilipoglycan serum.
[f] The specific activity is the inhibitory concentration of stroma divided by the inhibitory concentration of fraction. The probabilities that differences are significant are 0.95 or greater.
[g] ND, Not determined.

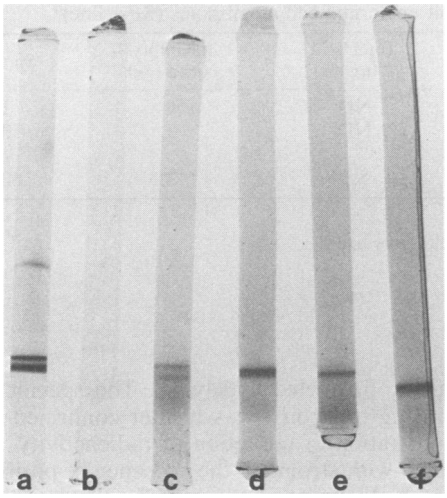

FIG. 1. Sodium dodecyl sulfate-polyacrylamide patterns of various fractions derived from sheep erythrocytes during purification of lipoglycan receptor. Gels: (a) α-lipoprotein, (b) 1 M NaCl eluate from glass bead column, (c) total methanol eluate from column, (d) 20- to 26-ml methanol eluate, (e) 36- to 40-ml methanol eluate, and (f) 42- to 48-ml methanol eluate. The negative electrode is at the top. Gels were stained with Coomassie blue.

exhibited a specific activity of 3.7 to 4.0, and the second fraction exhibited a specific activity of 125 to 160, depending upon the lot of starting material. Each fraction exhibited only one band on both 7 and 10% polyacrylamide gels and stained positively for protein, carbohydrate, and lipid. Chloroform-methanol extraction did not reduce the activity, which was retained in the methanol-water phase.

A similar but smaller quantity of active receptor could be obtained by passage of the β-lipoprotein fraction through the column. The fractionation into low- and high-density lipoprotein fractions could be bypassed, and the receptor could be purified on the glass column directly from the crude receptor fraction. The highly purified receptor is a white granular powder soluble in 0.1 M Tris-hydrochloride (pH 7.0). More than 50% of its activity was lost by heating at 70°C for 4 h; all of it was lost by heating at 100°C for 30 min. Treatment with 0.1 M HCl or 0.1 M NaOH for 15 min at 37°C destroyed 50% of the activity. Likewise, the enzymes trypsin, pronase, papain, and chymotrypsin destroyed its activity. Treatment with glucosidases and lipases had no effect on the coating inhibitory activity.

Isolation of receptor from other tissues. Normal and trypsin-treated rabbit kidney and epidermal cells as well as untreated membranes of both types of cells inhibited coating of erythrocytes by lipoglycans. Tables 2 and 3 present data on erythrocyte coating inhibition and the chemical composition of various fractions derived during the isolation of lipoglycan receptors from kidney and epidermal cell membranes. As with erythrocyte stroma, cyclic freezing and thawing resulted in partial release of receptor into a soluble form with concomitant loss from the membranes. However, upon extraction with *n*-pentanol, the coating-inhibitory activity appeared primarily in the organic phase (>90%). Extraction of the dry residue, after removal of *n*-pentanol, with petroleum ether left 80% of the inhibitory activity in the insoluble residue. All of the activity could be dissolved in methanol. Passage of the methanol-soluble material from kidney cell membranes through the controlled-pore-size glass bead column yielded several fractions with coating-inhibitory activity (Table 2). The most active fraction was F3 (28 to 30 ml) and represented about 10% of the dry weight of the total eluate. The specific activity correlated best with the amount of protein present in each fraction. Thin-layer chromatography of all fractions from the isolation steps of all tissues examined did not reveal any qualitative or quantita-

TABLE 2. Isolation of lipoglycan receptor from rabbit kidney cell membrane[a]

Fraction	Protein (μg/mg)	Carbohydrate (μg/mg)	Total lipid P (μg/mg)	Inhibitory concn (μg)	Sp activity
Membrane[b]	605 ± 42	0.04 ± 0.01	ND[c]	200 ± 16	1
Petroleum ether insoluble	180 ± 7	0.01 ± 0	ND	5 ± 1	40
Methanol soluble	1,057 ± 7	<0.01	4.4	4.5 ± 1	44.4
F1 (16 to 18 ml)	80 ± 7	<0.01	8.8	20 ± 3	10
F2 (20 to 26 ml)	30 ± 3	<0.01	0.8	50 ± 10	4
F3 (28 to 30 ml)	240 ± 10	<0.01	8.8	1.5 ± 0.4	133.3
F4 (32 to 38 ml)	30 ± 4	<0.01	7.2	20 ± 4	10
F5 (40 to 46 ml)	140 ± 14	<0.01	7.3	4 ± 0.7	50

[a] See Table 1 for explanation.
[b] The starting material was 100 mg.
[c] ND, Not determined.

TABLE 3. Isolation of lipoglycan receptor from rabbit epidermal cell membranes (SF-1 line)[a]

Fraction[b]	Protein (μg/mg)	Carbohydrate (μg/mg)	Total lipid P (μg/mg)	Inhibitory concn (μg)	Sp act
Membrane[c]	586	0.03	ND[d]	150	1
Petroleum ether insoluble	197	0.01	ND	3	50
Methanol soluble	138.5	<0.01	5.3	2.5	60

[a] See Table 1 for explanation.
[b] Only one lot consisting of a pool from three batches of cells was used.
[c] The starting material was 61 mg.
[d] ND, Not determined.

tive distinction in lipid composition. Treatment of each fraction from both kidney and epidermal cells with trypsin destroyed coating-inhibitory activity, whereas treatment with wheat germ lipase had no effect. Further fractionation of the methanol-soluble material from epidermal cells was not performed because of the small quantity of material obtained. As with the purified receptor from erythrocyte stroma, binding of kidney and epidermal cell membranes to lipoglycans did not block the antibody binding sites on the lipoglycans. Preincubation of 1 coating unit of lipoglycan with 10 inhibitory units to the receptor did not change the hemagglutination inhibition titers. Furthermore, purified receptors from both kidney and epidermal cell membranes did not block the receptor site for lipoglycans on erythrocytes. Erythrocytes were incubated with 10 inhibitory units of purified receptors from kidney and epidermal cell membranes for 45 min at 37°C. After four washings, the treated erythrocytes were exposed to 1 coating unit of lipoglycans. The hemagglutination titers were identical to those obtained with untreated erythrocytes.

Binding of radiolabeled lipoglycans to erythrocyte stroma. Erythrocyte stroma, rather than erythrocytes, were used to examine binding of radiolabeled lipoglycans since higher concentrations of lipoglycans, in particular that of *A. axanthum*, cause lysis of intact erythrocytes. The results of a typical experiment, in which the concentration of stroma are varied, are shown in Fig. 2. A typical dose-response curve was obtained, indicating a finite number of receptors per unit of stroma. This binding was not affected by temperature, but was somewhat dependent upon time of incubation reaching a maximum at 25 min (Fig. 3). Specific binding was corrected for nonspecific binding by incubation of stroma with 1.5 mg of unlabeled lipoglycan for 1 h followed by the addition of 50 μl labeled lipoglycan and further incubation for 30 min. Each experimental value has been corrected by subtraction of radioactivity bound under these conditions from the value obtained without preexposure to unlabeled lipoglycan. The specificity of binding to stroma was further confirmed by demonstration of reduction of radioactivity associated with stroma in the presence of purified receptor. Figure 4 shows that as the receptor concentration was increased, less radioactive lipoglycan sedimented with stroma, with 20 μg of receptor preventing attachment of 80% of the lipoglycan added.

Properties of lipoglycans. Attempts were made to define the portion of the lipoglycan molecules

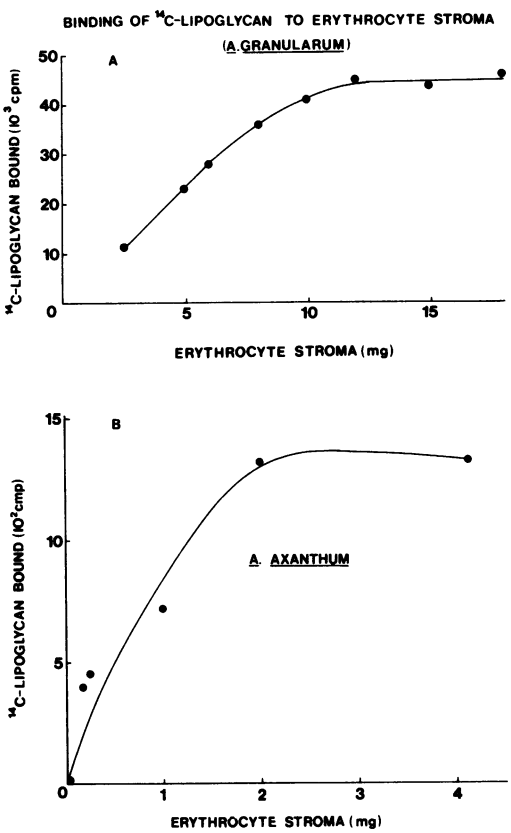

FIG. 2. Binding of [^{14}C]lipoglycans to erythrocyte stroma. (A) *A. granularum*, (B) *A. axanthum*.

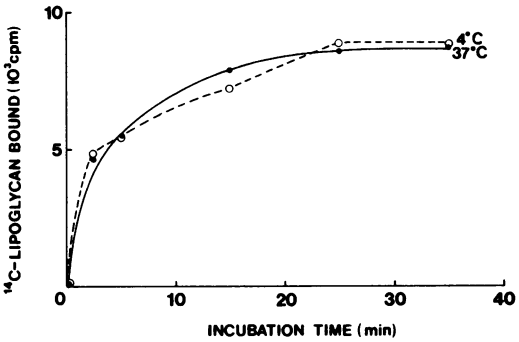

FIG. 3. Effect of incubation time and temperature on binding of [^{14}C]lipoglycan from *A. granularum* to erythrocyte stroma.

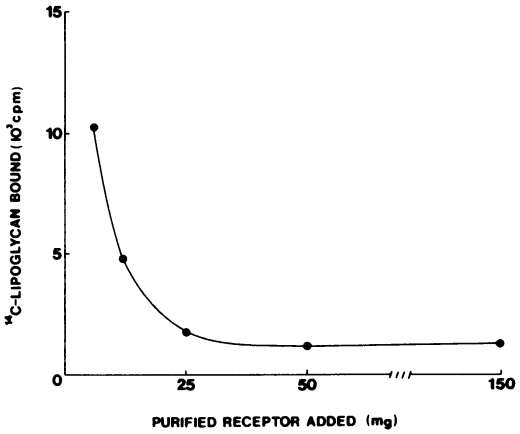

FIG. 4. Inhibition by purified lipoglycan receptor from erythrocytes on binding of [^{14}C]lipoglycan from *A. granularum* to erythrocyte stroma.

which is involved in their attachment to erythrocytes. Preincubation of lipoglycans with various compounds before the addition of erythrocytes was performed to assess their effect on attachment or competition for the binding sites on erythrocytes (Table 4). All of the carbohydrates tested had no effect on lipoglycan binding. Preincubation with bovine serum albumin at a concentration of 200 μg/ml completely inhibited binding of a maximum of 20 μg of lipoglycans. This concentration of albumin was 80-fold larger than the amount of purified receptor required, and the binding was therefore considered to be nonspecific. Hemoglobin (0.1 to 4 mg/ml) had no effect on the capacity of lipoglycan to coat erythrocytes. Glycophorin caused total inhibition at a concentration of 800 μg/ml, i.e., it was four times less active than serum albumin. Since nonspecific adsorption of lipoglycan to albumin and glycophorin was observed, a series of 17 different amino acids were tested at concentrations of 2 to 6 mg per ml. None of these had any effect.

Periodate-oxidized, borohydride-reduced lipoglycans appeared to bind to erythrocytes, although these coated erythrocytes no longer could agglutinate in the presence of specific antisera against lipoglycans. However, exposure of erythrocytes, pretreated with oxidized lipoglycan, to 1 coating unit of untreated lipoglycan no longer were capable of hemagglutination. Apparently receptor sites on erythrocytes were occupied with lipoglycans on which the antigenic determinants had been destroyed (17).

Deacylation of lipoglycan from *A. granularum* destroyed its capacity to bind to erythrocytes as assessed by hemagglutination and competition with intact lipoglycan. However, deacylation of lipoglycan from *A. axanthum* had no effect on its binding. Since *A. axanthum* lipoglycan contains *N*-acyl fatty acid residues, these would not be removed by mild alkali. Therefore, deacylated lipoglycan from another species, *Acholeplasma modicum*, was examined. This deacylated lipoglycan exists as a monomer, a dimer, and a tetramer (18). The dimer and the tetramer retained the ability to attach to erythrocytes. Hence, it would appear that size rather than fatty acid residues is of importance for binding. The monomeric weight of the lipoglycan from *A. granularum* is 20,000, that of the lipoglycan from *A. axanthum* is 100,000, and that of the lipoglycan from *A. modicum* is 36,000 (18).

DISCUSSION

Preliminary examination of the nature of the interaction between acholeplasmal lipoglycans and intact sheep erythrocytes suggested that lipoglycan binding was not ionic in character since variations in ionic strength and pH had no effect. However, periodate oxidation of intact erythrocytes increased the hemagglutination titer directed against lipoglycans. It is possible that such treatment removed glycophorin or other surface carbohydrates, thereby exposing more lipoglycan receptor sites. On the other hand, treatment of erythrocytes with formaldehyde has caused a reduction in hemagglutination capacity by specific antiserum to lipoglycans. Formaldehyde treatment might have altered the surface of the erythrocytes masking the availability of the receptors or have caused some conformational change in the receptor itself. Pretreatment of erythrocytes with trypsin caused a twofold increase in capacity to hemagglutinate. As with periodate oxidation, trypsin digestion apparently unmasked the receptor, possibly by the removal of glycophorin, which was found to have no significant inhibitory activity toward binding of lipoglycans. Likewise

TABLE 4. Potential inhibitors of binding of acholeplasmal lipoglycan to sheep erythrocytes[a]

Potential inhibitor tested	Range of concn (mg/ml)	Inhibitory concn (mg/ml)
Glycophorin	0.05–1.0	0.8
Bovine serum albumin	0.01–1.0	0.2
Phosphotidyl ethanolamine	0.1–0.5	NI[b]
Phosphotidyl choline	0.1–0.5	NI
Hemoglobin	0.1–4.0	NI
Lipopolysaccharide (*E. coli*)	0.01–0.5	NI

[a] Various amounts of potential inhibitors were added to 15 μg of lipoglycan in phosphate-buffered saline and incubated 1 h at 37°C. The mixture was added to 0.1 ml of packed sheep erythrocytes, incubated 45 min, washed three times with phosphate-buffered saline, and suspended to a concentration of 2%. A twofold decrease in hemagglutination titer with rabbit antilipoglycan serum was considered significant inhibition. The following amino acids were not inhibitory at concentrations of 2 to 6 mg/ml: L-glycine, D,L-serine, D,L-valine, D,L-alanine, D,L-threonine, D,L-methionine, D,L-phenylalanine, D,L-aspartic acid, L-tyrosine, L-histidine, L-asparagine, L-glutamic acid, L-arginine, L-lysine, L-cystine, L-proline, and L-isoleucine. The following carbohydrates were not inhibitory at concentrations of 0.5 to 8.0 mg/ml: D-ribose, D-mannose, D-fucose, D-galactose, N-acetylglucosamine, kojibiose, sophorose, nigeran, laminaran, maltose, cellobiose, isomaltose, gentibiose, lactose, melibiose, and stachyose.

[b] NI, Not inhibitory.

preexposure of erythrocytes to a wide variety of carbohydrates, lipids, and proteins failed to interfere with lipoglycan binding.

A scheme for the isolation and purification of the erythrocyte receptor for lipoglycans was devised, based upon the work of others (2, 11, 12, 23, 24). *n*-Pentanol was chosen for extraction of erythrocyte stroma because this procedure resulted in better recovery of receptor. Zwaal and Van Deenen (24) reported that the aqueous phase from *n*-pentanol extraction, i.e., the fraction containing the lipoglycan receptor, consisted of lipoproteins. These were separated further into an α-lipoprotein and a β-lipoprotein fraction (7). The concentration of lipoglycan receptor was found to be fivefold higher in the α-lipoprotein fraction. However, upon development of the next fractionation step, it was found that this separation into α- and β-lipoproteins could be bypassed. A logical further step was an attempt to separate the proteins in the α-lipoprotein fraction on the basis of size. When a column of controlled-pore-size glass beads was employed, neither water nor 1 M NaCl would elute any activity. The use of methanol to strip the column resulted in a useful fractionation. Apparently, the proteins were more soluble in methanol than in water. However, once eluted and freed of methanol, the proteins were readily soluble in water or buffer. Although activity was found in several fractions, only one possessed high specific activity and exhibited one band on sodium dodecyl sulfate-polyacrylamide gels of different concentrations. Its ability to stain with Coomassie blue, periodate-Schiff reagent, and Sudan black suggested that it was a lipoglycoprotein. This purified receptor did not interact with the receptor site on intact erythrocytes or with the antibody binding site of the lipoglycans. This receptor appears distinct from the erythrocyte receptor for gram-negative bacterial lipopolysaccharides. Springer et al. (22) purified a receptor for lipopolysaccharide from aqueous *n*-butanol extracts of human erythrocyte stroma and characterized it as a lipoglycoprotein. Although this receptor inhibited coating of gram-negative lipopolysaccharide onto erythrocytes, the purified acholeplasmal lipoglycan receptor had no such effect. Furthermore, the lipopolysaccharide receptor is concentrated in the β-lipoprotein fraction, whereas the lipoglycan receptor separates into the α-lipoprotein fraction. The comparative composition of the two receptors also differs.

A receptor for acholeplasmal lipoglycans also was isolated from rabbit kidney cell and cultured epidermal cell membranes. It appears to possess physicochemical characteristics differing from those of the receptor from erythrocytes. Apparently these differences are related to a higher content of lipids as judged from thin-layer chromatography and its extraction into the organic phase with *n*-pentanol. However, only trypsin destroys its coating-inhibitory activity. Springer and Adye (21) isolated a receptor for gram-negative lipopolysaccharide from human leukocytes and platelets. However, in contrast to their receptor from erythrocytes, the receptor from these blood cells was lipoidal in nature as it was found to be soluble in petroleum ether.

The receptor for acholeplasmal lipoglycans also appears different from that reported for *Mycoplasma* species. For two organisms which have been examined in some detail, *Mycoplasma gallisepticum* (4) and *Mycoplasma pneumoniae* (5), the sialoglycoprotein, glycophorin, of the erythrocyte membrane appears to be involved. In the case of *Acholeplasma* species, glycophorin and bovine serum albumin possessed very low activity and were considered nonspecific inhibitors of lipoglycan coating of erythrocytes. The specificity of the receptor for lipoglycans was confirmed by demonstration of binding of radiolabeled lipoglycans to erythrocyte stroma and the competition for this binding by purified receptor. Attempts to produce antibody against the receptor which would prevent this competition were unsuccessful. However,

similar difficulties have been experienced with the receptor for lipopolysaccharides (22).

It is probable that the intact lipoglycoprotein molecule is required for binding of lipoglycans, since destruction of polysaccharide moiety by periodate oxidation and proteolysis with trypsin destroys its activity. Removal of extractable lipids and treatment with lipases, however, has no effect. Nevertheless, neither procedure ensures total destruction of the lipid moiety. A partial examination of the lipoglycan segment involved in binding to the receptor was performed. The inability of a wide variety of lipids to interfere with binding and the retention of binding capacity upon deacylation suggests that the lipid radicals of the lipoglycans are not involved. Neither is the specific antibody binding site on the lipoglycan, since coating of erythrocytes does not mask the interaction between the terminal sugar units and antibody. Presumably, internal segments of the polysaccharide chain are involved. The size of the polysaccharide chain also is critical since monomers of the smaller lipoglycans, i.e., *A. granularum* and *A. modicum*, can no longer bind.

The biological significance of this specific binding of lipoglycans from *Acholeplasma* species, which are considered nonpathogenic, is not clear. It may be involved in modulation of the immune response (9) and initiation of their endotoxin-like activities (17) since these organisms are found associated with certain disease processes.

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