Membrane Vesicles Containing the Sendai Virus Binding Glycoprotein, but Not the Viral Fusion Protein, Fuse with Phosphatidylserine Liposomes at Low pH†

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ABSTRACT: Membrane vesicles containing the Sendai virus hemagglutinin/neuraminidase (HN) glycoprotein were able to induce carboxyfluorescein (CF) release from loaded phosphatidylserine (PS) but not loaded phosphatidylcholine (PC) liposomes. Similarly, fluorescence dequenching was observed only when HN vesicles, bearing self-quenched *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE), were incubated with PS but not PC liposomes. Thus, fusion between Sendai virus HN glycoprotein vesicles and the negatively charged PS liposomes is suggested. Induction of CF release and fluorescence dequenching were not observed when Pronase-treated HN vesicles were incubated with the PS liposomes. On the other hand, the fusogenic activity of the HN vesicles was not inhibited by treatment with dithiothreitol (DTT) or phenylmethanesulfonyl fluoride (PMSF), both of which are known to inhibit the Sendai virus fusogenic activity. Fusion was highly dependent on the pH of the medium, being maximal after an incubation of 60–90 s at pH 4.0. Electron microscopy studies showed that incubation at pH 4.0 of the HN vesicles with PS liposomes, both of which are of an average diameter of 150 nm, resulted in the formation of large unilamellar vesicles, the average diameter of which reached 450 nm. The relevance of these observations to the mechanism of liposome–membrane and virus–membrane fusion is discussed.

Fusion of liposomes, either among themselves (Wilschut et al., 1980; Struck et al., 1981) or with biological membranes such as viral envelopes (Eidelman et al., 1984), has been extensively used as a model system to study the molecular mechanism of membrane–membrane fusion. Papahadjopoulos and his colleagues have demonstrated that bivalent metals such as Ca^{2+} (Wilschut et al., 1980) and, to a lesser extent, Mg^{2+} (Düzgüneş et al., 1981) induce fusion of membrane vesicles made entirely of phospholipid molecules. Fusion was observed only when negatively charged phospholipids such as phosphatidylserine (PS)[1] or cardiolipin were included in the composition of the liposomes. Fusion between negatively charged phospholipid vesicles was also observed upon incubation with polycations such as polylysine (Gad & Eytan, 1983).

Recently (Eidelman et al., 1984; Haywood & Boyer, 1984) it has been observed that fusogenic virions are able to interact and probably fuse with liposomes made of or containing negatively charged phospholipid molecules. Haywood and Boyer (1984) claimed that Sendai virions are able to fuse with liposomes lacking any specific virus receptors, provided that negatively charged phospholipid molecules are included in the liposomes.

Fusion between Sendai virions and negatively charged phospholipid vesicles lacking virus receptors has been inferred from experiments showing virus-induced release of carboxyfluorescein from loaded liposomes. Lysis was observed only when negatively charged phospholipid molecules were included in the liposome membrane but did not occur with trypsinized virions, indicating the involvement of a viral polypeptide in the process (Haywood & Boyer, 1984; Amselem et al., 1985). Fusion between viral envelopes and negatively charged liposomes was also inferred from experiments showing intermixing of the viral and liposomal membranes (Amselem et al., 1985).

Fusion between viral envelopes containing the G protein of vesicular stomatitis virus (VSV) and negatively charged liposomes was demonstrated with the use of energy-transfer methods (Eidelman et al., 1984). The fusion process was pH-dependent, being highly efficient at low pH, as indeed is expected from enveloped viruses that, after internalization by endocytosis, fuse with the endosomal membranes (White et al., 1983). Maximal fusion, i.e., reduction in energy transfer, was obtained when fluorescent vesicles containing the G protein were incubated with PS liposomes at a pH as low as 3.0 (Eidelman et al., 1984).

Similar characteristics of fusion have recently been observed upon interaction between fluorescent influenza virions and phospholipid vesicles (Stegmann et al., 1985). A high degree of fusion was observed only at low pH and with liposomes composed of cardiolipin, while at neutral pH or with liposomes of uncharged phospholipid molecules such as phosphatidylcholine (PC), fusion was minimal. In this regard it should be mentioned that PS liposomes have been shown to specifically inhibit infection of VERO cells by VSV (Schlegel et al., 1983). Thus, it appears that negatively charged liposomes are able to fuse either among themselves when incubated with positively charged metal ions or polycations (Wilschut et al., 1980; Gad & Eytan, 1983) or with viral envelopes, preferably at low pH (Eidelman et al., 1984; Haywood & Boyer, 1984). However, despite these observations, it is still questionable whether fusion of virus envelopes with negatively charged liposomes does reflect biological fusion processes such as those occurring during virus infection.

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; HN, hemagglutinin/neuraminidase glycoprotein; F, fusion glycoprotein; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; CF, carboxyfluorescein; RSVE, reconstituted Sendai virus envelopes; N-NBD-RSVE, RSVE labeled with N-NBD-PE; N-NBD-HN, HN vesicles labeled with N-NBD-PE; N-NBD-F, F vesicles labeled with N-NBD-PE; SFV, Semliki Forest virus.

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Fusion of enveloped viruses belonging to the paramyxovirus and myxovirus groups with cell plasma membranes is absolutely dependent upon the presence of sialic acid containing components such as sialoglycoproteins or sialoglycolipids, which serve as virus receptors (Rott & Klenk, 1977). From studies on the interaction between Sendai and phospholipid liposomes, it has been suggested (Haywood & Boyer, 1984) that the specific interaction between enveloped virions such as Sendai virus and membrane sialic acid residues may be due to the fact that the latter possess a high density of negative charges. Since it has been shown that Sendai virions are able to interact with liposomes composed of negatively charged phospholipids, the possibility was raised (Haywood & Boyer, 1984) that the negatively charged phospholipid molecules can substitute for the sialic acid residues as very efficient functional virus receptors.

From studies about the distribution of phospholipid molecules in biological membranes, it seems that most of the membrane negatively charged phospholipid molecules are located on the inner face of the membranes (Op den Kamp, 1979). Conversely, upon infection, enveloped viruses encounter first the external face of the membrane, which is poorly endowed with negatively charged phospholipid molecules. It is conceivable that fusion processes in which membrane proteins participate are more complex than those observed with PS liposomes.

In this work we have followed the interaction of fluorescently labeled, reconstituted Sendai virus envelopes with PS liposomes by fluorescence dequenching techniques (Chejanovsky & Loyter, 1985). The use of Sendai virions as a model system offers several advantages over the use of other enveloped viruses: (i) Infection of cells by this virus is mediated by a fusion process between the viral envelope and the cell plasma membrane, a process whose pH optimum is neutral (Peretz et al., 1974). (ii) Fusion and infection of Sendai virions can be specifically inhibited by various inhibitors such as phenylmethanesulfonyl fluoride (PMSF) or dithiothreitol (DTT) (Nussbaum et al., 1984). (iii) Infection of cells and fusion of the viral envelopes require the presence of the two viral envelope polypeptides, namely, the hemagglutinin/neuraminidase (HN) and the fusion (F) glycoproteins (Nussbaum et al., 1984). The HN glycoprotein, in addition to being the viral binding protein, has also been shown to actively participate in the membrane fusion step itself (Miura et al., 1982; Nussbaum et al., 1984).

The results of this work clearly demonstrate that reconstituted Sendai virus envelope and membrane vesicles containing only the viral HN glycoprotein are able to fuse with PS liposomes. Surprisingly, fusion was pH-dependent, being maximal at low pH. However, all our results show that fusion of the viral envelopes or of their isolated components with the negatively charged phospholipid vesicles does not reflect the virus's biological fusogenic activity required for infection of living cells.

Materials and Methods

Chemicals. Phosphatidylcholine (PC) (from egg yolk) and phosphatidylserine (PS) (from bovine brain) as well as dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), and Pronase were obtained from Sigma. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) was purchased from Avanti Biochemicals (Birmingham, AL), and carboxyfluorescein (CF) was from Eastman Kodak. Ammonyx-LO was from Millmaster-Onyx Int., Fairfield, NJ.

Virus. Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutinating units and hemolytic activity were determined as previously described (Peretz et al., 1974).

Cells. Human blood, type O, was obtained from the blood bank of Hadassah Hospital, Jerusalem. The blood was washed 5 times with solution A (160 mM NaCl, 20 mM Tricine, pH 7.4) and finally suspended in solution A to give 2–3% (v/v).

Reconstitution of Sendai Virus Envelopes or Vesicles Containing the Viral Hemagglutinin/Neuraminidase (HN) or Fusion (F) Glycoproteins. Reconstituted Sendai virus envelopes (RSVE) were obtained by solubilization of intact, pelleted Sendai virions (10 mg) with 20 mg of Triton X-100 (500 μL, 4% w/v), following removal of the detergent by direct addition of SM-2 Bio-Beads, as described before (Vainstein et al., 1984). With [^{3}H]Triton, it has been demonstrated that, under the conditions used, very little (0.005% w/v) Triton is left in the virus envelope preparations (Vainstein et al., 1984). This amount does not affect the fusogenic activity of these envelopes.

Membrane vesicles, containing only the HN or the F glycoproteins, were prepared essentially as described before (Nussbaum et al., 1984). Briefly, for the preparation of F-containing vesicles, the pH of a detergent solution (1 mL) containing the two viral envelope glycoproteins was adjusted to 5.3 by dialyzing against a buffer (0.5 L) containing sodium acetate (10 mM), pH 5.3, and 0.25% Triton X-100. After 2 h at 4 °C, a volume of up to 300 μL of the turbid dialysate was loaded on 5 mL of dry CM-Sepharose CL-6B beads. The Sepharose beads were washed and equilibrated with a buffer containing 10 mM sodium acetate and 4% Triton X-100, and then packed by centrifugation in a 5-mL syringe. After loading, the column was centrifuged (500*g*, 3 min) and the eluent obtained contained only the F glycoprotein, as revealed by gel electrophoresis (Figure 1). Detergent was removed from the eluate by direct addition of SM-2 Bio-Beads (Vainstein et al., 1984). The membrane vesicles obtained were centrifuged (100000*g*, 60 min) and suspended in solution A.

Membrane vesicles containing only the HN glycoprotein were prepared by dialyzing 1 mL of the detergent solution of the viral glycoproteins against a buffer containing 10 mM sodium phosphate, pH 6.0, and 0.25% Triton X-100. After 2 h at 4 °C, a volume up to 300 mL of the dialysate was loaded on 5 mL of dry DEAE-52-cellulose (5-mL syringe) (Fukami et al., 1980). The cellulose beads were washed and equilibrated with buffer containing 10 mM sodium phosphate, pH 6.0, and 4% Triton X-100. All other steps of centrifugation of the columns, removal of detergent from the eluents, and formation of reconstituted vesicles were as described above for the preparation of F vesicles. Gel electrophoresis analysis revealed (Figure 1) that the eluate contained only the HN glycoprotein. Both the CM-Sepharose and DEAE-cellulose columns can be reused up to 5 times on the same day.

Hybrid vesicles, containing both the F and HN glycoproteins, were prepared by mixing detergent solutions of the F and HN glycoproteins in the appropriate weight ratios. Detergent was removed from the mixture as described above and before (Vainstein et al., 1984).

Preparation of Phospholipid Liposomes. The desired amount of lipids (usually 2 μmol of PS or PC) was dried from their chloroform solution under nitrogen at 4 °C. After addition of 1 mL of either solution A or a solution containing only 160 mM NaCl, the suspension obtained was vigorously shaken. The turbid mixture was then sonicated in a sonicator bath for 3 min, until a clear, opalescent solution was obtained. Electron microscopy observations, using the negative-staining technique, revealed the formation of large liposomes (see

Figure 6) with an average diameter of 150 nm.

Preparation of RSVE, HN, and F Vesicles Labeled with N-NBD-PE. A chloroform solution of N-NBD-PE (50 μg) was evaporated under nitrogen to give a thin layer of the fluorescent probe, to which a detergent solution of the viral envelope glycoproteins or Triton X-100 solubilized HN or F glycoproteins (150 μL of 4% Triton X-100 containing 200 μg of viral glycoproteins), prepared as above, was added. After solubilization of the fluorescent probe by vigorous shaking, the detergent was removed from the various systems by incubation with SM-2 Bio-Beads (40 mg), as described above. All subsequent steps resulting in the formation of vesicles that contained the viral glycoproteins were as described above and before (Chejanovsky & Loyter, 1985).

Entrapment of Carboxyfluorescein (CF) in Phospholipid Vesicles. An aqueous solution of CF (adjusted to pH 7.4) was added to a thin layer of the dried phospholipid molecules to give a final concentration of 80 mM CF. After the addition of solution A to give a final volume of 1 mL, the mixture was vigorously vortexed and then sonicated for 3 min in a sonicator bath. A light, opalescent suspension was obtained. For removal of the free, untrapped CF, the liposomes (200 μL of 500–800 μg in PS or PC) were loaded on a syringe containing 5 mL of dried Sephadex G-25 (fine, Pharmacia) as previously described (Citovsky & Loyter, 1985). For determination of virus-induced CF release, the liposomes were used immediately.

Fluorescence Measurements of the Interaction between Reconstituted Viral Vesicles and Liposomes. (A) N-NBD Fluorescence Dequenching. Fluorescent, N-NBD-PE-containing, reconstituted viral vesicles (RSVE, HN, and F, 0.3–0.6 μg of each) were incubated at the indicated concentrations with liposomes composed of PC or PS, usually in a final volume of 1.6 mL of the appropriate buffer, for 15 min at 37 °C. Fluorescent measurements were performed on an MPF-4 Perkin-Elmer spectrophotometer, using an excitation wavelength of 473 nm and an emission wavelength of 545 nm. A high-pass filter of 520 nm was used, and the excitation and emission were kept narrow so as to reduce light scattering. Fluorescent dequenching was used to follow intermixing of the viral vesicles and liposome phospholipid molecules as described before (Chejanovsky & Loyter, 1985). Total fluorescence dequenching (maximum dilution of the fluorescent probe) was considered as the fluorescence obtained after solubilization of the vesicles with Ammonyx-LO (0.1% w/w). The percentage of fluorescence dequenching was calculated as described before (Chejanovsky & Loyter, 1985).

(B) Viral-Induced CF Release from Loaded Liposomes. A sample of CF-containing liposomes was incubated with either RSVE or vesicles containing either the viral HN or F glycoproteins, at 37 °C, in a final volume of 100 μL, essentially as described before (Citovsky & Loyter, 1985). For fluorescence measurements, the suspension was diluted with 1.5 mL of cold solution A, and the fluorescence degree was measured before and after addition of Ammonyx-LO (0.1% final concentration). The degree of fluorescence obtained in the presence of Ammonyx-LO (total lysis of liposomes) was considered as 100% CF release. Fluorescence measurements were performed on an MPF-4 Perkin-Elmer spectrophotometer (CF excitation at 490 nm and emission at 520 nm). The fluorescence self-quenching property of CF (Weinstein et al., 1977) was used to monitor liposome integrity as well as extent and rate of CF release.

Preparation of Fluorescent Vesicles Made of Sendai Virus Extracted Lipids. Lipids were extracted from Sendai virus particles (10 mg) by the method of Folch et al. (1957). The organic solvents were evaporated by nitrogen, and the dry layer of lipid obtained was redissolved with chloroform–methanol (2:1). After removal of a sample for phospholipid analysis, the lipid extract was dried under nitrogen, and the dried layer obtained was dissolved in 500 μL of 4% (w/w) Triton X-100. Following 5 min of vigorous shaking, the detergent-solubilized viral lipids were added to a thin layer of fluorescent molecules, obtained after a chloroform solution of N-NBD-PE was dried under nitrogen, to give a ratio of viral lipids:N-NBD-PE of 94:6 (Chejanovsky & Loyter, 1985). Subsequent to another 5 min of vigorous shaking, the detergent was removed by the direct addition of SM-2 Bio-Beads, as described above and before (Vainstein et al., 1984). Electron microscopy observations, using the negative-staining technique, revealed the formation of liposomes with an average diameter of 150–200 nm.

Incubation of the fluorescent lipid vesicles with liposomes composed of PS or PC and determination of fluorescence dequenching were performed as described above for fluorescent, reconstituted Sendai virus envelopes.

Protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. Phospholipid concentrations were estimated by the method of Stewart (1980), with PC as a standard.

Table I: Susceptibility of Loaded PS Liposomes, but Not of Human Erythrocytes, to the Lytic Activity of Sendai Virus HN Glycoprotein

expt	system	hemolysis (% of total)	CF release from PS liposomes (% of total)[a]
I[b]	RSVE	80.0	69.5
	Pronase-treated RSVE	3.5	8.3
	F vesicles	0	11.0
	Pronase-treated F vesicles	<1.0	7.4
	HN vesicles	<1.0	60.6
	Pronase-treated NH vesicles	<1.0	7.4
II[c]	HN vesicles	1.0	ND[d]
	F vesicles	0	ND
	HN + F vesicles (1:0.025 w/w)	13	ND
	HN + F vesicles (1:0.05 w/w)	30	ND
	HN + F vesicles (1:0.1 w/w)	52	ND
	HN + F vesicles (1:1 w/w)	68	ND

[a] Incubation of only loaded PS vesicles under the same conditions resulted in release of 6–8% of the enclosed CF [see also Amselem et al. (1985)]. [b] RSVE, HN, or F vesicles (6 μg of protein of each) were incubated with human erythrocytes (100 μL of 2.5% v/v) or with liposomes composed of PS (200 μM PS in 100 μL of solution A, pH 7.4) for 30 min at 37 °C, after which homolysis and CF release were determined as described under Materials and Methods and before (Peretz et al., 1974; Citovsky & Loyter, 1985). Proteolytic treatment was performed by incubating RSVE, HN, or F vesicles for 30 min at 37 °C with Pronase (30 μg of pronase/100 μg of viral proteins). The treated viral vesicles were added immediately to the various systems to give a final concentration of 6 μg of protein. [c] HN, F (8 μg of protein of each), or hybrid vesicles (8-μg total), containing the indicated weight ratios between HN and F glycoproteins, were incubated with human erythrocytes (100 μg of 2.5% v/v) for 30 min at 37 °C. Hemolysis was determined as described before (Peretz et al., 1974). [d] ND, not determined.

Results

Interaction of RSVE or Vesicles Containing the Viral HN and F Glycoproteins with Liposomes Composed of PS. The results in Table I, experiment I, confirm previous observations (Amselem et al., 1985; Citovsky & Loyter, 1985) showing that RSVE are able to induce release of CF from loaded liposomes composed of negatively charged phospholipids such as PS vesicles. Treatment of RSVE with Pronase abolished the lytic ability (the ability to lyse loaded PS liposomes) almost completely, as well as their hemolytic activity (Table I, experiment

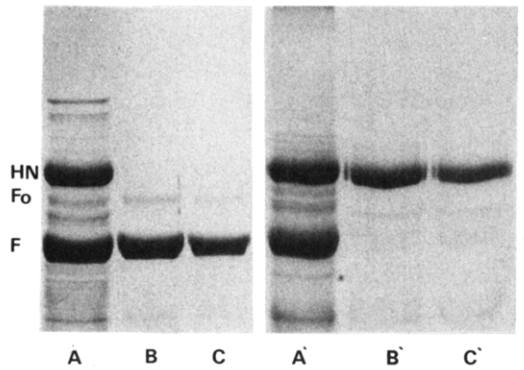

FIGURE 1: Gel electrophoresis analysis of purified Sendai viral HN and F glycoproteins. Sendai viral F and HN glycoproteins were purified from the detergent solution of the viral glycoproteins as described under Materials and Methods. A volume of 20 μL, containing about 25 μg of the detergent-solubilized viral glycoproteins (A and A′) or of the eluents from CM-Sepharose (B and C) or from DEAE-cellulose (B′ and C′) columns, was analyzed by gel electrophoresis (7.5% acrylamide) according to the method of Laemmli (1970). B, C and B′, C′ show purified F and HN glycoproteins obtained from two successive loadings on CM-Sepharose and DEAE-cellulose columns, respectively, emphasizing the reproducibility of this method. Note that the HN preparations are devoid of any F or F_0 glycoproteins. Some residual F_0 glycoproteins can be seen in the preparation of detergent-solubilized viral glycoproteins (A and A′) or F polypeptides (B and C).

I), indicating the involvement of a viral-associated polypeptide in both processes. It has previously been shown (Hsu et al., 1979) and can also be inferred from the results in Table I that neither HN nor F vesicles (membrane vesicles containing the viral HN or F glycoproteins, respectively) are able to induce hemolysis when incubated at 37 °C with human red blood cells. On the other hand, HN vesicles were able to induce a high degree of lysis in loaded PS vesicles, most of which was abolished after treatment of the HN glycoproteins with Pronase (Table I, experiment I).

Gel electrophoresis analysis revealed that no cross-contamination can be detected in the purified preparations of either the HN or the F glycoproteins. The patterns seen in Figure 1 (B′ and C′) clearly show that the HN preparations obtained by the method described in this work are devoid of any detectable F glycoproteins. This can also be inferred from the results presented in Table I, experiment II. As mentioned above and also seen in experiment II (Table I), neither the purified HN nor F vesicles induced hemolysis when incubated with human erythrocytes. However, reconstitution of the HN glycoprotein in the presence of as little as 2.5% of the F glycoprotein (HN:F, w/w ratio of 1:0.025) resulted in the formation of hybrid vesicles that, under the conditions used, promoted 13% hemolysis (Table I, experiment II). Hybrid vesicles containing HN and F in a weight ratio of HN:F of 1:0.05 (95% of HN glycoproteins and only 5% of F glycoproteins) induced a 30% hemolysis (Table I, experiment II). These observations strengthen the results obtained by gel electrophoresis analysis (Figure 1) showing that our HN vesicle preparations are practically devoid of any contamination by the F glycoprotein. Should any F polypeptides be present in the HN glycoprotein preparations, this would amount to less than 2.5%, a quantity that does not restore the fusogenic and hemolytic activities of such vesicles.

Virus-induced hemolysis of red blood cells and lysis of loaded liposomes are considered to reflect virus–membrane or virus–liposome fusion processes, respectively (Hosaka & Shimizu, 1977; Amselem et al., 1985). Therefore, it should be concluded that neither the F nor the HN vesicles are able to fuse with human erythrocyte membranes. However, the results

Table II: Induction of Fluorescence Dequenching upon Interaction of Fluorescent RSVE with PS, but Not with PC, Liposomes

expt	system	dequenching (%) for liposomes composed of PS	PC
I[a]	RSVE	60.0	18.2
	PMSF-treated RSVE	40.2	ND[b]
	Pronase-treated RSVE	15.2	9.3
II[c]	RSVE	52.0	15.0
	glutaraldehyde RSVE	8.4	6.0
	vesicles made of Sendai virus lipids (0.3 μg)	7.0	6.0
	vesicles made of Sendai virus lipids (0.6 μg)	8.0	6.0

[a] Fluorescent, untreated, or treated (with PMSF, DTT, or Pronase) RSVE (0.4 μg of viral protein) were incubated with liposomes composed of either PS or PC (2 mM each) for 30 min at 37 °C, in a final volume of 100 μL of solution A. Insertion of N-NBD-PE into the viral reconstituted envelopes and determination of fluorescence dequenching (after dilution to 1.6 mL with solution A, pH 7.4) were as described under Materials and Methods. RSVE (100 μg) were treated with DTT (4 mM) or PMSF (6 mM) for 30 min at 37 °C, as described. Treatment with Pronase was performed as described in Table I. [b] ND, not determined. [c] Fluorescent RSVE were incubated with 0.05% glutaraldehyde at 4 °C exactly as described before (Toister & Loyter, 1973). Fluorescent, untreated, or treated RSVE (0.4 μg of viral protein) were incubated with PS or PC liposomes as described above. Fluorescent liposomes composed of the viral phospholipids were prepared as described under Materials and Methods. The fluorescent liposomes (0.3 and 0.6 μg) were incubated with liposomes composed of either PS or PC (2 mM of each) for 30 min at 37 °C, as described above for RSVE. The degree of fluorescence dequenching was determined as described under Materials and Methods for the interaction between fluorescent RSVE and liposomes.

in Table I, experiment I, raise the possibility that the HN vesicles are able to fuse with vesicles composed of PS molecules.

The results in Table II show that incubation of fluorescent (bearing N-NBD-PE) RSVE with PS liposomes results in fluorescence dequenching. Increase in fluorescence dequenching reflects, in the present system, the formation of fused vesicles in which the viral phospholipids were diluted by and intermixed with the PS molecules (Chejanovsky & Loyter, 1985). Very little fluorescence dequenching was observed when fluorescent RSVE were incubated with liposomes composed of only PC (Table II, experiment I). Treatment of RSVE with either PMSF or DTT, reagents that have been shown to inactivate the viral hemolytic and fusogenic activities (Nussbaum et al., 1984), did not significantly affect its ability to interact with PS loposomes. As can be seen (Table II, experiment I), incubation of either DTT- or PMSF-treated fluorescent RSVE with PS liposomes resulted in fluorescence dequenching, the extent of which was only slightly lower than that obtained with untreated RSVE. Only treatment with Pronase affected the RSVE ability to interact with the PS liposomes, as can be inferred from the results showing that only a small extent of fluorescence dequenching as observed by incubation of Pronase-treated fluorescent RSVE with PS liposomes. Pronase treatment abolishes the ability of either intact virions or HN vesicles to bind to and agglutinate human erythrocytes (not shown).

The results in Table II, experiment II, show that very little fluorescence dequenching (about 6–7%) was observed when glutaraldehyde-treated fluorescent RSVE were incubated with either PS or PC liposomes. It has been shown earlier (Toister & Loyter, 1973) that glutaraldehyde-treated RSVE are able to bind to human erythrocytes and probably to promote lip-

Table III: Interaction of HN and F Vesicles with Phospholipid Vesicles As Monitored by Fluorescence Dequenching[a]

expt	system	dequenching (%) for liposomes composed of PS	PC
1	HN vesicles	49.5	
	F vesicles	13.4	
	HN + F vesicles	58.3	
2	HN vesicles	66.1	10.2
	PMSF-treated HN vesicles	65.2	ND[b]
	DTT-treated HN vesicles	45.7	15.1
	F-vesicles	11.4	3.4

[a] HN vesicles, F vesicles, or vesicles obtained by coreconstitution of HN and F (HN + F vesicles) were obtained and labeled with N-NBD-PE as described before (Chejanovsky & Loyter, 1985) and under Materials and Methods. HN vesicles (100 μg) were treated with DTT and PMSF as described for RSVE in Table II. The fluorescent vesicles (0.4 μg of viral protein) were incubated either with PS or with PC liposomes (2 mM) in a final volume of 100 μL of solution A, pH 7.4, for 30 min at 37 °C, after which the various systems were diluted with solution A to give 1.6 mL for fluorescence determination. [b] ND, not determined.

id–lipid exchange (Maeda et al., 1977) but not to fuse with them. Very little fluorescence dequenching was also observed when fluorescent vesicles made of viral-extracted lipids were incubated with PS or PC liposomes (Table II, experiment II). It should be added that the vesicles made of the viral lipids neither induce hemolysis nor promote fluorescence dequenching when incubated with intact human erythrocytes or human erythrocyte ghosts, respectively (not shown).

On the basis of these results, it may be assumed that the high degree of fluorescence dequenching observed by incubation of RSVE and liposomes composed of PS is not due to lipid–lipid exchange between the viral lipids and the added liposomes.

Our results show that the fluorescence dequenching observed upon incubation of RSVE with PS liposomes is due to the activity of the viral HN glycoprotein (Table III). As can be seen, incubation of fluorescent HN vesicles with PS liposomes resulted in fluorescence dequenching, the extent of which was similar to that observed with either RSVE (compare results in Table II with those in Table III) or with HN + F vesicles, namely, vesicles formed after coreconstitution of HN and F viral glycoproteins (for experimental details, see Materials and Methods). However, a very low degree of fluorescence dequenching was observed when vesicles, containing the Sendai virus fusion protein, i.e., the F glycoprotein, were incubated with PS liposomes (Table III, experiment 1). Neither treatment with PMSF nor treatment with DTT markedly affected the fluorescence dequenching observed upon incubation of the HN vesicles with PS liposomes. A very low degree of fluorescence dequenching was obtained when fluorescent HN vesicles were incubation with liposomes composed of only PC (Table III, experiment 2). From these results it can be deduced that HN but not F vesicles are able to fuse with PS liposomes.

Interaction of HN and F Vesicles with PS Liposomes: Effect of pH. The fusogenic activity of several enveloped viruses such as influenza, vesicular stomatitis virus (VSV), or Semliki Forest virus (SFV) is expressed only at low pH such as pH 5.0–6.0 (White et al., 1983). It was therefore of interest to find out the effect of pH on the fusogenic activities of RSVE as monitored by fluorescence dequenching and to study whether the fusogenic ability of the F vesicles could be activated at low pH.

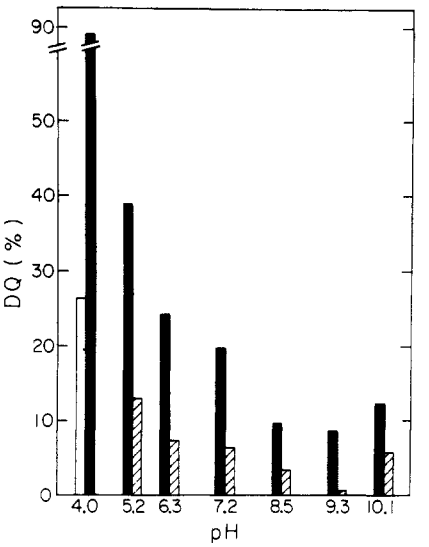

FIGURE 2: Interaction of HN and F vesicles with PS liposomes as revealed by fluorescence dequenching: effect of pH. Fluorescent HN or F vesicles (0.4 μg) were incubated at various pHs with PS liposomes (100 μM) in a final volume of 100 μL for 15 min at 37 °C, after which the fluorescence degree was determined in a final volume of 1.6 mL, as described under Materials and Methods. HN was treated with Pronase, as described for RSVE in Table II. In order to achieve the various pHs, the following buffers (10 mM final concentrations) were added to a solution containing 140 mM NaCl: pH 4.0, sodium acetate; pH 5.2–8.5, sodium phosphate; pH 9.0–10.1, glycine–NaOH. (▨) F vesicles, (■) HN vesicles, and (□) Pronase-treated HN vesicles. Note that in these experiments a concentration of 100 μM PS was used (see also Figure 3).

The results in Figure 2 indeed show that the low degree of fluorescence dequenching observed upon incubation of fluorescent F vesicles with PS liposomes was increased by lowering the pH of the incubation medium. As can be seen, the low degree of fluorescence dequenching (5%) observed upon incubation of F vesicles and PS liposomes at pH 7.2 increased to above 12% when the incubation was performed at pH 5.2. An increase in fluorescence dequenching was also observed when the HN vesicles were incubated at pHs lower than 7.2. The results in Figure 2 clearly demonstrate that, with a relatively low concentration of PS (100 μM), about 20% of fluorescence dequenching was obtained with fluorescent HN vesciles at pH 7.2, while about 40% fluorescence dequenching was observed with the same system (100 μM PS) at pH 5.2. A much higher increase in fluorescence dequenching was observed with HN vesicles at pH 4.0, at which the degree of fluorescence dequenching reached about 90% (Figure 2). Incubation of F vesicles at pH 4.0 (0.4 μg in a 100-μL volume) resulted in a high degree of turbidity, and therefore, it was difficult to follow by fluorescence dequenching the interaction between F vesicles and PS liposomes at this low pH. It should be noted that the incubation of fluorescent vesicles made of lipids extracted from Sendai virions (see Materials and Methods and Table II, experiment II) with PS liposomes at the different pHs resulted in very little fluorescence dequenching, never exceeding 5–8% (not shown). In subsequent experiments the interaction between HN vesicles and PS liposomes was studied at pH 4.0, since under these conditions a maximum fluorescence dequenching was observed.

The results in Figure 3A show that, when the fluorescent HN vesicles were incubated at pH 4.0 with increasing concentrations of PS liposomes, maximum fluorescence dequenching (80–85%) was obtained with as little as 20 μM PS. No fluorescence dequenching was observed when HN veiscles were incubated with liposomes composed only of PC molecules

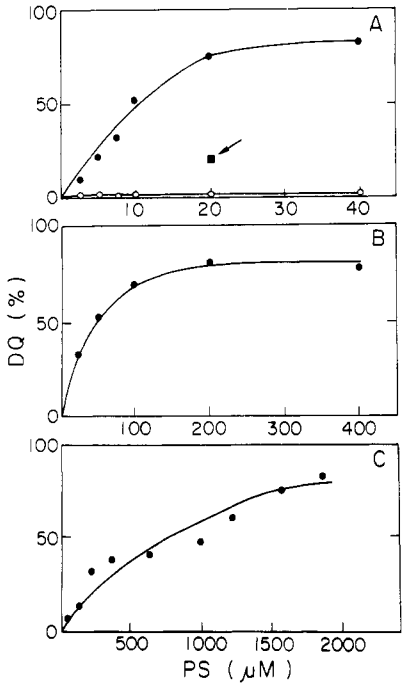

FIGURE 3: Increase in the affinity between HN vesicles and PS liposomes at low pH. Fluorescent HN vesicles (0.5 μg) were incubated with increasing concentrations of PS (●) or PC [(○) in (A)], in a final volume of 100 μL for 15 min at 37 °C. Fluorescence determinations were performed after dilution to 1.6 mL. Incubation and dilution to 1.6 mL for fluorescence determinations were performed by using a solution containing 140 mM NaCl and 10 mM of the following buffers: (A) sodium acetate, pH 4.0 [arrow indicates the degree of fluorescence obtained by incubating Pronase-treated HN vesicles with PS (20 μM)]; (B) sodium phosphate, pH 5.2; (C) sodium phosphate, pH 7.2.

(Figure 3A). A low degree of fluorescence dequenching was obtained upon incubation of Pronase-treated HN vesicles with 20 μM PS liposomes at pH 4.0 (Figure 3A, arrow). About 10 times more phospholipids were required to reach maximum fluorescence dequenching when incubation of the HN vesicles with PS liposomes was performed at pH 5.2 rather than at pH 4.0 (Figure 3B). Maximum fluorescence dequenching was reached with only as much as 2 mM PS liposomes when the fluorescent HN vesicles were incubated with the PS liposomes at pH 7.2 (Figure 3C).

Kinetic studies revealed that the increase in the fluorescence degree at pH 7.2 (Figure 4A, curve c) is extremely slow, while at pH 4.0 it is very rapid and linear during the first 30–60 s of incubation (Figure 4A, curve a). No fluorescence dequenching was observed upon incubation of the fluorescent HN vesicles with PC liposomes (Figure 4A, curve d) or of fluorescent F vesicles with PS liposomes, even after 10 min of incubation at pH 4.0 (Figure 4A, curve e). Similarly, very little increase in the degree of fluorescence dequenching was observed, even after long incubation periods, of Pronase-treated fluorescent HN vesicles with PS liposomes (Figure 4B, curve b).

The interaction between the HN vesicles and the PS liposomes was temperature-dependent, as can be inferred from the results in Figure 5. Maximum fluorescence dequenching was observed between 32 and 38 °C, although a relatively high degree of fluorescence dequenching was also observed between 16 and 30 °C (Figure 5). A sharp decrease in fluorescence dequenching was observed only below 16 °C. However, even at 4 °C, about 18% of fluorescence dequenching was obtained, as compared to a maximum of 60% fluorescence dequenching at 37 °C (Figure 5).

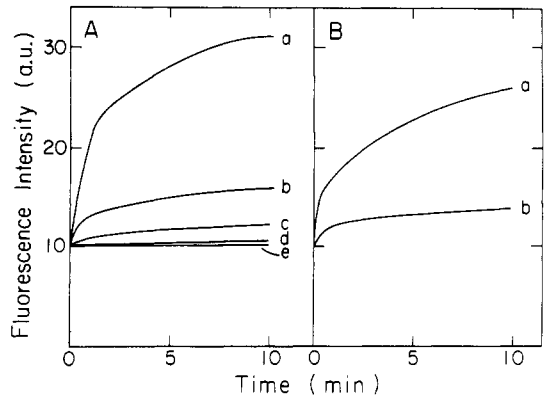

FIGURE 4: Interaction between HN vesicles and PS liposomes: kinetic studies. (A) Fluorescent HN (a–d) or F (e) vesicles (0.4 μg of protein) were preincubated at the various pHs, at a final volume of 1.6 mL, for 5 min at 37 °C, after which 20 μM (16 μL of 2 mM) PS (a–c, e) or PC (d) was added, and the fluorescence intensity was continuously followed for 10 min of incubation. All experimental conditions were as described in Figure 2 and under Materials and Methods. (a) Sodium acetate, pH 4.0; (b) sodium phosphate, pH 5.2; (c) sodium phosphate, pH 7.2; (d) sodium acetate, pH 4.0; (e) sodium acetate, pH 4.0. (B) Fluorescent untreated (a) or Pronase-treated (b) NH vesicles were incubated at 37 °C with PS (20 μM) at pH 4.0, as described in (A).

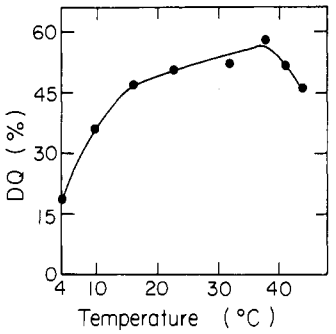

FIGURE 5: Effect of temperature on the interaction between HN vesicles and PS liposomes. Fluorescent HN vesicles (0.4 μg of protein) were incubated with 20 μM PS, in a final volume of 1.6 mL of NaCl (140 mM) and acetate buffer (10 mM), pH 4.0, at the various temperatures for 15 min. Fluorescence determinations were performed at room temperature, except for the systems incubated at 4 and 10 °C, in which fluorescence was determined at 4 °C.

The electron micrographs in Figure 6 show that large vesicles appeared only in samples containing HN vesicles that were incubated with PS liposomes at 37 °C at pH 4.0. It is conceivable that these large vesicles are formed as a result of fusion between the HN vesicles and the PS liposomes. It appears that the average diameter of the large vesicles is about 450 nM (Figure 6C) as compared to 150 nM of the HN vesicles (Figure 6A) and the PS liposomes (Figure 6B).

Discussion

Several reports have recently been published demonstrating that intact virions or reconstituted virus envelopes, containing viral glycoproteins, can fuse efficiently with negatively charged phospholipid vesicles (Stegmann et al., 1985; Eidelman et al., 1984; Haywood & Boyer, 1984; Amselem et al., 1985). Furthermore, in some cases as with influenza virions (Stegmann et al., 1985) or envelopes bearing the G protein of VSV (Eidelman et al., 1984), fusion was more pronounced at low pHs, namely, at pH 5.5 and below. The requirement for low pH is compatible with the observations that such virions fuse with the endosomal membranes after being internalized into the targeted cells (White et al., 1983). Fusion is induced by the intraendosomal low pH, as increasing the pH by lysoso-

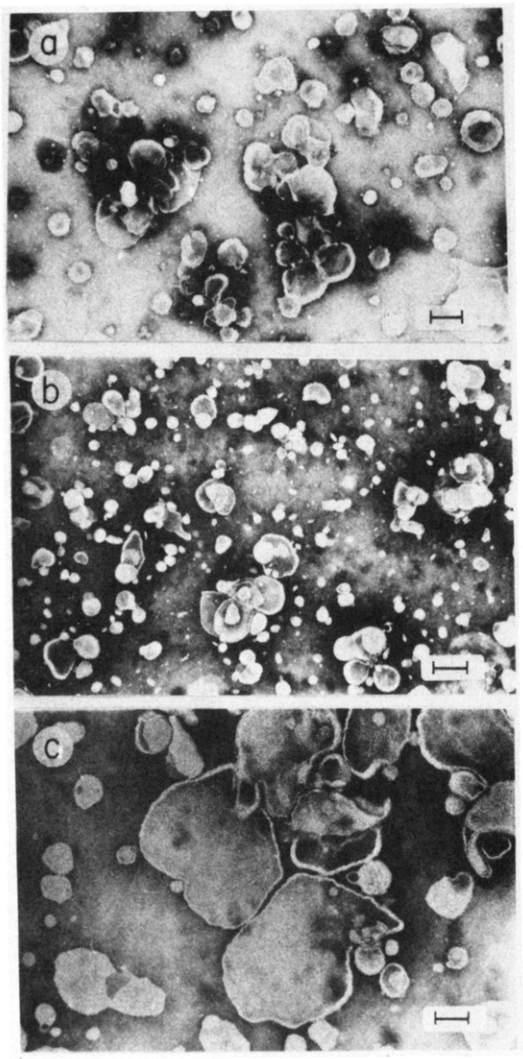

FIGURE 6: Electron micrographs of large vesicles formed upon fusion between HN vesicles and PS liposomes. HN vesicles (10 μg of protein) were incubated with PS (20 μM) for 15 min at 37 °C at pH 4.0, after which a sample was stained with PTA for electron microscopy observations, as described before (Vainstein et al., 1984). (a) HN vesicles, (b) PS liposomes, and (c) PS liposomes incubated with HN vesicles. Bar = 136 μm.

motropic reagents inhibits fusion of and infection by enveloped viruses such as SFV (White et al., 1983).

The results of this work show that, by using fluorescence-dequenching methods, Sendai virions effectively fuse with negatively charged liposomes, i.e., liposomes made up only of PS. Dequenching of virus-associated fluorescent probes has been used by us and by others as a highly sensitive method to follow, on a quantitative basis, virus–membrane fusion processes (Stegmann et al., 1985; Eidelman et al., 1984; Chejanovsky & Loyter, 1985).

The results presented here indeed reflect a process of virus–liposome fusion and not lipid–lipid exchange, as can be inferred from the following experiments: (i) A high degree of fluorescence dequenching was obtained mainly following interaction with PS liposomes, whereas very little or no fluorescence dequenching was observed upon interaction of fluorescent viral envelopes with PC liposomes. Lipid–lipid exchange is expected to occur at an equal rate and extent with liposomes of both compositions (Nichols & Pagano, 1983). (ii) At low pH, fluorescence dequenching displayed fast kinetics, which are uncharacteristic for lipid–lipid exchange. (iii) Fluorescence dequenching was greatly inhibited after treatment of the viral envelopes with proteolytic enzymes such as Pronase. However, the above considerations and experiments do not completely exclude the possibility that the results obtained are due to contact mediated by lipid–lipid exchange. Pronase treatment of the RSVE may abolish the ability of the viral envelopes to interact with or bind to the PS liposomes and, therefore, no contact mediated by lipid–lipid exchange will be observed when such Pronase-treated RSVE are incubated with PS liposomes.

Our observation (Table II, experiment II) that glutaraldehyde-fixed virions show very little fluorescence dequenching when incubated with PS liposomes, either at pH 7.4 or 4.0, completely rules out the possibility of contact mediated by lipid–lipid exchange. It has been shown before (Toister & Loyter, 1975) that such treated virions are able to agglutinate and bind to chicken erythrocytes but not to fuse with them. Furthermore, it has been demonstrated that fixation by glutaraldehyde, under the conditions used, allows lipid–lipid exchange (Maeda et al., 1977). Moreover, our electron microscopic studies (Figure 6), showing the formation of large vesicles upon incubation of the vesicles with PS liposomes, support our conclusion that the fluorescence dequenching observed is due to a fusion process and not to contact mediated by lipid–lipid exchange.

On the basis of these considerations and the present results, it can be inferred that Sendai virions fuse effectively at low pHs with liposomes composed of PS and lacking viral receptors. These results confirm previous observations by Haywood and Boyer (1984), using differential centrifugation methods, to demonstrate fusion between Sendai virions and negatively charged liposomes. It has been reported before (Chejanovsky & Loyter, 1985) that the dequenching value can be considered as a direct measure of the degree of fusion, namely, the percent of the viral vesicles that fuse with biological membranes or with liposomes. From the present data, it appears that the HN vesicles are highly fusogenic and, at low pH, up to 90% of these vesicles can fuse with PS liposomes. However, the results of this work as well as that of Haywood and Boyer (1984) contradict previous observations showing that fusion of Sendai virions with phospholipid vesicles is absolutely dependent upon receptors such as sialoglycolipids or sialoglycoproteins (Oku et al., 1982; Kundrot et al., 1983). Only liposomes containing cholesterol in addition to PC and bearing components containing sialic acid residues effectively fuse with Sendai virions (Oku et al., 1982; Kundrot et al., 1983).

It has been well established that fusion of Sendai virus occurs with the cell plasma membrane at pH 7.5 (or above) and requires the presence of the two viral envelope glycoproteins, the HN and F polypeptides (Peretz et al., 1974; Rott & Klenk, 1977; Miura et al., 1982; Nussbaum et al., 1984). Furthermore, fusion and infection of living cells by these virions can be blocked by several reagents such as DTT, PMSF, or proteolytic enzymes (Nussbaum et al., 1984). Nevertheless, only the last were able to inhibit fusion of RSVE or HN with PS liposomes, while neither DTT nor PMSF had any effect on fusion of fluorescent viral envelopes with the negatively charged phospholipid vesicles.

All these contradictory results raise the question whether fusion of viral envelopes with negatively charged phospholipids is indeed an appropriate model system for processes such as membrane–membrane fusion and, especially, for virus–membrane interactions. This question is further supported by previous reports showing that negatively charged phospholipid molecules are located mainly on the inner face of biological membranes and not on the external side with which viruses interact (Op den Kamp, 1979).

Fusion of negatively charged phospholipid vesicles such as PS or cardiolipin, as was already mentioned above, could be induced by positively charged polypeptides that possess hydrophobic moiety and exhibit detergent-like activity (Gad & Eytan, 1983). It has been well established that Sendai viral glycoproteins as well as glycoproteins of other animal viruses possess such properties (White et al., 1983).

Taking into consideration the isoelectric point of the viral HN and F glycoproteins, it is expected that they will be positively charged below pH 6.5 and 4.9, respectively (Shimizu et al., 1974). Hence, both glycoproteins will interact and associate with negatively charged liposomes at low pH. Due to the difference in the isoelectric point, the HN glycoprotein will probably express this property at pH 6.5 and below, while the F glycoprotein will require much lower pH in order to be positively charged and allow its interaction with negatively charged liposomes. We suggest here that, due to this property and the fact that these glycoproteins possess a hydrophobic sequence, they will be able to fuse with negatively charged liposomes at low pH. Therefore, we suggest that the fusion process reported here and also before (Haywood & Boyer, 1984; Amselem et al., 1985) between Sendai virions and negatively charged phospholipid vesicles does not reflect the biological fusogenic activity of the virus. This biological activity is probably expressed only when liposomes composed of PC are used, the fusion with which requires the presence of cholesterol (Hsu et al., 1983; Citovsky & Loyter, 1985). It is possible that the same is true for previous observations regarding the pH-dependent fusion of VSV or influenza virions and negatively charged liposomes (Stegmann et al., 1985; Eidelman et al., 1984).

White and Helenius (1980) have shown that SFV fuses with liposomes composed of neutral phospholipids only at low pH. Fusion was absolutely dependent on the presence of cholesterol (White & Helenius, 1980). This is very similar to the observations made with Sendai virions, although with the latter, fusion occurred at neutral pH (Kundrot et al., 1983). This may suggest that the molecular mechanism of the virus–membrane interaction process is the same for both groups but that the activity of the viral fusogenic proteins is expressed at different pHs.

Registry No. Neuraminidase, 9001-67-6.

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