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Sendai Virus Induced Leakage of Liposomes Containing Gangliosides†

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ABSTRACT: Sendai virus induced liposome leakage has been studied by using liposomes containing a self-quenching fluorescent dye, calcein. The liposomes used in this study were prepared by a freeze and thaw method and were composed of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (1:2.60:1.48 molar ratio) as well as various amounts of gangliosides and cholesterol. The leakage rate was calculated from the fluorescence increment as the entrapped calcein leaked out of the liposomal compartment and was diluted into the media. It was shown that the target liposome leakage was virus dose dependent. Trypsin-treated Sendai virus in which the F protein had been quantitatively removed did not induce liposome leakage, indicating that the leakage was a direct result of F-protein interaction with the target bilayer membrane. The activation energy of this process was approximately 12 kcal/mol below 17 °C and approximately 25 kcal/mol above 17 °C. Gangliosides GM_1, GD_{1a}, and GT_{1b} could serve as viral receptor under appropriate conditions. Liposome leakage showed a bell-shaped curve dependence on the concentration of ganglioside in the liposomes. No leakage was observed if the ganglioside content was too low or too high. Inclusion of cholesterol in the liposome bilayer suppressed the leakage rate of liposomes containing GD_{1a}. It is speculated that the liposome leakage is a consequence of fusion between Sendai virus and liposomes.

Sendai virus and other paramyxoviruses can interact with a wide spectrum of cells resulting in the following consequences: (a) virus–cell fusion; (b) cell membrane changes which lead to leakage of small intracellular constituents such as ions; (c) swelling of cells; (d) lysis of cells, e.g., hemolysis; (e) cell–cell fusion and/or polykaryon formation. The interrelationships among these processes are of great interest. It has been suggested that cellular permeability change is the

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consequence of virus–cell fusion and the cause of both hemolysis and swelling of cells which lead to polykaryon formation (Wyke et al., 1980; Impraim et al., 1980; Knutton & Bachi, 1980). Osmotic stress induced by the permeability changes may be the driving force which leads either to cell lysis or to polykaryon formation depending on the ability of the cell to retain or restore the membrane integrity after the stress (Peretz et al., 1974; Maeda et al., 1977; Knutton & Bachi, 1980).

Although this causal relationship seems reasonable, the true mechanism remains unclear. There exists a strong need for mechanistic studies at the molecular level to supplement the morphological studies. The use of a model target membrane seems particularly suitable for this purpose due to its simple composition. Using this approach, it has been shown that membrane fusion induced by Sendai virus is not dependent on metabolic energy, divalent cations, or cytoskeletons and is not dependent on any protein in the target membrane other than the Sendai virus receptor glycophorin (Umeda et al., 1983; Oku et al., 1982). Furthermore, gangliosides can replace glycophorin as Sendai virus receptor and mediate the fusion process (Haywood & Boyer, 1981, 1982). However, because of the lack of a sensitive assay, systematic studies were not easy to carry out.

Calcein, 2′,7′-[[bis(carboxymethyl)amino]methyl]-fluorescein, is a self-quenching dye. At concentrations greater than 5 μM, progressive fluorescence quenching takes place. It has been widely used for the liposome leakage assay because it is not necessary to remove liposomes for leakage measurements (Allen & Cleland, 1980; Kundrot et al., 1983). We have encapsulated calcein in the aqueous compartment of the ganglioside-containing liposomes. When the liposomes interact with the Sendai virus, the fluorescence increases as the dye leaks out and is diluted in the medium. Because the fluorescence increment can be continuously measured, kinetic studies can easily be done.

In this study, we have characterized the optimal conditions for the liposome leakage induced by Sendai virus. We have also shown the relevance of this process to the activities of the F protein of Sendai virus. The dependence of the liposome leakage process on temperature, osmotic pressure, receptor concentration, and the presence of cholesterol in the target membranes has also been studied.

Materials and Methods

Materials. Egg phosphatidylcholine and egg phosphatidylethanolamine were purchased from Avanti (Birmingham, AL). Bovine brain phosphatidylserine, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated (TPCK-treated)[1] bovine pancreas trypsin, turkey egg white trypsin inhibitor, and calcein were purchased from Sigma. Bovine brain gangliosides GD_{1a}, GT_{1b}, and GM_1 were purchased from Supelco. Thirty micrograms of each ganglioside was applied to a TLC plate and developed with chloroform–methanol–2.5 N ammonium hydroxide (60:40:9) to check the purity (Skipski, 1975; Kundu, 1981). Only one spot was found for each kind of gangliosides. Ficoll 400 was purchased from Pharmacia.

Liposome Preparations. Mixtures of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine (1:2.60:1.48 molar ratio), and various amounts of gangliosides and cholesterol were evaporated free of the solvent under a stream of nitrogen, desiccated under reduced pressure overnight, and suspended by vortexing in HEPES-buffered saline (150 mM NaCl, 5 mM KCl, 5 mM HEPES, and 0.02% sodium azide, pH 7.4) to a final concentration of 16 mg/mL. Lipid suspensions were sonicated and mixed with the same volume of either 140 or 80 mM calcein in HEPES-buffered saline. The mixture was sonicated thoroughly in a bath sonicator (Laboratory Supplies, Hicksville, NY) and then carried through nine cycles of freeze–thaw in an acetone–dry ice bath. The final lipid concentration was 8 mg/mL, and the final calcein concentration was either 70 mM for experiments concerning temperature, F-protein, and osmotic dependence or 40 mM for experiments concerning the membrane composition. Column chromatography on Bio-Gel A0.5M (1 × 12 cm) was used to separate free calcein from calcein-loaded liposomes. The liposomes were oligolamellar vesicles and had an average diameter of 700 nm as estimated by negative-stain electron microscopy. Lipid concentration was determined by phosphate assay (Ames & Dubin, 1960).

Virus Preparation. Sendai virus was grown in 10-day-old embryonated chicken eggs. The allantoic fluid was harvested and centrifuged at 3000*g* for 20 min to remove particulate material. The virus in the supernatant was pelleted by centrifugation at 13300*g* for 4 h. It was further purified by discontinuous sucrose density gradient centrifugation at the interface of 20–50% sucrose after centrifugation at 52200*g* for 2 h. The virus was washed by dilution with standard sodium citrate solution (150 mM NaCl and 30 mM sodium citrate, pH 7.4) and repelleted at 52200*g* for 70 min.

Trypsin Treatment of Sendai Virus. Four hundred microliters of TPCK–trypsin (1 mg/mL) was added to 200 μL of Sendai virus (10 mg of protein/mL) and incubated at 37 °C for 1.5 h. Four hundred microliters of trypsin inhibitor (1 mg/mL) was added to the latter solution, and Sendai virus was separated from reagents by using a Bio-gel A0.5M column. A hemagglutination assay was used to check the binding activity of HN protein (see below), while a hemolysis assay was used to check the activity of F protein (see below). SDS–PAGE (7.5% acrylamide) was used to check the integrity of both HN and F proteins.

Hemagglutination Assay and Hemolysis Assay. Fifty microliters of 0.5% human red blood cells was added to each of the 50-μL 2-fold serially diluted Sendai virus solutions in a V-bottomed Costar 96-well serocluster and incubated at room temperature for 5 h. The titers of hemagglutination and hemolysis can be easily determined by visual inspection of the formation of a red blood cell network and by the clearness of the suspension, respectively. The highest dilutions at which hemagglutination and hemolysis occurred were recorded. One hemagglutination unit is defined as the amount of Sendai virus required to hemagglutinate 1 mL of 1% red blood cells. The hemolysis unit is defined similarly. The results of the hemagglutination assay were identical with those of the conventional hemagglutination assay, which was performed in the cold over 2 h.

Liposome Leakage Assay. Two milliliters of HEPES-buffered saline containing 750 μM EDTA was preequilibrated to a desired temperature. Five microliters of liposomes was then added to a final concentration of 1.75 μg/mL. After 1-min incubation with constant stirring, 5 μL of Sendai virus preequilibrated to the same temperature was added to the mixture to a final concentration of 12.5 μg of viral protein/mL. To monitor calcein leakage, a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermostated cuvette holder

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TLC, thin-layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

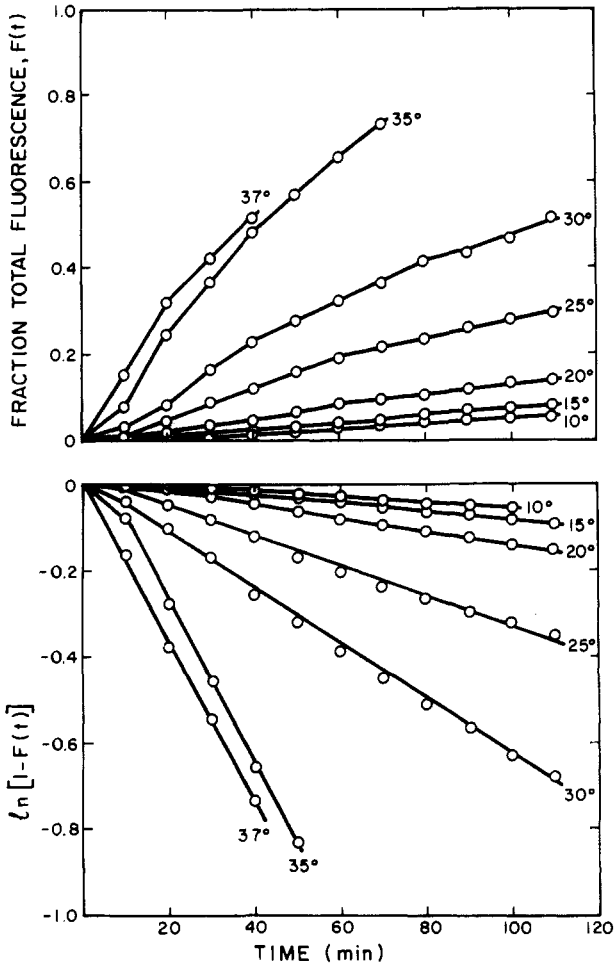

FIGURE 1: (Top panel) Time-dependent increases of fluorescence as a result of calcein leakage induced by Sendai virus at different temperatures. (Bottom panel) First-order plot of the above data.

connected to a Neslab RTE-8 constant-temperature circulator was used. Excitation was at 490 nm; emission was measured at 520 nm. The excitation slit was 5 nm; the emission slit was 3 nm. At the end of the experiment, concentrated deoxycholate solution was added to a final concentration of 0.06% to obtain complete dye release. The reading of the total fluorescence was about 100–130 at a fixed scale of 0.2 depending on the temperature used for the liposomes containing 70 mM calcein. The fraction of total fluorescence, $F(t)$, is defined as

$$F(t) = \frac{I_t - I_0}{I_f - I_0}$$

where I_0 = the initial fluorescence, I_f = the total fluorescence measured in the presence of deoxycholate, and I_t = the fluorescence at time t.

Viscosity and Osmolarity Measurements. Relative viscosity was measured as follows: A 9-in. Pasteur pipet was filled with the test solution up to the constriction ring. The pipet was held vertically, and the solution was allowed to flow downward by gravity. The time in seconds it took the solution to flow from the constriction ring to 8 cm downward toward the nose was used as a relative measure of viscosity. Osmolarity of solutions was measured with a Wescor 5130A vapor pressure osmometer. To measure the effect of osmolarity on liposome leakage, liposomes and buffer were preequilibrated in the medium with the desired osmolarity at 37 °C. Then 5 μL of Sendai virus was added to start the reaction. The measurement was the same as described in the previous section.

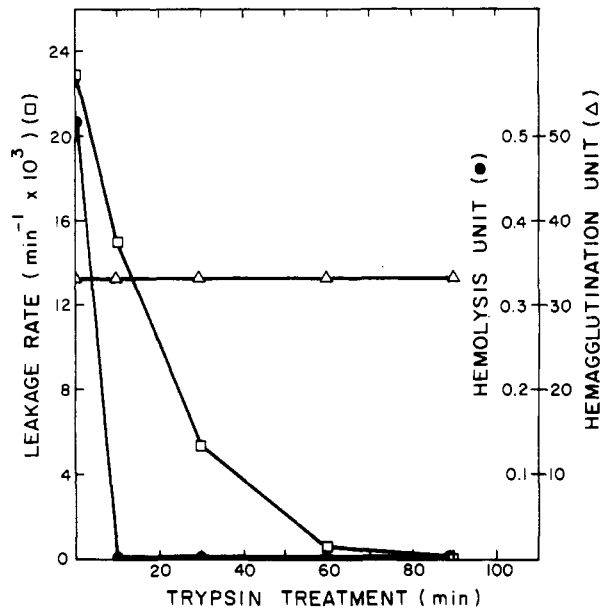

FIGURE 2: Effect of trypsin digestion of Sendai virus on calcein leakage (□) from liposomes containing 6.5% GD_{1a} at 37 °C. Hemolysis (●) and hemagglutination (△) activities of the treated virus were also measured.

RESULTS

Temperature Dependence of the Leakage Rate. Fluorescence intensity changes as the result of Sendai virus induced leakage of liposomes containing 6.5% GD_{1a} showed a dependence on both time and temperature (Figure 1, top panel). To avoid the inaccurate initial rate determination from the curvilinear profiles (Figure 1, top panel), we have determined the first-order leakage rate. The latter was obtained from the slope of the plots ln [1 − $F(t)$] vs. t (Figure 1, bottom panel). It showed that the leakage had a brief lag period of less than 10 min at 10–35 °C and then followed first-order kinetics at all temperatures tested. When the first-order rate of liposome leakage was plotted in an Arrhenius plot, two broken straight lines were obtained. The activation energy for the leakage process was obtained from the slopes. It was approximately 25 kcal/mol for leakage at higher temperatures and approximately 12 kcal/mol at lower temperatures. The transition temperature was at 17 °C. Trypsin-treated Sendai virus, from which part of F protein was cleaved, did not induce the leakage of liposomes at any temperature tested.

For all other experiments, we recorded the fluorescence changes as a result of liposome leakage at 37 °C for 2 h and then plotted ln [1 − $F(t)$] vs. t, ignoring the lag period, to obtain leakage rates from the slopes.

F-Protein Dependence of the Leakage Rate. Trypsinized virus was used to show that liposome leakage was not due to nonspecific interactions between the virus and the liposome. Sendai virus has two major membrane proteins: (a) HN protein, which is responsible for the binding (hemagglutination) activity, and (b) F protein, which is responsible for the fusion and membrane leakage activities (Ishida & Homma, 1978; Wyke et al., 1980). TPCK–trypsin was used to gradually and specifically cleave the F protein but leave HN protein intact (Neurath et al., 1973; Shimizu & Ishida, 1975). After as short as 10-min of digestion, the hemolytic activity of the virus was lost while residual levels of the liposome lytic activity of the virus still remained (Figure 2). SDS–PAGE analysis of the trypsin-treated virus indicated a gradual loss of the F protein but not the HN protein upon protease digestion (data not shown). By 30 min of digestion, no intact F protein could be

Table I: Osmotic vs. Viscosity Effect on Liposome Leakage Rate

medium	rel viscosity (s)	osmolarity (mOsm/L)	leakage rate ($min^{-1} \times 10^{-3}$)
1.86% sucrose	3.5	360	11.0
18.00% sucrose	5.0	980	1.5
1.86% Ficoll	5.0	278	14.2
18.00% Ficoll	49.0	305	5.3

detected. These results indicate that both the liposome leakage activity and the hemolysis activity of the virus are closely dependent on the presence of the intact F protein of the virus and not the HN protein. It is not clear whether the apparent higher sensitivity of the hemolysis activity than the liposome leakage activity to the trypsin treatment is due to the difference in the two target membranes or due to the size difference of the two marker molecules, i.e., hemoglobin vs. calcein.

All trypsin-treated Sendai viruses used in the following experiments were prepared as described under Materials and Methods.

Sendai Virus Dose Dependence of the Leakage Rate. For liposomes containing 6.5% GD_{1a} (1.75 μg of lipid/mL), the leakage rate linearly increased with increasing amount of intact Sendai virus up to about 12.5 μg of viral protein/mL and reached a plateau at about 25.0 μg of viral protein/mL. The second-order rate constant was $(1.18 \pm 0.12) \times 10^{-3}$ mL^2 g^{-2} min^{-1}. The trypsin-treated Sendai virus could cause a minute amount of liposome leakage only at very high concentrations (50 μg of viral protein/mL).

For all other experiments, both intact virus and trypsin-treated Sendai virus were used at the concentration of 12.5 μg of viral protein/mL, at which nonspecific interactions between virus and liposomes were negligible.

Osmotic Pressure and Viscosity Dependence of the Leakage Rate. Seventy millimolar calcein in HEPES-buffered saline, which had an osmolarity of 592 mOsm/L, was entrapped inside the liposomes. The osmolarity of the medium was increased from that of the HEPES-buffered saline, which had an osmolarity of 242 mOsm/L, by adding sodium chloride, sucrose, or Ficoll to the medium. The osmolarity of the medium less than, equal to, or greater than 592 mOsm/L is defined as hypoosmotic, isoosmotic, or hyperosmotic, respectively.

As shown in Figure 3, the leakage rate decreased as the osmolarity of the medium increased. Slight hyperosmolarity of the medium drastically decreased the rate. Sucrose suppressed the leakage rate much more effectively than sodium chloride could at the same osmolarity. The discrepancy can be attributed to a viscosity effect as shown by the data in Table I. Eighteen percent Ficoll as opposed to 1.86% Ficoll was only slightly higher in osmolarity (305 vs. 276 mOsm/L) but had much higher viscosity (49 vs. 5 s in relative viscosity units). This viscosity change greatly decreased the leakage rate. On the other hand, 18% sucrose had the same viscosity as 1.86% Ficoll (5 s) but had much higher osmolarity (980 vs. 278 mOsm/L). This osmolarity change also caused a large decrease in the leakage rate. Thus, adding sucrose to medium had both an osmotic effect and a viscosity effect on the liposome leakage, while sodium chloride had only an osmotic effect. It is reasonable to expect that the leakage rate is reduced in a high viscosity medium since the frequency of collision between the virus and liposomes would be reduced.

For all other experiments, HEPES-buffered saline (242 mOsm/L) was used as the medium. For the experiments concerning temperature, F-protein, and osmotic dependence,

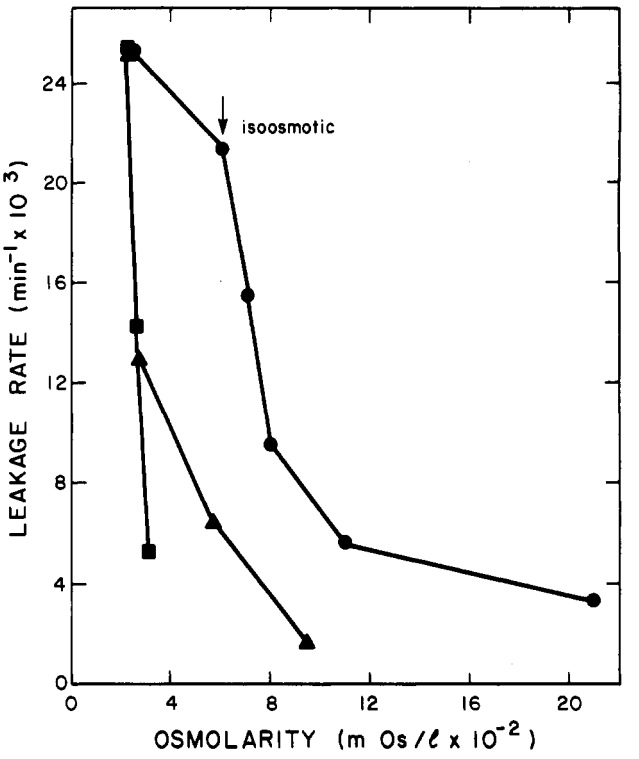

FIGURE 3: Osmotic pressure dependence of calcein leakage from liposomes containing 6.5% GD_{1a} at 37 °C. The osmolarity of the medium was varied by adding sodium chloride (●), sucrose (▲), or Ficoll (■). The isoosmotic point of liposomes was at 592 mOsm/L.

70 mM calcein was entrapped inside the liposomes. For experiments concerning the membrane composition, only 40 mM calcein was entrapped inside liposomes so as to reduce the chance of spontaneous leakage. Some liposomes with certain compositions were not as stable as others.

Receptor Species and Concentration Dependence of the Leakage Rate. There were three types of liposome leakage in these experiments: (a) spontaneous leakage; (b) leakage induced by the binding of the trypsin-treated virus to the liposomes; (c) leakage induced by the binding of the intact virus. In Figures 4–6, we show five profiles in each figure: (a) liposome leakage induced by intact virus; (b) liposome leakage induced by trypsin-treated virus; (c) spontaneous leakage; (d) F-protein-dependent leakage which resulted from subtracting (b) from (a); (e) F-protein-independent leakage which resulted from subtracting (c) from (b). In doing so, we have assumed that F-protein-dependent leakage and F-protein-independent leakage were independent of each other. Figure 4 shows that both F-protein-dependent leakage and F-protein-independent leakage have a bell-shaped dependence on GD_{1a} concentration which peaked at 6.5 and 1.6 mol %, respectively. The shoulder of the intact virus profile at 1.6 mol % could be due to the F-protein-independent leakage. The leakage occurring at GD_{1a} concentrations greater than 4 mol % was only F-protein dependent. We routinely used liposomes containing 6.5 mol % GD_{1a} for all the experiments in other sections to obtain optimized and purely F-protein-dependent leakage.

F-protein-dependent and F-protein-independent leakage also showed a bell-shaped dependence on GT_{1b} and GM_1 concentrations (Figures 5 and 6). However, both leakage rates peaked at about 3 mol % of GT_{1b} and at about 8 mol % of GM_1, which were approximately the same concentrations at which F-protein-independent leakage occurred. The F-protein-dependent leakage was the only process or the predominant process at all GT_{1b} or GM_1 concentrations except

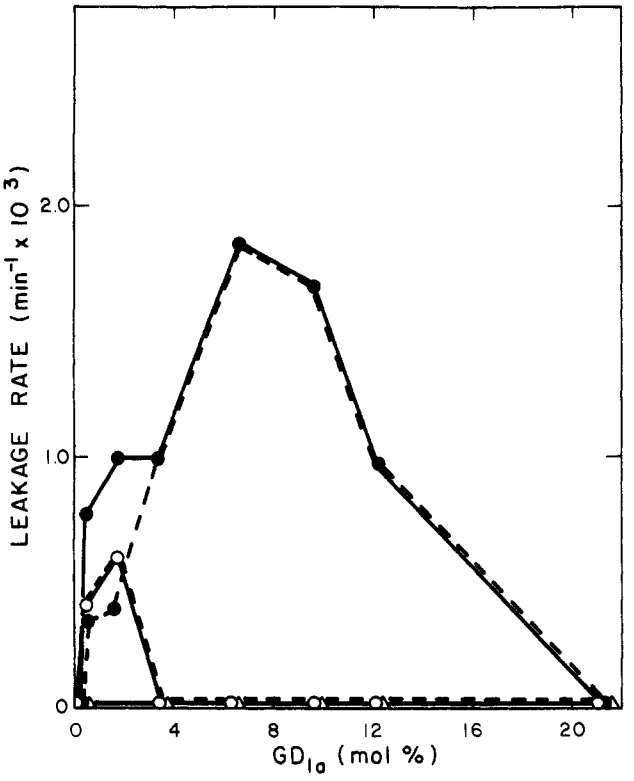

FIGURE 4: Rate of Sendai virus induced calcein leakage as a function of GD_{1a} concentration in the liposome membrane: leakage induced by intact virus (●—●); leakage induced by trypsin-treated virus (○—○); spontaneous leakage (△—△); F-protein-dependent leakage (●---●); F-protein-independent leakage (○---○).

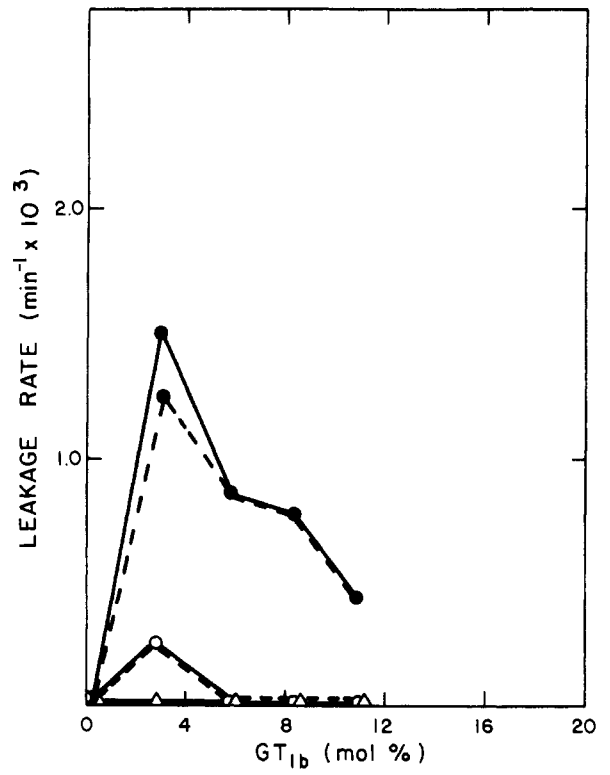

FIGURE 5: Rate of Sendai virus induced calcein leakage as a function of GT_{1b} concentration in the liposome membrane. Symbols same as those in Figure 4.

at 4 mol % of GM_1, at which the spontaneous leakage was predominant.

Effect of Cholesterol on the Leakage Rate. Inclusion of cholesterol into liposomal membrane suppressed the leakage

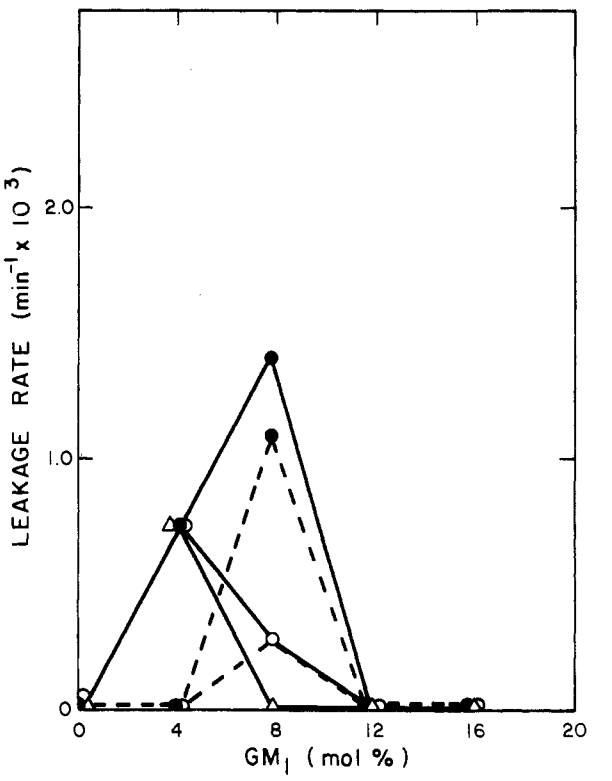

FIGURE 6: Rate of Sendai virus induced calcein leakage as a function of GM_1 concentration in the liposome membrane. Symbols same as those in Figure 4.

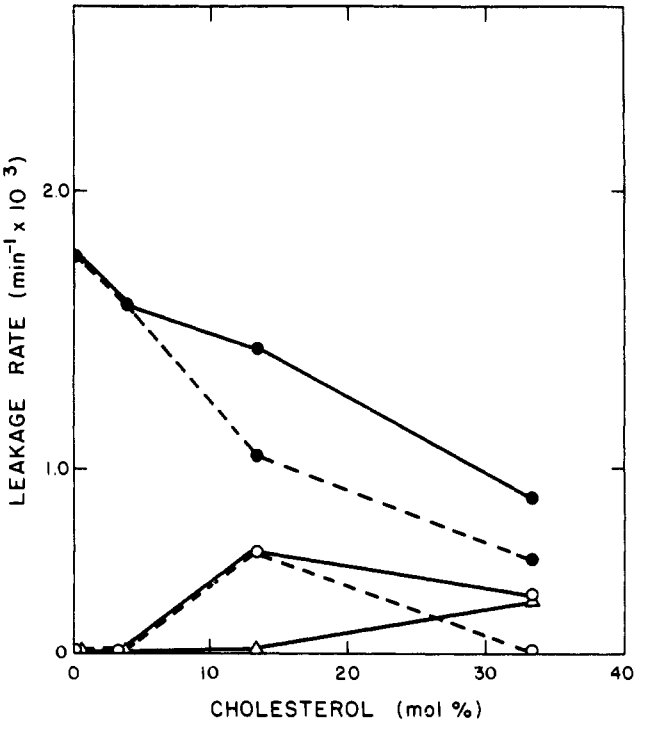

FIGURE 7: Rate of Sendai virus induced calcein leakage as a function of cholesterol concentration in the liposome membrane containing 6.5% GD_{1a}. Symbols same as those in Figure 4.

rate induced by intact virus (Figure 7). At 33 mol % cholesterol, the F-protein-dependent leakage rate was only about 23% of that of control liposomes without cholesterol. On the other hand, 13% cholesterol increased the F-protein-independent leakage rate. At 33% cholesterol, spontaneous leakage also became significant. For other experiments, we did not incorporate cholesterol into liposomes to eliminate the

complications induced by cholesterol.

Divalent Cation and the Leakage Rate. Divalent cations are not required in Sendai virus induced liposome leakage. All the experiments were done without divalent ions. In addition, EDTA was presented in all experiments to chelate the possible contaminating divalent cations from Sendai virus preparations.

Discussion

By using ganglioside-containing liposomes as well-defined, easily manipulated target membranes, we have studied in detail the Sendai virus induced lytic process. Although the major receptor on the cell membranes for Sendai virus was considered to be sialic acid bearing glycoproteins, e.g., glycophorin in erythrocyte membrane (Kundrot et al., 1983; Wu et al., 1980), gangliosides can also be receptors. The ability of GD_{1a} and mixed gangliosides as receptors for Sendai virus was supported by the virus–liposome fusion studies (Haywood & Boyer, 1981, 1982). However, Markwell et al. (1981) postulated that only GD_{1a} and GT_{1b}, not GM_1, could function as natural receptors for Sendai virus in host cells. The postulation was based on the ability of these gangliosides to render the sialidase-treated cells susceptible to Sendai virus. Contradictorily, we found that GM_1 could also serve as a receptor. Failure to detect the receptor activity of GM_1 (Markwell et al., 1981) may be due to the narrow effective range of GM_1 content (Figure 6). Ganglioside concentrations which allow the maximal leakage rate are 7.7 mol % for GM_1, 6.5 mol % for GD_{1a}, and 3 mol % for GT_{1b}. Thus, the receptor activity of the ganglioside follows the order $GT_{1b} > GD_{1a} > GM_1$. It is interesting to note that GT_{1b}, GD_{1a}, and GM_1 contain respectively three, two, and one sialic acid residues, which is the virus binding moiety of gangliosides. The apparent correlation suggests that the number of sialic acid residues determines the receptor activity of the ganglioside.

The F-protein-independent leakage process occurs over a very narrow range of ganglioside content for all the gangliosides tested, i.e., GM_1, GD_{1a}, and GT_{1b}. When Sendai virus binds to liposomes via an HN protein–ganglioside linkage, gangliosides would be clustered in the contact area and phase separated from other lipids in the liposomal membrane. Ganglioside clusters could form hydrophobic pores and be susceptible to the permeation of calcein as proposed by Delmelle et al. (1980). Alternatively, the phase junctions between ganglioside clusters and the rest of the membrane could also allow the permeation of calcein, as suggested by Papahadjopoulos et al. (1973), who postulated that sodium ion could permeate through the lipid phase boundary region of membranes.

It is generally accepted that the cellular leakage induced by Sendai virus is the consequence of virus–cell fusion (Homma et al., 1976; Impraim et al., 1980; Lyles & Landsberger, 1979). This is because the addition of leaky viral membrane to the cell membrane as a result of fusion makes cells leaky. F-protein-dependent liposome leakage may also be the consequence of liposome–virus fusion (Kundrot et al., 1983). In this case, the rate-limiting step measured in our experiments may be the fusion step. It is interesting to note that the F-protein-dependent liposome leakage has an activation energy of 25 kcal/mol, which is similar to 26 kcal/mol found for erythrocyte–Sendai virus fusion (Lyles & Landsberger, 1979). Nonbilayer structure formation has been suggested to cause leakage of cellular or liposomal membranes (Cullis & de Kruijff, 1978; Janoff et al., 1983) and may be an intermediate step in the fusion process (Hope & Cullis, 1981; Nir et al., 1983; Verkleij et al., 1980; de Kruijff et al., 1980; Siegel, 1984). Nonbilayer structure formation, which might be enhanced by the presence of F protein in the contact region, could also be important in F-protein-dependent liposome leakage because there are two good candidates of nonbilayer-forming lipids in our liposomes, i.e., phosphatidylethanolamine (Cullis & de Kruijff, 1978) and gangliosides (Tomasi et al., 1982). Alternatively, bilayer perturbation by the hydrophobic segment near the NH_2-terminal region of the F protein could also play an important role in the induction of membrane fusion (Hsu et al., 1981; Richardson et al., 1980). In fact, recent studies by Richardson & Choppin (1983) have shown that peptides of sequence analogous to the NH_2-terminal of the F protein bind to the target cell membrane in a specific and saturable manner. Although it is not clear about the molecular identity of the F-protein receptor, our results suggest that it may be a lipid(s).

Although the present model study has revealed a number of molecular details concerning Sendai virus membrane leakage, it is different from other model studies. It is known that cholesterol is required for the lytic response of glycophorin-incorporated liposomes induced by Sendai virus (Kundrot et al., 1983). In the present study, we did not observe any cholesterol requirement in the F-protein-dependent leakage of the liposomes. In fact, the presence of cholesterol suppresses the leakage response. The discrepancy is probably due to the difference in the viral receptor used. Glycophorin could require cholesterol for the proper expression of the viral binding sites, whereas the gangliosides, having a much simpler oligosaccharide structure, do not. It has also been shown that the presence of cholesterol enhances the fusion between Sendai virus and liposomes containing phosphatidylcholine and mixed gangliosides (Hsu et al., 1983). Thus, the effect of cholesterol on the action of Sendai virus depends on the lipid composition of the liposomes. One possible involvement of cholesterol is to regulate the formation of the nonbilayer structure. Cholesterol enhances the nonbilayer formation in some cases and suppresses it in others, depending on the phospholipid composition (Cullis et al., 1978; Jensen & Schutzbach, 1984). Thus, the presence of cholesterol in the target membrane may positively or negatively modulate the membrane leakage depending on the lipid composition. We are currently studying this aspect in greater detail.

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Registry No. GM_1, 52930-43-5; GD_{1a}, 12707-58-3; GT_{1b}, 59247-13-1; cholesterol, 57-88-5.

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Molecular Basis of Superreactivity of Cysteine Residues 31 and 32 of Seminal Ribonuclease

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ABSTRACT: The molecular basis of the high reactivity toward reducing agents of intersubunit disulfides at positions 31 and 32 of dimeric bovine seminal ribonuclease was investigated by studying in the monomeric enzyme the fast reaction kinetics with disulfides of the adjacent cysteine-31 and -32, exposed by selective reduction of the intersubunit disulfides. Negatively charged and neutral disulfide reagents were used for measuring the thiol reaction rates at neutral pH. The kinetics studied as a function of pH permitted us to define pK values for the thiols of interest and indicated the possibility of determining pK values of SH groups in proteins indirectly by measuring the kinetics of reactivity of the SH groups with a disulfide reagent. The results were compared with those obtained under identical conditions with synthetic thiol peptides and model compounds. The data indicate that the superreactivity of intersubunit disulfides of seminal ribonuclease is matched by the high reactivity at neutral pH of adjacent cysteine residues 31 and 32, as compared to all small thiol compounds tested. The synthetic hexapeptide segment of seminal ribonuclease Ac-Met-Cys-Cys-Arg-Lys-Met-OH, which includes the two cysteine residues of interest, was even more reactive. These data, and the other results reported in this paper, led to the conclusion that the superreactivity at neutral pH of cysteine residues at positions 31 and 32 of bovine seminal ribonuclease is primarily dependent on the nearby presence of positively charged groups, particularly the ϵ-NH_2 of lysine-34, and is influenced by the adjacency of the two thiols and by the protein tertiary structure.

One of the main structural features of bovine seminal RNase is the presence of two interchain disulfide bridges linking the half-cystines at adjacent positions 31 and 32 of the two subunit chains (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975). There is an intriguingly high reactivity of these disulfides (D'Alessio et al., 1975) compared with that of the intrachain disulfides, four per subunit. Monomer $\rightleftharpoons$ dimer conversions may have physiological significance, as the cata-

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