JOURNAL OF VIROLOGY, Oct. 1984, p. 281–283
0022-538X/84/100281-03$02.00/0

Vol. 52, No. 1

Role of Cholesterol in Fusion of Semliki Forest Virus with Membranes

MARGARET C. KIELIAN* AND ARI HELENIUS

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Received 27 February 1984/Accepted 28 June 1984

The low pH-triggered membrane fusion activity of Semliki Forest virus is dependent on the presence of cholesterol in the target membrane. When liposomes containing phospholipids and cholesterol analogs were used, fusion activity was observed with steroids which did not have a planar nucleus or an isooctyl side chain at C-17, but fusion activity was not observed when analogs which lacked the 3β-OH group were used. Binding of virus to liposomes at low pH was similarly, but not totally, dependent on the presence of a 3β-OH sterol.

The entry of Semliki Forest virus (SFV; an alphavirus) into its host cells requires fusion between the virus envelope and the limiting membrane of endocytic vacuoles in the cell (12, 16). This fusion is catalyzed by the virus spike glycoproteins and triggered by the mildly acidic pH of prelysosomal endocytic vacuoles. In vitro fusion studies with liposomes have shown that no proteins are required in the target membrane for efficient fusion and that the phospholipid composition can vary. Fusion is, however, strictly dependent on the presence of cholesterol. Maximal fusion occurs when the mole fraction of cholesterol in the liposomes is 0.3 or higher (22). The fusion of SFV with cellular membranes implies, by analogy with the liposome system, the presence of cholesterol in various endocytic membrane compartments.

A cholesterol requisite is not unique to SFV; Sindbis virus (another alphavirus) shows a strong dependence on cholesterol for liposome binding at an acid pH (17), and two recent studies of Sendai virus (a paramyxovirus) indicate an absolute cholesterol requirement for lysis or fusion, with an optimum at 0.3 to 0.5 mole fraction of cholesterol (13, 14). In contrast, fusion of influenza virus (an orthomyxovirus) occurs efficiently in the absence of cholesterol (15, 23).

To analyze the cholesterol dependence of SFV in more detail, we determined whether other steroids can support virus binding and fusion. To assay for fusion, we mixed [^{3}H]uridine-labeled SFV with RNase-containing liposomes, lowered the pH to 5.0, and determined the digestion of viral RNA by trichloroacetic acid precipitation (22). In the published assay (22), liposomes were prepared in RNase-containing buffers, and the untrapped enzyme was removed by gel filtration. In most of the experiments reported here, the untrapped RNase was not removed. The liposome preparation in buffer plus enzyme was simply diluted to a final concentration of 0.75 mM lipid and 0.5 to 2 mg of RNase per ml before the addition of SFV. This procedure resulted in comparable fusion estimates. The percentages of fusion of SFV with liposomes were 9, 67, and 84% when the liposome mixture contained external RNase only (RNase diluted to 0.75 mg/ml), internal RNase only, and internal and external RNase (RNase diluted to 0.75 mg/ml), respectively. The percentage of fusion was calculated as [1 − (fraction of counts trichloroacetic acid-precipitable at pH 5/fraction of counts trichloroacetic acid-precipitable at pH 7)] × 100.

Nine different steroids were incorporated into liposomes consisting of steroid-phosphatidylethanolamine-phosphatidylcholine-sphingomyelin-phosphatidic acid in the molar ratio 1.5:1:1:1:0.2 (22). Cholesterol proved to be the most efficient in supporting the fusion activity of SFV (Table 1). Three other steroids (coprostanol, androstanol, and dihydrocholesterol) showed activities 40 to 90% that of cholesterol, whereas the remainder were inactive. A major distortion of the planar ring system, caused by the *cis* A/B ring arrangement of coprostanol, did not abolish fusion. The sterol 5,6 double bond did not appear essential, judging from the high activity of dihydrocholesterol. The isooctyl side chain at C-17 was also not a prerequisite for fusion, as androstanol supported fusion almost as well as cholesterol.

The common feature among the steroids which lacked fusion-supporting activity was an alteration in the 3β-OH group. Epicholesterol (which differs from cholesterol in having an axial 3α-OH), 5α-cholestane (with a hydrogen molecule in the 3 position), 5α-cholestan-3-one (with a carbonyl replacement), cholesterol methyl ether, and cholesterol acetate were all inactive. It can be concluded that a major variation in the ring structure and the aliphatic "tail" of the steroid are tolerated but that the 3β-hydroxyl group is absolutely necessary for fusion.

All steroids tested were judged free of impurities by thin-layer chromatography with three different solvent systems (6, 9). Although steroids of different structure are known to pack into bilayers to various extents, only in the case of cholesterol methyl ether was the mole fraction of incorporated steroid significantly lower than the 0.3 required for optimal fusion (Table 1 and reference 22). Thus, it is unlikely that the requirement for the 3β-OH group is due solely to the effect of this group on steroid incorporation into bilayers.

Membrane fusion probably involves several steps: initial attachment of the two membranes, their coalescence, and their subsequent separation (24). To determine whether sterol was required during the attachment stage, we assayed the association of SFV with cholesterol or epicholesterol liposomes. To separate liposome-associated viruses (including both bound and fused virus particles) from free virus particles, we used flotation in small, 0.7-ml sucrose gradients (Fig. 1; see also reference 17). At pH 7.0, ^{32}P-labeled liposomes containing 33 mol% cholesterol floated to the top of the gradient, with little [^{3}H]uridine-labeled virus associated. After pH 5.0 treatment, virtually all the virus, as expected from the fusion studies, floated with the liposomes (Fig. 1 and Table 2). Neutralization of the sample before flotation resulted in only slightly decreased association (Table 2). When epicholesterol was substituted for cholesterol in the liposomes, the amount of binding at pH 7.0 remained

* Corresponding author.

TABLE 1. Effect of steroid structure on SFV-liposome fusion

Steroid[a]	% Fusion[b] (no. of determinations)	% Activity relative to cholesterol control[c]	Mole fraction of steroid[d]
Cholesterol (cholest-5-en-3β-ol)	79.5 ± 11 (8)	100	0.312
Epicholesterol (cholest-5-en-3α-ol)	1.5 (2)	2	0.345
Coprostanol (5β-cholestan-3β-ol)	31 (2)	40	0.296
Dihydrocholesterol (cholestan-3β-ol)	46.5 (2)	53	0.339
5α-Cholestane	8 (2)	9	
Androstanol (5α-androstan-3β-ol)	59 (2)	88.5	0.326
5α-Cholestan-3-one	8.3 ± 7 (3)	13	0.358
Cholesterol methyl ether	0 (1)	0	0.236
Cholesterol acetate	11 (2)	13	0.289

[a] The starting liposome composition was as described in the text. Each steroid was substituted for cholesterol at the same ratio of 1 mol of steroid to 2 mol of phospholipid (0.33 mole fraction).
[b] The percentage of fusion was calculated as described in the text.
[c] As determined for individual experiments.
[d] The starting liposome preparation was centrifuged at 10,000 × g for 1.5 min (22). This step pelleted >80% of the free cholesterol. The phospholipid concentration of the preparation before and after centrifugation was monitored by the addition of trace amounts of ^{32}P-lipids (22). Steroid content was determined by quantitative thin-layer chromatography (9) with $CHCl_3$-acetone (90:10) or hexane-acetone (94:6) as the solvent system. 5α-Cholestane ran at the front of all solvent systems tested and was therefore not determined.

negligible. After acid treatment (with or without subsequent neutralization), binding amounted to about a third of that observed with cholesterol (Table 2). It seemed, therefore, that efficient attachment of the virus to the target membrane required a sterol with a 3β-OH group. However, it was also evident that some acid-dependent attachment was possible to membranes which do not support fusion. Thus, the acid-induced attachment of SFV to a membrane was not alone sufficient for fusion.

The effect of cholesterol on phospholipid membranes has been studied extensively (2, 7, 18). Cholesterol interacts with phospholipids to form complexes, thus decreasing the area occupied per phospholipid molecule—a phenomenon known as condensation (5, 11). It stabilizes bilayers, decreasing their permeability and increasing their osmotic resistance (3, 6). In lipid vesicles, cholesterol at mole fractions of >0.22 acts to decrease hydration (19). Finally, it affects the fluidity of bilayers, increasing the mobility of the gel phase, and above the phase-transition temperature, increasing the ordering of the liquid-crystal phase (7). Three structural features of the cholesterol molecule are generally believed to be important for these effects: a free β-hydroxyl group at C-3, an aliphatic side chain at C-17, and a planar (*trans*-fused) ring system (2, 7, 18). Of these three features, only the unblocked 3β-hydroxyl appeared to be an absolute requirement for SFV-liposome fusion. Both coprostanol and androstanol, which cause little or no condensation or permeability changes in bilayers, permitted significant levels of SFV fusion. The sterol structural requirements for SFV fusion thus do not correlate with those required to produce the physical effects described above.

In its very specific requirement for a 3β-OH group and its less stringent requirements for other aspects of sterol structure, SFV fusion resembles the thiol-activated cytolysins (1, 10, 21). These bacterial toxins insert into membranes and cause cell lysis, apparently by complexing specifically with cholesterol. Pretreatment of toxin with cholesterol before addition to membranes leads to stoichiometric complex

TABLE 2. Association of SFV with liposomes

Liposome type and pH conditions	% of virus liposome associated[a] (no. of expt)
Cholesterol	
pH 5	82 ± 7 (3)
pH 5, Neutralized	64.5 (2)
pH 7	8 (3)
Epicholesterol	
pH 5	26 ± 11 (3)
pH 5, Neutralized	21 (2)
pH 7	2 (1)

[a] Percentage of the total recovered virus found in fractions 1 through 4. The recovery ranged from 54 to 77%. Liposomes were RNase free.

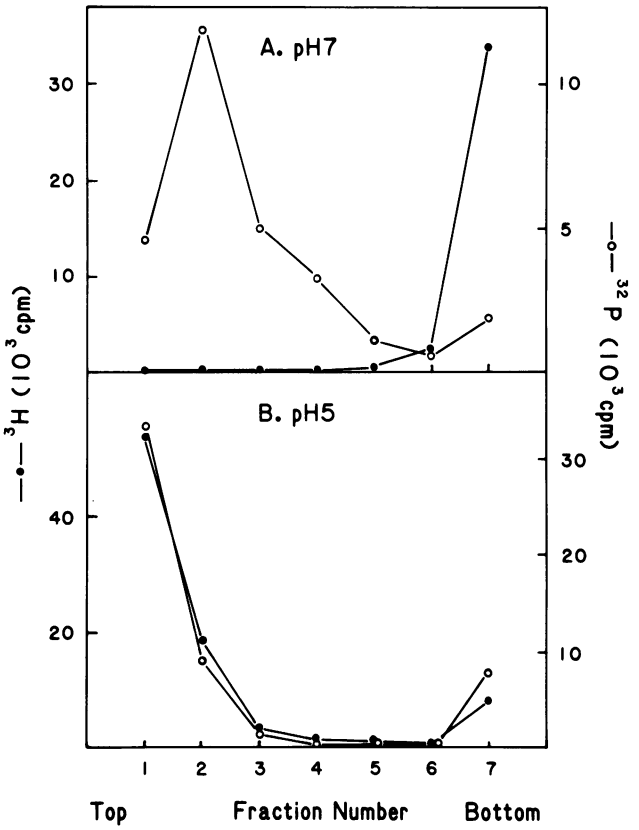

FIG. 1. Gradient separation of ^{32}P-labeled liposomes and ^{3}H-labeled SFV. ^{3}H-labeled SFV was mixed with RNase-free liposomes, as described in the text for the fusion assays. After treatment at pH 7.0 (A) or pH 5.0 (B) for 15 min at 37°C, 50 μl of the virus-liposome mixture was mixed with 100 μl of 66.6% sucrose to yield a final sucrose concentration of 44% and layered on the bottom of a 0.7-ml Beckman ultraclear centrifuge tube; 350 μl of 25% sucrose and 200 μl of 5% sucrose were then added. Sucrose solutions were weight to volume in 0.1 M NaCl containing either 50 mM Tris (pH 7.0) or 50 mM acetate (pH 5.0). After centrifugation for 4 h at 45,000 rpm in an SW55 rotor at 4°C, gradients were fractionated by hand from the top with a 100-μl pipette and assayed by liquid scintillation counting. Fraction 1 is the top of the gradient. This gradient system is similar to that described for influenza virus (R. Doms, A. Helenius, and J. M. White, manuscript in preparation).

formation and toxin inactivation. The toxin-sterol interaction has a similar stereospecificity for the 3β-OH group and also occurs with coprostanol; unlike the SFV fusion system, however, androstanol shows no activity (1, 10). This example demonstrates that sterol requirements for protein insertion into bilayers appear to be based on different structural criteria than those governing cholesterol-phospholipid interactions. Acid treatment of SFV may cause the insertion of spike glycoproteins into membranes by a cholesterol-dependent mechanism similar to that of the cytolysins.

The apparent cholesterol-dependent fusion of arthropod-borne viruses such as SFV and Sindbis virus poses an interesting paradox. Mosquitoes are the natural vector for these two alphaviruses in the wild (4), but the *Insecta* lack the ability to synthesize sterol and as sterol auxotrophs must obtain it from a dietary source (2, 18). Several insect cell lines in culture can nevertheless be maintained in the absence of exogenous cholesterol and under these conditions have dramatically reduced levels of sterol (20).

One can speculate that the presence of cholesterol in a blood meal might permit the initial infection of mosquitoes by SFV. It is well known that SFV infection in mosquitoes is chronic and nonlethal (8). A decreased sterol content in tissues other than the digestive tract could be a contributing factor in the control of the systemic infection.

This work was supported by Public Health Service grant AI-18582 from the National Institute of Allergy and Infectious Diseases and by an Anna Fuller postdoctoral fellowship to M.C.K.

We thank Robert Doms, Mark Marsh, and Judy White for reading the manuscript and Joy White for typing. We are especially grateful to David Castle for advice on the determination of steroid incorporation.

LITERATURE CITED

1. **Alouf, J. E.** 1980. Streptococcal toxins. Pharmacol. Ther. **11**:661–717.
2. **Bloch, K. E.** 1983. Sterol structure and membrane function. Crit. Rev. Biochem. **14**:47–92.
3. **Bruckdorfer, K. R., R. A. Demel, J. DeGier, and L. L. M. van Deenen.** 1969. The effect of partial replacements of membrane cholesterol by other steroids on the osmotic fragility and glycerol permeability of erythrocytes. Biochim. Biophys. Acta **183**:334–345.
4. **Chamberlain, R. W.** 1980. Epidemiology of arthropod-borne togaviruses, p. 75–227. *In* R. W. Schlesinger (ed.), The togaviruses. Academic Press, Inc., New York.
5. **Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen.** 1972. Structural requirements of sterols for the interaction with lecithin at the air-water interface. Biochim. Biophys. Acta **255**:311–320.
6. **Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen.** 1972. The effect of sterol structure on the permeability of liposomes to glucose, glycerol and Rb^+. Biochim. Biophys. Acta **255**:321–330.
7. **Demel, R. A., and B. DeKruyff.** 1976. The function of sterols in membranes. Biochim. Biophys. Acta **457**:109–132.
8. **Erwin, C., and D. T. Brown.** 1983. Requirement of cell nucleus for Sindbis virus replication in cultured *Aedes albopictus* cells. J. Virol. **45**:792–799.
9. **Fisher, K. A.** 1976. Analysis of membrane halves: cholesterol. Proc. Natl. Acad. Sci. U.S.A. **73**:173–177.
10. **Geoffroy, C., and J. E. Alouf.** 1983. Selective purification by thiol-disulfide interchange chromatography of alveolysin, a sulfhydryl-activated toxin of *Bacillus alvei*. J. Biol. Chem. **258**:9968–9972.
11. **Gershfeld, N. L.** 1978. Equilibrium studies of lecithin-cholesterol interactions. I. Stoichiometry of lecithin-cholesterol complexes in bulk systems. Biophys. J. **22**:469–488.
12. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
13. **Hsu, M.-C., A. Scheid, and P. W. Choppin.** 1983. Fusion of Sendai virus with liposomes: dependence on the viral fusion protein (F) and the lipid composition of liposomes. Virology **126**:361–369.
14. **Kundrot, C. E., E. A. Spangler, D. A. Kendall, R. C. MacDonald, and R. I. MacDonald.** 1983. Sendai virus-mediated lysis of liposomes requires cholesterol. Proc. Natl. Acad. Sci. U.S.A. **80**:1608–1612.
15. **Maeda, T., K. Kawasaki, and S. Ohnishi.** 1981. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. Proc. Natl. Acad. Sci. U.S.A. **78**:4133–4137.
16. **Marsh, M., E. Bolzau, and A. Helenius.** 1983. Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. Cell **32**:931–940.
17. **Mooney, J. J., J. M. Dalrymple, C. R. Alving, and P. K. Russell.** 1975. Interaction of Sindbis virus with liposomal model membranes. J. Virol. **15**:225–231.
18. **Nes, W. R., and M. L. McKean.** 1977. Biochemistry of steroids and other isopentenoids. University Park Press, Baltimore, Md.
19. **Newman, G. C., and C. Huang.** 1975. Structural studies on phosphatidylcholine-cholesterol mixed vesicles. Biochemistry **14**:3363–3369.
20. **Silberkang, M., C. M. Havel, D. S. Friend, B. J. McCarthy, and J. A. Watson.** 1983. Isoprene synthesis in isolated embryonic *Drosophila* cells. I. Sterol-deficient eukaryotic cells. J. Biol. Chem. **258**:8503–8511.
21. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183. *In* J. Jeljaszewitz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
22. **White, J., and A. Helenius.** 1980. pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. U.S.A. **77**:3273–3277.
23. **White, J., A. Helenius, and J. Kartenbeck.** 1982. Membrane fusion activity of influenza virus. EMBO J. **1**:217–222.
24. **White, J., M. Kielian, and A. Helenius.** 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. **16**:151–195.