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Sendai virus-mediated lysis of liposomes requires cholesterol

(model membrane/enveloped virus/membrane fusion/glycophorin/osmotic stress)

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ABSTRACT Vesicles were constituted with glycophorin, the Sendai virus receptor of human erythrocytes, and loaded with calcein, a polar derivative of fluorescein, at self-quenching concentrations. On exposure to Sendai virus and mild hypo-osmotic stress, vesicles of the appropriate composition released a significant portion of their internal contents, as indicated by an increase in calcein fluorescence. Susceptible liposomes were not induced to leak by heat-inactivated virus or by trypsin-treated virus. The response of the vesicles to virus attachment is thus analogous to virus-induced hemolysis and presumably involves fusion of the vesicle and virus membranes. In addition to glycophorin and phosphatidylcholine, cholesterol was absolutely required for the lytic response to the virus. The need for cholesterol was not attributable to inactivation of the virus by liposomes without cholesterol. The presence of gangliosides increased the encapsulated volume of the liposomes, but gangliosides did not effectively substitute for glycophorin. Thin-layer chromatography of lipid extracted from incubated virus and liposomes containing a small amount of a fluorescent phosphatidylcholine indicated that phosphatidylcholine in the vesicle is not chemically altered by functional interaction with the virus.

Fusion with cell membranes is an essential step in the infection of cells by many membrane-bounded viruses. Evidence is mounting that even those enveloped viruses that enter a cell by endocytosis eventually fuse—e.g., with the phagosome–lysosome membrane (1). The mechanism of membrane fusion is obscure, however, not only in the case of viral membranes but also in general, although considerable progress has been made in elucidating the structure of virus membranes (2). In comparison with virus membranes, target cell membranes are extremely complex. Investigators have therefore sought to reduce that complexity by using model target membranes for the analysis of the molecular mechanism of fusion. Investigations of the interactions of the highly fusogenic Sendai virus with model membranes, which could attach to (3–5), be lysed by (6), and fuse with (7, 8) the virus, have affirmed the feasibility of this approach.

Virus-induced hemolysis is generally accepted to represent the response of erythrocytes to fusion with Sendai virus. Using a simple and sensitive assay for lysis (9) of model target liposomes under hypo-osmotic conditions, we sought to define the composition of a vesicle that would provide a response analogous to hemolysis. We observed significant susceptibility to virus-induced lysis only with membranes containing both cholesterol and the virus receptor protein, glycophorin, in addition to a bilayer-forming lipid. The latter was not chemically altered during the interaction, and preincubation of the virus and liposomes without cholesterol did not affect the hemolytic activity of the virus. In defining the composition of a susceptible membrane, we have begun to limit the number of possible mechanisms by which Sendai virus fuses with cells.

MATERIALS AND METHODS

Lipids and Chemicals. Chromatographically pure phosphatidylcholine (type V-E), trypsin (type I), turkey egg white trypsin inhibitor, and cholesterol, which we recrystallized from ethanol, were from Sigma. 1-Acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine (NBD-PC) was from Avanti Biochemicals. Calcein was from Hach Chemical (Loveland, CO). Gangliosides were prepared from gray matter of bovine brain according to the method of Gammack (10).

Liposome Preparation. Liposomes, constituted with glycophorin, were prepared by a modification of a method described previously (4). First, 3.4 mg of phosphatidylcholine, 0.1 mg of glycophorin (10 mg/ml in water) isolated by the method of Marchesi and Andrews (11), and, as appropriate, other lipids were combined in 1–2 ml of chloroform/methanol (1:1, vol/vol) in a 150-ml beaker. The solvent was removed under nitrogen and subsequently under oil pump vacuum for 1 hr. Calcein at 50 mM and pH 7.0 was added, and the beaker was incubated at 37°C overnight. Untrapped calcein and unincorporated glycophorin were removed by centrifugation at 27,000 × *g* for at least 15 min in a Sorvall SS-34 rotor, by gel filtration chromatography, or by both procedures.

Virus Preparation and Treatment. Standard procedures were followed (12). Sendai virus (Z strain) was grown in the allantoic sac of 10- or 11-day chicken eggs. The eggs were incubated an additional 3 days after inoculation and the virus was collected from the allantoic fluid. The virus was purified by differential centrifugation. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate, according to a modification of the method of Fairbanks *et al.* (13), revealed no proteins other than those of the virus. Concentrated virus in 150 mM NaCl/5 mM sodium phosphate, pH 7.5 (P_i/NaCl), was divided into aliquots and stored at −70°C or at −20°C. Prior to use, the virus was disaggregated by passage through a microliter syringe 10 or more times. Hemagglutination activity of the virus was determined with the Salk method (14). For most experiments, the virus was used at a concentration of about 16,000 hemagglutination units (HAU)/ml.

To heat denature virus, an aliquot of stock virus was immersed in boiling water for 10 min. For trypsin treatment, 1 ml of stock virus suspension was diluted into 9 ml of 0.01 M sodium phosphate, pH 7.2, at 37°C and mixed with 0.06 mg of trypsin

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Abbreviations: P_i/NaCl, 150 mM NaCl/5 mM sodium phosphate, pH 7.5; Mops/NaCl, 150 mM NaCl/10 mM 4-morpholinepropanesulfonic acid, pH 7.5; HAU, hemagglutination units.

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and incubated at the same temperature for 20 min. Then 30 ml of chilled P_i/NaCl containing 0.12 mg of turkey egg white trypsin inhibitor was added, followed immediately by centrifugation in a Sorvall SS-34 rotor for 1 hr at 27,000 × *g*. The pellet was resuspended in 1 ml of P_i/NaCl.

Assay for Leakage of Liposome Contents. Fluorescence was measured in a Farrand spectrofluorometer with an optical accessory to focus the exciting light into the center of 5-mm sample tubes. The slits were removed and 490-nm and 520-nm interference filters of 10-nm bandpass were inserted in the excitation and emission beams. Monochromators were set accordingly. This procedure affords very high sensitivity for fluorescein derivatives and virtually eliminates contributions from light scattering.

The standard procedure for the leakage assay (9) was as follows. First, 400 μl of 150 mM NaCl buffered with 10 mM 4-morpholinepropanesulfonic acid at pH 7.5 (Mops/NaCl) was placed in a cuvette in the fluorometer and stirred magnetically. Then 2–20 μl of the stock liposomes in suspension was added, followed by 10 μl of stock virus, treated virus suspension, or P_i/NaCl. After 1.0–1.2 min 75 μl of water was added. Fluorescence was recorded for the next 4 min or more. Finally, 25 μl of 10% Triton X-100 was added to determine the fluorescence corresponding to 100% liposome leakage.

Extraction and Thin-Layer Chromatography of Liposome Lipids. Liposomes were prepared from glycophorin, phosphatidylcholine, cholesterol, gangliosides, and 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine in the molar proportions 0.001:1:0.5:0.125:0.1, according to the procedure described above except that Mops/NaCl replaced the calcein solution. Fifty microliters of the resulting liposome stock (5 μmol/ml) was incubated with 125 μl of stock virus suspension (16,000 HAU/ml), initially for 30 min at 0°C and subsequently for 2.5 hr at 37°C. The mixture was then extracted according to the Folch method (15). Aliquots of dried and redissolved extract in 1 ml of chloroform/methanol (2:1, vol/vol) were applied to a thin-layer plate, Polygram Sil G (Brinkmann) and developed in chloroform/methanol/28% aqueous ammonia (65:35:5, vol/vol). After development the plate was scanned under a fluorescence microscope equipped with a photomultiplier tube, the output of which was amplified and recorded.

Hemolysis Assay. Viral hemolytic activity was assayed with 1- to 3-day-old human erythrocytes, washed three times in P_i/NaCl. After preincubating 2 μl of disaggregated stock virus with 1–10 μl of liposomes composed of egg phosphatidylcholine/phosphatidylethanolamine (2:1 mole ratio, phospholipid at 10 mg/ml) with or without cholesterol (1:1 mole ratio with phospholipid when present) for 15 min at room temperature, samples were chilled in 1 ml of cold Mops/NaCl. Then 0.04 ml of a 25% (vol/vol) erythrocyte suspension was added, followed by mixing, incubation at 4°C for 10 min, and incubation at 37°C for 30 min. Samples were centrifuged at maximal speed in an International bench-top centrifuge for 5 min. Supernatants were removed and 0.1 ml of 10% Triton X-100 was added to solubilize any ghosts. The absorbances of the samples were read in the spectrophotometer at 540 nm.

RESULTS

Agglutination. Preliminary experiments were done on virus-mediated agglutination, because adhesion must precede fusion. Liposomes of a variety of compositions were prepared and examined for their ability to bind virus both by direct observation under the microscope and by hemagglutination inhibition. It was evident from these experiments that glycophorin was essential for agglutination but that it acted synergistically with bovine brain gangliosides. Liposomes containing only the gangliosides as a source of sialic acid, the moiety recognized by virus, were not agglutinated by the virus. Inclusion of gangliosides in glycophorin-containing vesicles, however, reduced by $^1/_2$ or more the amount of liposomes required to inhibit hemagglutination and generated vesicles that were noticeably better agglutinated by virus, as observed with a light microscope.

Lysis of Liposomes Induced by Virus. Information gained from preliminary agglutination experiments dictated the selection of components appearing to play some role in the interaction between liposome and virus. Testing mixtures from which individual components were omitted indicated the extent to which each of these contributed to the response. Dicetyl phosphate was included in the tested mixtures, because there was some indication from the work of Oku *et al.* (6) that it might be beneficial. An important feature of erythrocytes as targets for Sendai virus action is their high content of hemoglobin, which is responsible for colloid osmotic stress. To closely mimic conditions necessary for hemolysis, most of our experiments involved imposition of a slight hypo-osmolarity subsequent to liposome–virus interaction.

Fig. 1 illustrates the response of liposomes of different compositions to Sendai virus and osmotic stress. Differences in trapping efficiency among the different liposomes were small in comparison with the differences in susceptibility to lysis. After incubation of calcein-containing vesicles and virus for 1 min in a fluorometer cuvette at room temperature, the contents of the cuvette were diluted 15% with water (zero time). The increase in fluorescence is due to release of calcein from the aqueous compartment of the lipid vesicles. Calcein is intensely fluorescent at concentrations below millimolar, but at 50 mM, the concentration at which it was loaded into the liposomes, it is self-quenched and only weakly fluorescent (9). Because the external phase is several orders of magnitude larger than the internal volume of the vesicles, any calcein that leaks from them will exhibit intense fluorescence.

Fig. 1*A* shows that in the absence, as well as in the presence, of virus there is some increase in fluorescence on the introduction of hypo-osmotic stress. This increase is probably due to leakage of large vesicles; bilayers have low surface tensions and in the absence of a cytoskeletal support some vesicles must burst. In these controls there is little increase in fluorescence after the initial small burst. In contrast, liposomes in the presence of intact virus not only leaked more upon the introduction of osmotic stress but continued to release calcein for many minutes. Most responses were recorded for 4 min, at which time virus-treated samples are releasing marker at a steady rate. An osmotic stress of 15% was chosen largely on the basis of expedience. This gave a substantial difference between experimental and control samples in a determination that could be carried out reasonably rapidly. The response was inconveniently slow at 7% and the background was undesirably large at 22%. It should be emphasized that an osmotic stress is not essential for leakage of marker from receptive vesicles. We can detect leakage without stress, as have Oku *et al.* (6), but the release rate, relative to control values, is substantially larger with the stress (see below, Fig. 1*C*). Inclusion of dicetyl phosphate did not significantly affect the susceptibility of vesicles to viral lysis (not shown).

Fig. 1*B* shows that glycophorin is essential for a lytic response, because vesicles containing gangliosides as the only sialic acid-containing constituent do not exhibit sustained leakage in the presence of Sendai virus, and the response to hypo-osmolarity in the presence of virus is the same as in the presence of P_i/NaCl. The omission of gangliosides from an otherwise complete system (phosphatidylcholine, cholesterol, and glycophorin) reduces the encapsulated marker to $^1/_3$ to $^1/_5$ and correspondingly reduces the sensitivity of the assay.

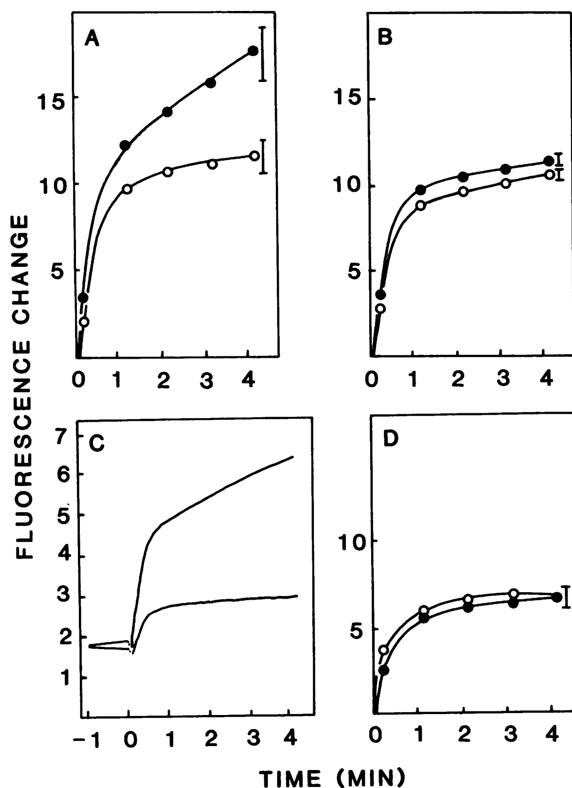

FIG. 1. Composition dependence of virus-induced lysis of liposomes. (*A*) Complete system. Liposomes contained phosphatidylcholine/cholesterol/gangliosides/glycophorin (1:0.5:0.125:0.001, mole ratio). The vertical axis represents the amount of calcein released from the liposomes as percentage of the amount encapsulated. Twenty microliters of stock liposomes and 10 μl of stock virus (●) or P_i/NaCl (○) were added to buffered saline in the fluorometer cuvette at 28–32°C and 1 min later (0 time) an osmotic stress corresponding to a 15% reduction in external solute concentration was imposed. There were 100 nmol of phosphatidylcholine (approximate value, but constant for all samples) and 160 HAU of Sendai virus in a final volume of 575 μl. The bar indicates maximal deviation around the mean of duplicate values. (*B*) Glycophorin omitted. Liposomes contained phosphatidylcholine/cholesterol/gangliosides (1:0.5:0.125, mole ratio). Otherwise, as in *A*. (*C*) Time course of release from vesicles with (upper curve) and without (lower curve) glycophorin. Composition of glycophorin-containing sample was as in *A*, above. The other sample contained phosphatidylcholine, cholesterol, and gangliosides in the same proportions. Recording began with addition of virus to the fluorometer cuvette containing liposomes. At 0 time, water was added to decrease the osmolarity by 15%. Fluorescence intensity is given in arbitrary units. (*D*) Cholesterol omitted. Liposomes contained phosphatidylcholine/gangliosides/glycophorin (1:0.125:0.001). Otherwise, as in *A*.

Fig. 1*C* presents the complete time course of the response of vesicles with and without glycophorin to virus. The record begins with the combination of vesicles and virus in P_i/NaCl. A small but distinct linear increase in fluorescence is observed with glycophorin-containing vesicles, but the difference between the two vesicle types is markedly accentuated by an osmotic stress (time zero). In contrast to the other records of this figure, in which percent of maximal leakage is plotted, fluorescence in Fig. 1*C* is given in arbitrary units.

Like glycophorin, cholesterol is essential (Fig. 1*D*). Vesicles that do not contain cholesterol are no more responsive to osmotic shock in the presence of virus than in its absence. As shown in Fig. 2, the cholesterol requirement is met at a cholesterol-to-phospholipid mole ratio of 1:2. A cholesterol-to-phospholipid mole ratio of 1:4 does not suffice. If liposomes without cholesterol were inactivating the virus, the cholesterol requirement

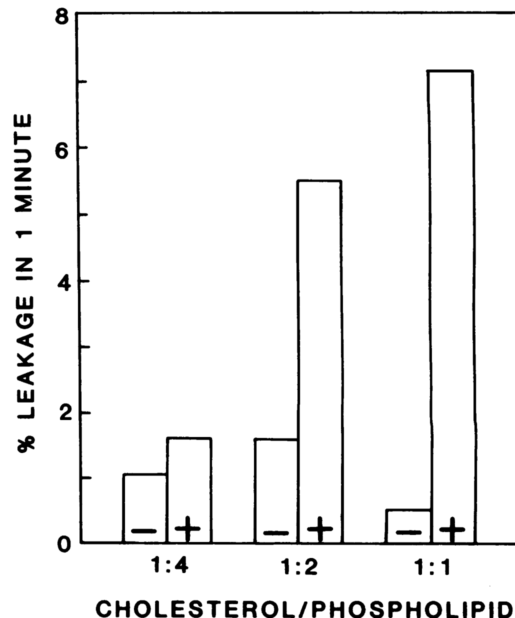

FIG. 2. Effect of cholesterol-to-phospholipid ratio on viral lysis of liposomes. Liposomes (3.5 μl of stock solution) contained phosphatidylcholine/ganglioside/glycophorin in the mole ratio 1:0.125:0.001 and cholesterol in the mole ratio to phosphatidylcholine as given in the figure. Liposomes and virus or P_i/NaCl were mixed and subjected to osmotic stress as described for Fig. 1. The percent leakage of the liposomes in the first minute after imposition of osmotic stress is given as + or − for liposomes treated with virus or P_i/NaCl, respectively.

would merely represent the inability of cholesterol-containing liposomes to inactivate the virus. Fig. 3 shows that virus that had been preincubated with phospholipid vesicles with or without cholesterol under lysis assay conditions retained as much hemolytic activity as virus not preincubated with liposomes. Thirteen micrograms of phospholipid corresponds to the maximal liposome-to-virus ratio employed in the liposome lysis experiments. When virus was preincubated with glycophorin- and

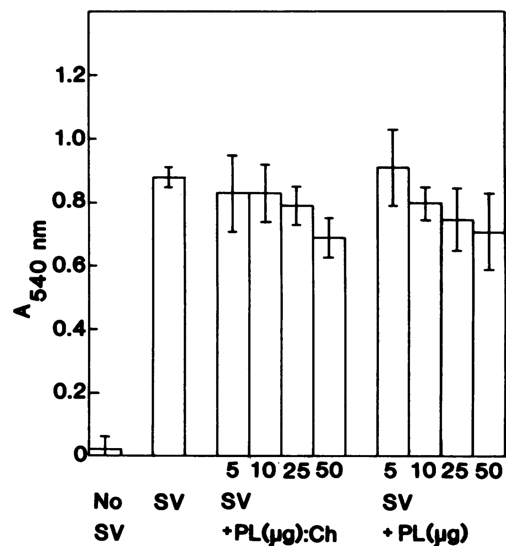

FIG. 3. Lack of effect of preincubation of Sendai virus with liposomes containing phospholipid/cholesterol (1:1 mole ratio) or phospholipid alone on hemolytic activity. Two microliters of virus stock was incubated for 15 min at room temperature with various amounts of liposomes prior to assay of hemolytic activity. Bars indicate SEM. SV, Sendai virus; PL, phospholipid; Ch, cholesterol.

ganglioside-containing liposomes with and without cholesterol, the presence of cholesterol had no effect on the residual hemolytic activity, which in both cases was 15–20% less than that of virus not preincubated with liposomes (not shown). This decrement is due to binding of glycophorin- and ganglioside-containing liposomes to some of the virus, which then became unavailable for interaction with erythrocytes.

To demonstrate that the responses described require active viral proteins—i.e., hemagglutinin for attachment and the F protein for fusion (review, ref. 2)—heat-treated and trypsin-treated viruses were assayed for their ability to induce lysis of target liposomes. Because the viral hemagglutinin is inactivated by heat (16), the heat-treated control (not shown) indicated that viral hemagglutinin is necessary for release of marker from target liposomes. F protein, on the other hand, can be selectively inactivated by trypsin without affecting the hemagglutinin (17). As shown in Fig. 4, trypsin-treated virus, which retained 70% of its hemagglutinating activity but only 5% of its hemolytic activity, did not induce significant release of marker from susceptible liposomes. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate confirmed that almost all of the F band was eliminated by trypsin treatment. In the experiments of Fig. 4, the amount of liposomes was reduced to about $^1/_3$ that used for the experiments of the previous two figures. Although the magnitude of the response is reduced, the higher ratio of virus to liposomes provokes a larger difference between intact and trypsin-treated virus. Response of liposomes to addition of P_i/NaCl was, within 2%, the same as that to trypsin-treated virus. These records, which cover the entire time course of the response, reveal that most of the leakage occurs within 20 min. Complete release of liposome contents does not occur, and the residue is presumed to have been entrapped within multilamellar liposomes, which exist in these preparations (4).

The possibility that phospholipid hydrolysis is necessary for virus–cell fusion has been tested and, although no evidence of hydrolysis has been obtained (18), a small amount of local hydrolytic activity at the limited area of contact of virus with cell could be obscured by the background metabolism of the cells. Fusion-specific phospholipid hydrolysis should be easier to identify in a virus–liposome system with no metabolic capabilities. Nevertheless, we could obtain no evidence that Sendai virus exerts lipolytic activity on phosphatidylcholine, the "matrix" lipid of the liposomes. Susceptible liposomes were constituted with a few percent of a phosphatidylcholine bearing a fluorescent acid at the 2 position. Thin-layer chromatography of the lipids extracted from incubated virus and liposomes revealed a single fluorescent band that migrated with phosphatidylcholine (Fig. 5).

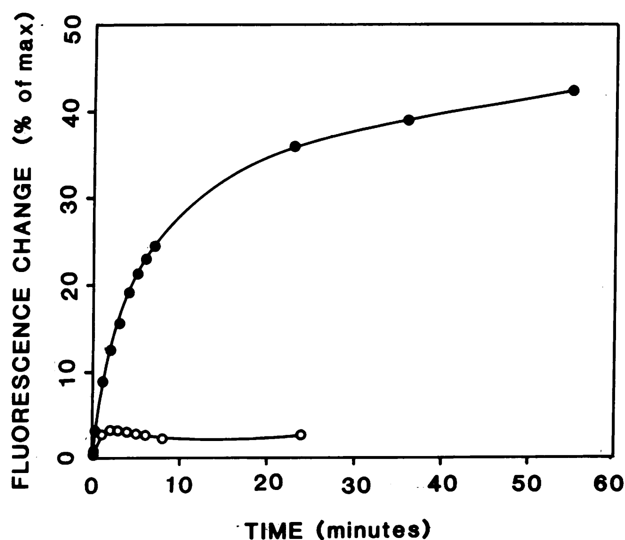

FIG. 4. Lysis of liposomes requires intact F protein. Liposomes (3.5 μl of stock solution) containing phosphatidylcholine/cholesterol/gangliosides/glycophorin (1:0.5:0.125:0.001 mole ratio) were treated with intact virus (●) or virus pretreated with trypsin (○). The latter was nearly devoid of hemolytic activity but still active in hemagglutination. Other conditions were as given for Fig. 1.

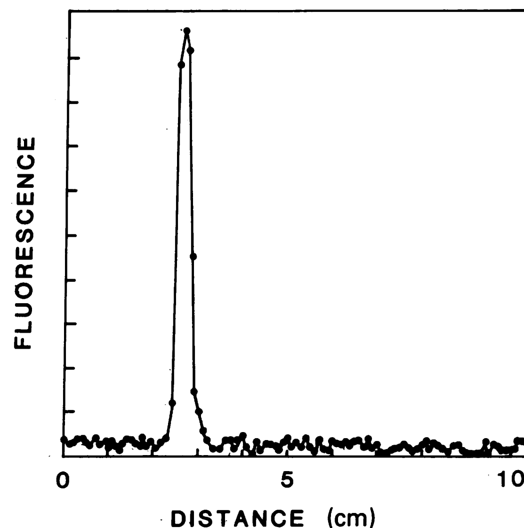

FIG. 5. Lack of effect of Sendai virus on receptor membrane phosphatidylcholine. Sendai virus (2,000 HAU) was incubated for 30 min at 0°C then for 2.5 hr at 37°C with liposomes (0.25 μmol as phosphatidylcholine) containing phosphatidylcholine/cholesterol/gangliosides/glycophorin/1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl phosphatidylcholine (1:0.5:0.125:0.001:0.1 mole ratio). Total lipids were extracted and chromatographed on a thin-layer plate developed in chloroform/methanol/28% aqueous ammonia (65:35:5, vol/vol). The fluorescence intensity was measured every millimeter and plotted in the figure. The position of the fluorescence maximum was identical in a control sample from which the virus was omitted.

DISCUSSION

It is generally accepted that leakage is a consequence of fusion of the virus membrane with the cell membrane (19). We suggest, therefore, that "lysis" of a liposome by Sendai virus may involve the same or similar molecular interactions as does, for example, lysis of an erythrocyte. Thus, liposomes that are induced to leak their contents upon treatment with Sendai virus are useful models for the study of membrane fusion.

Employing an assay that is analogous to hemolysis, we confirmed that Sendai virus induces release of a small molecular weight marker from liposomes constituted with the virus receptor, glycophorin, as well as gangliosides, cholesterol, and phospholipid (6). In contrast to the absolute requirement for glycophorin, the need for gangliosides is less stringent. It is likely that some of the more complex gangliosides, which have much greater virus receptor activity than the simpler gangliosides (20), are present in such small amounts in certain cell types that glycoproteins, rather than glycolipids, must serve as the principal virus receptors therein. In confirmation of the work of Oku *et al.* (6), we report here that target membranes containing bovine brain gangliosides but no glycophorin were only slightly more permeable in the presence of Sendai virus than in its absence. On the other hand, liposomes composed only of gangliosides, phospholipids, and cholesterol have been depicted in electron micrographs as fusing with (7) and being "penetrated" by (21) Sendai virus.

Sendai virus does not exhibit a lipase activity directed at the phosphatide of potentially responsive membranes (18), even when—as under the circumstances reported here—any products of lipase activity should be most easily detected—i.e., in a liposome with no metabolic capabilities. Cholesterol, except with respect to esterification with fatty acids, is relatively inert metabolically. Although we have not ruled out esterification of cholesterol, it is clear from the lack of change in the fluorescent phospholipid that the latter was not the source of an acyl group.

Our assay system features the hypo-osmotic condition, which greatly increased the leakage induced by Sendai virus. Because osmotic stress has been shown to promote fusion in other systems involving bilayer vesicles (22, 23), it was necessary to determine whether the responses we obtained were, in fact, dependent on functional viral proteins—particularly, the fusion-promoting F protein of Sendai virus. The trypsin-treated virus with substantial hemagglutinating but almost no hemolytic activity did not cause liposome lysis under hypo-osmotic conditions. Even in the presence of osmotic stress, therefore, liposome lysis was due to active F protein.

Another finding was the dependence of virus-mediated lysis from target liposomes under hypo-osmotic conditions on their cholesterol content. It was necessary to rule out the possibility that cholesterol-deficient liposomes were not susceptible to Sendai virus because they inactivated the virus by removing its cholesterol. This possibility was raised by the transfer of cholesterol from vesicular stomatitis virus (24) and from spikeless influenza virus (25), which paralleled an 86% loss in infectivity in the case of vesicular stomatitis virus. Because the hemolytic activity of virus was the same regardless of whether it had been preincubated with liposomes containing cholesterol or with liposomes deficient in cholesterol, the cholesterol was required in this instance in the target membrane and not in the virus membrane. Using a different lysis assay without osmotic stress, Oku *et al.* (6) did not observe a cholesterol requirement.

Like the glycophorin requirement, the cholesterol requirement constitutes an important parallel between the model and the natural target membrane of Sendai virus, because erythrocytes also become refractory to Sendai virus after they have been depleted of cholesterol (26). Furthermore, cholesterol-depleted erythrocytes regain their susceptibility to viral hemolysis upon the replacement of cholesterol. The sensitivity of other model target membranes to other viruses—i.e., Sindbis (27), cyanophage AS 1 (28), and Semliki Forest (29)—also depended on their cholesterol content. Both cholesterol and gangliosides have been reported to enhance the susceptibility of membranes to penetration by complement (30) and by thyroid-stimulating hormone (31) and tetanus toxin (32), respectively. These findings and the cholesterol requirement demonstrated here raise the intriguing possibility that membrane components in or near the cell surface, like cholesterol and gangliosides, may play *specific* roles in a variety of processes involving transmembrane communication.

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