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Identification of Cholesterol in the Receptor Site for Rickettsiae on Sheep Erythrocyte Membranes

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Cholesterol was identified as an essential component of the receptor on the sheep erythrocyte to which *Rickettsia prowazeki* adsorbs before lysing the cell. Erythrocytes or ghosts, derived by hypotonic lysis, were treated with proteolytic enzymes, sialidase, sulfhydryl reagents, and periodate without affecting their ability to adsorb rickettsiae. Lipid extracts of ghosts and erythrocytes, on the other hand, contained receptor activity. Fractionation of the lipid extracts by silicic acid column chromatography resulted in the isolation of receptor activity in a neutral lipid fraction. The lipid fractions demonstrated receptor activity at 34 C but not at 0 C. These properties are also characteristic of the receptor activity with erythrocytes and ghosts. Cholesterol, co-lyophilized with palmitic acid, was found to possess receptor activity. Palmitic acid alone, cholesterol-lecithin, cholestane-palmitic acid, and various phospholipids and glycolipids had no receptor activity. Ghosts treated with amphotericin B or digitonin, compounds that bind to cholesterol in the membrane, lost their ability to adsorb rickettsiae.

When typhus rickettsiae and sheep erythrocytes are incubated together for a few minutes at 34 C and then centrifuged (under conditions in which erythrocytes but not free rickettsiae are sedimented), the rickettsiae are found not in the supernatant fluid but associated with the erythrocyte pellet. If, on the other hand, the incubation is at 0 C or contains KCN or another inhibitor of rickettsial metabolism, the rickettsiae are found in the supernatant fluid and not associated with the erythrocytes. This temperature-dependent, energy-requiring, rapid ($t_{\frac{1}{2}}$ = 4 min), and essentially irreversible association of rickettsiae with erythrocytes we have termed the adsorption step of rickettsial hemolysis (17, 18, 27).

Adsorption can be set apart from hemolysis by using: (i) short incubations; (ii) fluoride-inhibited erythrocytes, which adsorb rickettsiae but do not lyse; or (iii) ghosts, derived from sheep erythrocytes by hypotonic lysis. The adsorption to ghosts can be assayed by incubating ghosts and rickettsiae to form ghost-rickettsia complexes and then adding intact erythrocytes. Less lysis of erythrocytes will occur when these complexes have been formed, since the rickettsiae are irreversibly adsorbed to the ghosts and are hence unavailable to the erythrocytes (27).

In principle, any system containing the receptor activity can be assayed for that activity in the same manner as the ghosts. Accordingly, in this study we have examined erythrocytes and ghosts, treated in a variety of ways, as well as fractions extracted from these cells, to determine the identity of the receptor on the surface of the erythrocyte to which rickettsiae adsorb.

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MATERIALS AND METHODS

Rickettsial preparation and growth. *Rickettsia prowazeki*, Madrid E strain, was propagated in 6-day embryonated, antibiotic-free hen eggs by inoculation with 0.2 ml of a 10^{-5} dilution of a seed pool (yolk sac passage no. 273 and 274). Rickettsial suspensions were prepared from heavily infected yolk sacs by a modification of the methods of Bovarnick and Wisseman as previously described (4, 25, 26). Only fresh unfrozen rickettsiae were used.

The diluent for the rickettsial inoculum and rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate (SPG) solution originally devised by Bovarnick et al. (3), consisting of 0.218 M sucrose, 0.00376 M KH_2PO_4, 0.0071 M K_2HPO_4, and 0.0049 M glutamic acid, pH 7. The diluent for the sheep erythrocytes and receptor activity assays was SPG-Mg (SPG containing 0.01 M $MgCl_2$).

The hemolysis tests used were modifications of the method of Snyder et al. (22) as previously described (26).

Preparation of erythrocyte ghosts. The procedure used was essentially that of Dodge et al. (9) as

previously described (27). The concentration of ghosts is expressed as a percentage of the original packed-cell volume of the erythrocytes; i.e., if 4 ml of ghosts were derived from 1 ml of packed erythrocytes, the suspension would be referred to as 25%.

Receptor assay. The receptor activity of normal and treated ghosts, that is, their ability to adsorb rickettsiae, was assayed by combining 2 volumes of rickettsiae (usually at 20%, i.e., 0.2 g of original yolk sac equivalents per ml) with 1 volume of ghosts, allowing time for adsorption (30 min at 34 C), and then assaying unadsorbed rickettsiae with intact erythrocytes, as described previously (27). Rickettsiae once adsorbed to ghosts are unable to adsorb to and subsequently lyse erythrocytes (27). Samples, resuspended in SPG from the lyophilized state, from lipid extractions of erythrocytes and ghosts and from individual lipids were assayed for receptor activity in the same way as ghosts. Since rickettsiae do not adsorb to erythrocytes at 0 C, no receptor activity in any fraction was either anticipated or observed at this temperature. Control incubations of putative receptors and rickettsiae at 0 C eliminated artifactual effects of the fractions per se on the hemolytic process.

Receptor activity of treated ghosts was usually normalized as a percentage of the receptor activity found in untreated ghosts. The receptor activity of lipids and lipid extracts was expressed by a receptor index, which normalized the receptor activity at 34 C to the control activity at 0 C.

Enzymatic digestion of ghosts and erythrocytes. Ghost suspensions (50%) or erythrocytes (25%) were incubated for enzymatic digestion in the following buffers at 37 C: 0.135 M NaCl, 0.02 M sodium phosphate, pH 6.4, for sialidase (0.1 U/ml) experiments; 0.135 M NaCl, 0.01 M sodium phosphate, 0.0016 M $CaCl_2$, 0.0012 M $MgCl_2$, pH 7.4, for Pronase (1 mg/ml); and 0.135 M NaCl, 0.01 M $NaPO_4$, pH 7.4, for subtilopeptidase (1 mg/ml). After incubation, the ghosts were washed twice by centrifuging at 27,000 $\times$ *g* for 20 min at 4 C and resuspended in cold SPG-Mg to the original concentration. Similarly, erythrocytes were washed three times by centrifugation at 750 $\times$ *g* for 10 min at 4 C and resuspended in cold SPG-Mg to 5% concentration. The supernatant fluids, portions of treated and untreated erythrocytes, and ghosts were analyzed for the presence of sialic acid. Portions of the ghosts and erythrocytes were assayed for receptor activity and hemolytic activity, respectively.

NEM and periodate treatment of ghosts. Ghost suspensions (50%) in 0.25 M glycylglycine buffer (pH 7.0) were incubated with *N*-ethylmaleimide (NEM) for 10 min at room temperature. The ghosts were washed twice by centrifugation at 27,000 $\times$ *g* for 20 min and resuspended in SPG-Mg to the original concentration. Mercaptoethanol (1 mM) was added to all samples to stop the reaction, and the treated ghosts were assayed for receptor activity. Ghost suspensions (50%) in 0.5 M sodium acetate buffer (pH 5.5) were incubated at 4 C in the dark for 3 days with 25 mM sodium metaperiodate. Sorbitol (100 mM) was added after the incubation period to terminate the reaction. The ghost suspensions were washed, resuspended, and assayed as above.

Extraction procedures. To obtain lipid extracts, 1 volume of 50% sheep erythrocytes in physiological saline (or 50% ghost suspensions in distilled water) was added dropwise to 0.5 volume of methanol and mixed rapidly for 20 s. One volume of chloroform was added, and the emulsion was mixed vigorously for 1 min and incubated for 15 min at 0 C. The mixture was then centrifuged at 27,000 $\times$ *g* for 4 min. The resulting phases and interface were separated and evaporated to near dryness with a stream of dry nitrogen at room temperature. The upper and lower phases were resuspended in distilled water and benzene, respectively, and lyophilized. The lyophilized material was resuspended in SPG-Mg to a volume equivalent to the original 50% ghost suspension.

To extract glycolipids from sheep erythrocytes, the chloroform-methanol method of Nelson was used (16), which employed a solvent-to-sample ratio of 50:1 (vol/vol).

The lower (chloroform) phase from chloroform-methanol-extracted erythrocytes was mixed with cold acetone to precipitate phospholipids, according to the method of Kates (11). The acetone-insoluble and -soluble material were lyophilized, resuspended in SPG-Mg to a volume equivalent to the original concentration, and assayed for receptor activity and chemical composition.

Silicic acid column chromatography. A portion of the lower (chloroform) phase from chloroform-methanol-extracted erythrocytes or ghosts was separated by silicic acid column chromatography. The procedure was essentially that of Rouser et al. (19) with slight modifications. Columns made from 2-cm^3 glass syringes were packed with Unisil (200- to 325-mesh sialic acid, activated by heating at 60 C for 30 min; Clarkson Chemical Co.) in chloroform and washed with 5 volumes of chloroform. Fraction 1 was eluted with 10 ml of chloroform, fraction 2 was eluted with 10 ml of acetone-methanol (9:1, vol/vol), and fraction 3, the polar lipids, was eluted with 15 ml of methanol.

Portions of each fraction were evaporated to near dryness, resuspended in benzene, lyophilized, resuspended in SPG-Mg to a volume equivalent to the original 50% ghost suspensions, and assayed for receptor activity. Portions of each fraction were also assayed for cholesterol and phosphate and analyzed by thin-layer chromatography.

Thin-layer chromatography. Lipid extracts and the fractions obtained from column chromatography were analyzed by one-dimensional thin-layer chromatography on Silica Gel F254 (Brinkmann Co.), using chloroform-methanol-water (65:35:4, vol/vol/vol). Compounds were visualized by exposure to iodine vapor and identified by the α-naphthol spray method (21) by comparison to known standards.

Chemical analysis. Protein was determined by the method of Lowry et al. (13). Sialic acid released from erythrocytes and ghosts by sialidase or proteolytic enzymes was assayed by the periodate method (24). Samples to be assayed were first hydrolyzed for 1 h at 80 C to liberate sialic acid. Phosphorus was determined by the ashing method of Ames and Dubin (1). Cholesterol was analyzed by the method of Zlatkis et al. (28). Neutral carbohy-

drates were determined by the anthrone method of Strauss et al. (23).

Materials. Sodium metaperiodate was obtained from Fisher Scientific Co.; palmitic acid, cholestane, Pronase, subtilopeptidase A, *N*-acetylneuraminic acid (NANA), neuraminidase (from *Clostridium perfringens*), ceramides, cerebrosides, gangliosides, and sphingomyelin from Sigma Co.; NEM from Eastman Organic; mercuripapain from Worthington; cholesterol from Mann Research; sorbitol from Pfanstiehl Chemical Co.; and mercaptoethanol from Calbiochem. Influenza virus, WS strain, was donated by R. R. Wagner, University of Virginia. Egg lecithin was donated by T. E. Thompson, University of Virginia.

RESULTS

Effect of removal of surface glycopeptides and sialic acid. The possibility that the rickettsial receptor on the surface of the erythrocyte involved sialic acid (NANA) was tested by incubating either ghosts or erythrocytes with sialidase and then assaying these NANA-depleted cells for their ability to adsorb rickettsiae or be hemolyzed by rickettsiae. Rickettsial adsorption to sialidase-treated ghosts was unaffected, even with the loss of 80% of the ghosts' sialic acid residues (Table 1). Similarly, when intact erythrocytes were treated with sialidase, the removal of 70% of the sialic acid residues had a negligible effect on rickettsial hemolysis of these erythrocytes. However, if these sialic acid-depleted erythrocytes were incubated with influenza virus, the virus no longer caused hemagglutination. Therefore, it is unlikely that the site for rickettsial adsorption to the erythrocyte is NANA, and it is certainly not the influenza virus receptor.

Treatment of sheep erythrocytes with Pronase, papain, or subtilopeptidase removed, glycopeptides from the cell surface as indicated by the release of about 40% of the sialic acid. However, there was less than 10% difference in the extent of hemolysis of these treated erythrocytes as compared with normal erythrocytes. Likewise, when ghosts were treated with proteolytic enzymes, there was no alteration in their ability of adsorb rickettsiae even with approximately half of the sialic acid-containing glycoproteins removed. Cell receptors for plant agglutinins, insulin, and colicins have been characterized and shown to involve glycoproteins (6, 10, 15, 20); however, glycoproteins are probably not involved in the receptor site for rickettsiae.

TABLE 1. *Effect of enzymatic digestion of erythrocyte surface on rickettsial adsorption*

Treatment[a]	Ghosts		Erythrocytes	
	Sialic acid removed (%)	Adsorption (% of control)	Sialic acid removed (%)	Hemolysis (% of control)
Control	0	(100)	0	(100)
Sialidase	80	97	70	94
Pronase	51	105	53	105
Papain	45	113	44	101
Subtilopeptidase	ND	ND	39	90

[a] Ghosts or erythrocytes were incubated with sialidase at 37 C for 30 min or 60 min, respectively. All other incubations were at 37 C for 60 min.

Effect of periodate and sulfhydryl reagents on receptor activity of ghosts. Ghosts were incubated with either NEM or sodium metaperiodate to examine the involvement of thiol groups and carbohydrates in receptor activity. Neither NEM nor periodate had any effect on the extent of adsorption of rickettsiae to ghosts (Table 2). In these experiments, mercaptoethanol and sorbitol were used to terminate the reactions, and these reagents had no adverse effect on rickettsial hemolysis.

Identification of receptor activity in lipid fractions. To examine whether the lipids of the erythrocyte had receptor activity, they were extracted from the cells with chloroform-methanol, and the resulting upper and lower phases were lyophilized, resuspended in SPG-Mg, and assayed for receptor activity as described in Materials and Methods. The upper (methanol-water) phase contained protein and some water-soluble lipid material but negligible receptor activity (Table 3). Reextraction of this upper phase and interface with chloroform did not yield any receptor activity in the organic phase.

The lower (chloroform) phase from extracted erythrocytes contained receptor activity. The activity of this fraction was comparable to that obtained with intact ghosts, which had a receptor index of 50 to 70. Both cholesterol and phosphate were detected in the lower phase, indicating the presence of neutral lipids and phospholipids. This fraction was shown not to contain protein, sialic acid, or carbohydrate

TABLE 2. *Effect of NEM and periodate treatment of ghosts on rickettsial adsorption*

Treatment	Adsorption (% of control)
Control	(100)
NEM (0.1 mM)	99
NEM (1.0 mM)	90
Periodate (25 mM)	96

TABLE 3. *Fractionation of rickettsial receptor activity in chloroform-methanol-extracted erythrocytes*

Fraction[a]	Receptor activity (absorbancy at 545 nm)			Composition (μmol/ml of packed cells)	
	0 C	34 C	Receptor index[b]	Cholesterol	Phosphorus
Upper phase	1.07	1.12	−5	ND[c]	ND
Lower phase	1.07	0.64	40	1.27	0.44
Lower phase	1.25	0.94	25	1.31	0.51
Acetone soluble	1.18	0.61	48	1.19	0.25
Acetone insoluble	1.16	0.99	15	0.04	0.25
Lower phase	0.89	0.45	49	2.40	0.75
Silicic acid fraction 1 ...	0.82	0.53	35	0.90	0.00
Silicic acid fraction 2 ...	0.86	0.10	88	1.40	0.00
Silicic acid fraction 3 ...	0.75	0.64	15	0.00	0.70

[a] Eight milliliters of erythrocytes (50%) was extracted with chloroform-methanol (2:1, vol/vol) as detailed in the text. Three separate experiments are shown.

[b] Expressed as 100 × [(activity at 0 C − activity at 34 C)/activity at 0 C].

[c] ND, Not done.

(13, 23, 24), suggesting that this procedure did not extract glycolipids into the lower phase. Receptor activity was also observed in the lower phase from chloroform-methanol extractions of ghosts and the lipid-rich phase from *n*-butanol-extracted ghosts, using the method of Maddy (14).

Fractionation of the lower phase with cold acetone demonstrated that the receptor activity was associated with the acetone-soluble fraction that contained over 90% of the cholesterol and half of the phosphate. The other half of the phosphate of the lower phase (i.e., phospholipid) was in the acetone-insoluble fraction that did not contain receptor activity.

Fractionation of the lower phase on silicic acid columns afforded better separation of neutral lipids and phospholipids. Receptor activity was found in fractions 1 and 2, fractions containing cholesterol but no phospholipid. Fraction 3, which contained only phospholipid, had negligible receptor activity. The lipid compositions of the lower phase and the fractions eluted from the silicic acid column were also analyzed by thin-layer chromatography. No glycolipids were present in the lower phase, confirming that glycolipids were not extracted by this chloroform-methanol extraction procedure.

To provide further evidence that glycolipids are not involved in receptor activity, the chloroform-methanol extraction procedure of Nelson (16) was used to extract glycolipids from sheep erythrocytes. The lipid extract was fractionated by silicic acid chromatography and analyzed by thin-layer chromatography. Receptor activity was associated only with the fractions containing cholesterol and not with the glycolipid fraction.

Direct evidence that cholesterol is involved in the membrane receptor for rickettsiae is shown by the receptor activity of cholesterol emulsions. Cholesterol, co-lyophilized with palmitic acid and resuspended in SPG-Mg, had excellent receptor activity (Table 4). The cholesterol concentration was 0.3 mg/ml, approximately the concentration of cholesterol present in receptor assays of the neutral lipid fraction from silicic acid columns. Neither palmitic acid alone nor cholestane, a saturated cholesterol steroid with no hydroxyl group, co-lyophilized with palmitic acid had receptor activity. Interestingly, cholesterol co-lyophilized with phosphatidyl choline had no receptor activity. Mixed glycolipids, ceramides, gangliosides, and cerebrosides as well as phosphatidyl choline and sphingomyelin had no effect at the same concentrations at which cholesterol exhibited activity.

Effect of amphotericin B and digitonin. One would predict that substances known (8, 12) to bind cholesterol would interfere with the adsorption of rickettsiae to the receptor (i.e., cholesterol) on the ghost membrane. To test this hypothesis ghosts were incubated with amphotericin B (Calbiochem) or digitonin (Calbichem) for 1 h at 37 C or 30 min at 0 C, respectively. They were washed three times, resuspended in SPG-Mg, and tested for their ability to adsorb rickettsiae. Both compounds either markedly reduced or completely blocked the receptor activity of the ghost (Table 5). In

TABLE 4. *Test of various lipids for receptor activity*

Test substance[a]	Concn[b] (mg/ml)	Receptor activity (absorbancy at 545 nm)		Receptor index[c]
		0 C	34 C	
Gangliosides	0.3	0.86	0.87	−1
Ceremides	0.3	0.86	0.94	−9
Cerebrosides	0.3	0.83	0.84	−1
Sphingomyelin	0.3	0.90	0.93	−3
Phosphatidylcholine (PC)	0.3	1.02	1.03	−1
Palmitic acid (PA)	0.15	0.95	0.97	−2
PC plus cholesterol	0.5 + 0.6	0.97	1.09	−12
PA plus cholesterol	0.15 + 0.3	0.84	0.20	76
PA plus cholestane	0.15 + 0.3	0.95	0.87	8

[a] Lipids were suspended in benzene, lyophilized, resuspended in SPG-Mg, and assayed for receptor activity.
[b] Refers to final concentration of lipid in lipid-rickettsiae mixtures.
[c] See footnote *b*, Table 3.

TABLE 5. *Inactivation of ghost receptors with amphotericin B and digitonin*

Test system[a]	Adsorption (%)		
	1[b]	2	3
Ghosts	40	28	88
Ghost plus amphotericin B (10 μM)	0	5	14
Ghosts	51	43	76
Ghosts plus digitonin (10 mg/ml)	14	0	30
Ghosts plus digitonin (5 mg/ml)		9	77

[a] Digitonin experiments 1 and 2 were performed with 25% ghosts. In all other experiments ghosts were 50%.
[b] Experiment number.

digitonin experiments 1 and 2, 25% ghosts were used whereas 50% were used in experiment 3; this probably accounts for the diminished effects of digitonin in experiment 3.

DISCUSSION

Previous studies have demonstrated that metabolically active typhus rickettsiae adsorb to sheep erythrocytes and their ghosts formed by hypotonic lysis (17, 18, 27). The rickettsiae adsorbed equally well to vesicles with normal or reverse orientation, indicating that the receptor was not specialized on the outer aspect of the cell membrane (27). In experiments reported here, cholesterol was shown to be an essential component of the membrane receptor site. Although the location and distribution of cholesterol in the erythrocyte membrane is unknown, there are many lines of evidence demonstrating the ability of membrane cholesterol to interact with substances exterior to the cell. These include the exchange of cholesterol in the membrane with the cholesterol of serum lipoproteins (5) and the binding of polyene antibiotics (8, 12) and lysins (2) to membrane cholesterol.

Cholesterol exhibited receptor activity when solubilized in the presence of palmitic acid but not phosphatidyl choline. This may be explained by the difference in the type of artificial liposome formed by cholesterol with either palmitic acid or lecithin. Palmitic acid, a C_{16} fatty acid, forms micelles in solution with its negatively charged polar ends facing outward. Cholesterol probably is interdispersed between the fatty acid molecules and, therefore, is fairly mobile. On the other hand, lecithin and cholesterol form lipid bilayers in solution with no net charge on the lipids. Lecithin acyl chains are restricted in these liposomes (7), and perhaps cholesterol mobility is restricted as well. Therefore, the orientation of the cholesterol molecule is probably important in the sterol's capacity to adsorb rickettsiae. The inability of cholesterol-lecithin complexes to function as receptor may also be due to the low solubility of cholesterol in the liposomes used in these experiments. Palmitic acid may simply increase the solubility of cholesterol in solution.

The accessibility of the 3β-hydroxyl group of cholesterol appears to be necessary for adsorption as suggested by the inability of cholestane-palmitic acid complexes to inhibit rickettsial hemolysis. Cholestane, a saturated sterol, has no free hydroxyl group. Polyene antibiotics (8, 12) and streptolysin O (2) have also shown a requirement for sterols with a 3β-OH group for binding.

It is highly unlikely that the protein compo-

nents of the membrane protein components function as the receptor site. Experiments with proteolytic enzymes indicated that the depletion of erythrocytes and ghosts of their glycoproteins and/or sialic acid did not effect rickettsial adsorption. If only one site per erythrocyte is necessary for adsorption and lysis of a particular cell, one could argue that all of the receptor sites would have to be removed before differences in receptor activity would be detected. However, protein-rich extracts from ghosts treated with *n*-butanol or chloroform-methanol did not contain receptor activity.

Kinsky et al. have suggested that cholesterol is a stabilizer of the bilayer configuration of phospholipids in the membrane (12). When typhus rickettsiae binds to membrane cholesterol, perhaps this complex causes an initial instability of the membrane that would eventually lead to lysis of the cell. However, since adsorption of rickettsiae to metabolically poisoned erythrocytes or ghosts does not result in lysis, the rickettsia-cholesterol binding is obviously not the only process involved with cell lysis. The penetration of rickettsiae into mammalian host cells in vitro is analogous in certain respects to the rickettsia-erythrocyte system (18). Since these cells also contain cholesterol in their membranes, the initial process in host infection may be the adsorption of typhus rickettsiae to membrane cholesterol.

Further evidence that cholesterol is the receptor site for rickettsiae is demonstrated by the ability of amphotericin B and digitonin to interfere with rickettsial adsorption to ghosts. Amphotericin B and digitonin bind to membrane cholesterol (12). Thus, amphotericin B and digitonin saturate the receptor sites on the ghost and thereby prevent adsorption of rickettsiae.

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