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The interaction of Sendai virus with negatively charged liposomes: virus-induced lysis of carboxyfluorescein-loaded small unilamellar vesicles

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The interaction of Sendai virus with small, unilamellar vesicles, lacking virus receptors and loaded with self-quenched 6-carboxyfluorescein, was studied. Sendai virions induced release of carboxyfluorescein from vesicles composed of negative charged phospholipids, despite the fact that they did not contain virus receptors. Preliminary experiments indicate that the carboxyfluorescein release is accompanied by mixing of the virus and liposome lipids and their entrapped contents, suggesting liposome-virus fusion. No release of carboxyfluorescein was observed with vesicles containing only phosphatidylcholine. The rate of virus-induced carboxyfluorescein release was temperature dependent; the lytic activity of the virus was greatly enhanced above 25°C. This effect was not due to a thermal phase transition of the lipids in either the lipid vesicles or the virions. Virus-induced carboxyfluorescein release was inhibited by the presence of calcium ions in the medium and of cholesterol in the lipid vesicles. It increased with increasing concentrations of either the lipid vesicles or the virions. Pretreatment of virions with increasing concentrations of three different proteolytic enzymes (trypsin, chymotrypsin and proteinase) inhibited the virus' ability to cause release of carboxyfluorescein from negatively charged liposomes. Inhibition of the viral lytic activity was also observed after virions were incubated above 56°C.

Introduction

Fusion is a major event in the infection of eukaryotic cells by enveloped viruses, although in various viruses it represents a different step in the overall process of infection [1,2]. In paramyxoviruses such as Sendai virus, the fusion with the cell plasma membrane is the event leading to the introduction of viral nucleocapsid into the cell cytosol [2].

In the course of the fusion process of Sendai virions with cells, a profound change in passive membrane permeability generally occurs [3]. The virus-induced increase in membrane permeability is related to the virus-cell fusion process [4–8] and, therefore, its study may lead to a better understanding of the detailed mechanism of membrane-membrane fusion. Indeed, virus-induced lysis of

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Abbreviations: DPA, dipicolinic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; SUV, small unilamellar vesicle(s).

cells was used as a semiquantitative assay for the process of virus-cell fusion such as, for example, Sendai virus-induced hemolysis of red blood cells [9].

It is well established that the envelope of Sendai virus contains two integral glycoproteins: the hemagglutinin/neuraminidase (HN) which mediates attachment of the virus to cell membranes [10,11], and the fusion protein (F) which plays a major role in the fusion of the viral envelope with host cell membranes [12,13]. Sialic acid-containing membrane glycoproteins or glycolipids have been shown to interact specifically with the Sendai virus HN glycoprotein [14,15]. On the other hand, there is no information on the requirements of the F protein for any specific site of action in the plasma membrane.

Due to the complexity of both, the whole viral membrane and plasma membrane of intact cells, it appears that an elucidation of the molecular mechanism of virus-membrane interaction requires studies with simplified model systems in which composition, structure and function can be related. Indeed, recently liposomes made of well-defined phospholipids have been used as models for host cell membranes in studies of virus-membrane interactions [15–19].

It has been shown that Sendai virus particles can interact and fuse with phosphatidylcholine-cholesterol liposomes [18]. However, this interaction is greatly enhanced by the presence of viral receptors in the liposomes such as sialoglycolipids [16] or sialoglycoproteins [19]. It is possible that after the viral HN glycoprotein is attached to the sialic acid-containing molecules, the F glycoprotein interacts directly with the phospholipid bilayer, thus promoting membrane-membrane fusion.

Negatively charged phospholipid vesicles made of phosphatidylserine have been extensively used during the past few years in studies of liposome-liposome fusion (for review, see Ref. 20). The presence of bivalent metal ions such as Ca^{2+} [20] or positively charged polypeptides such as polylysine, have been shown to be essential for promoting fusion between these phospholipid vesicles [21].

In the light of these observations, it was of interest to determine whether Sendai virus particles interact, fuse and lyse negatively charged phospholipid vesicles lacking viral receptors. The present work is aimed at clarifying these points through studies of the interaction between Sendai virus particles and liposomes made of negatively charged phospholipids, especially phosphatidylserine. It is shown that at temperature above 30°C, Sendai virus induces the release of carboxyfluorescein from these phospholipid vesicles. The activity of Sendai virions on these liposomes is compared with its hemolytic activity.

Materials and Methods

Chemicals. Phosphatidylcholine (PC) was obtained from egg yolk, as described elsewhere [22]. Phosphatidylserine (PS) was prepared from egg PC by transesterification, using partially purified phospholipase D from Savoy cabbage [23]. The PS obtained was purified by silicic acid chromatography and was better than 99% pure. Egg phosphatidylglycerol (PG) egg phosphatidylethanolamine (PE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE) and *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Biochemicals, Inc.

Trypsin (Type III), chymotrypsin (Type I-S), soybean trypsin inhibitor (Type I-P), proteinase (repurified Type VI from *Streptomyces griseus*), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, soybean phosphatidylinositol (PI), cholesterol and dipicolinic acid (DPA) were all obtained from Sigma (St. Louis, MO). Triton X-100 was from Koch-Light Laboratories (Colnbrook, U.K.). Diphenylhexatriene was obtained from Molecular Probes (Junction City, OR). $TbCl_3 \cdot 6H_2O$ (99, 9%) was from Aldrich Chemical CO., Inc. (Milwaukee, WI).

Cells. Human blood, type O, was obtained from the blood bank of Hadassah Hospital, Jerusalem. Blood aged 4–8 weeks was used. The blood was washed five times with Solution A (160 mM NaCl/20 mM Tricine (pH 7.4)) and finally suspended in solution A to give a concentration of 2.0% (v/v).

Virus. Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutination titer and hemolytic activity were

determined as previously described [9,24].

Preparation of reconstituted Sendai virus envelopes. Reconstituted envelopes and Tb-loaded reconstituted envelopes were prepared essentially as described before [25]. After solubilization of intact Sendai virus particles with Triton X-100 and precipitation of detergent-insoluble material [25], the detergent was removed by dialysis in Spectrapor tubings (Spectrum Medical Industries, Inc.) For the Tb-loaded reconstituted envelopes, $TbCl_3$ (50 mM) was added to the solubilized virus suspension. Removal of the detergent resulted in the formation of fusogenic vesicles containing the two viral glycoproteins (HN and F). The reconstituted Sendai virus envelopes formed were collected by centrifugation ($100\,000 \times g$, 60 min) and then suspended in Solution A and kept at $-70°C$ until use.

Entrapment of carboxyfluorescein in phospholipid liposomes. The desired amount of the various phospholipid preparations was dried from the chloroformic solution, using a stream of nitrogen at 4°C. The dry phospholipid was then dispersed in Solution A, containing 5 mM EDTA and 80 mM purified 6-carboxyfluorescein (a gift of Mrs. R. Cohen from the Hebrew University-Hadassah Medical School). The final pH of the suspension was adjusted to 7.4 using sodium hydroxide. Small unilamellar vesicles (SUV) were prepared by ultrasonic irradiation at 4°C, under nitrogen atmosphere, as described elsewhere [26]. The resultant liposome suspension was centrifuged for 2 min at $10\,000 \times g$ to remove any residual metallic particles remaining in the suspension from the sonicator probe, using an Eppendorf microfuge.

The SUV, containing encapsulated carboxyfluorescein, were separated from the non-encapsulated carboxyfluorescein using Sephadex G-25 (fine). A volume of 0.2 ml of the SUV dispersion was loaded on 5 ml of the swollen Sephadex G-25 packed in a small column (5 ml syringe). After loading, the column was centrifuged ($500 \times g$, 5 min) and the eluent obtained contained the liposomes with encapsulated carboxyfluorescein. This SUV dispersion was used in all experiments within one day of its preparation.

Preparation of liposomes co-labelled with N-NBD-PE and N-Rh-PE. The method of resonance of energy transfer described by Struck et al. [41] was used to detect mixing of membrane lipids. In this assay two fluorescent lipids *N*-NBD-PE and *N*-Rh-PE with favorable spectral overlap are included in the same liposome membranes (PS 2 mM) at a relatively high concentration (2 mol% each) resulting in an efficient resonance energy transfer. When these liposomes fuse with virus particles, dilution of the fluorescent lipids occurs which results in a decrease in resonance energy transfer and a consequent 'dequenching' of the donor emission. *N*-NBD-PE was used as the donor and *N*-Rh-PE as the acceptor fluorescent lipid. The efficiency of energy transfer was measured by the emission at 530 nm of *N*-NBD-PE when the samples were excited at 470 nm. As a result of fusion, the fluorescence of *N*-NBD-PE was enhanced.

Fluorescence measurements. Carboxyfluorescein solution was excited at 490 nm and emission of fluorescence was detected at 520 nm [27]. Measurements were carried out in a final volume of 1.7 ml of Solution A containing 5 mM EDTA. Complete release of carboxyfluorescein from the liposomes was obtained by the addition of Triton X-100 to a final concentration of 0.1% (v/v) [27]. The fluorescence intensity observed after the Triton X-100 addition was considered as the maximal value (100%). All routine measurements were performed at 37°C. All fluorescence experiments were performed with a Perkin-Elmer MPF-44 spectrofluorimeter. Steady-state fluorescence depolarization of diphenylhexatriene was measured using the above instrument modified as described by Barenholz et al. [28], and steady-state fluorescence anisotropy was calculated as described previously [29].

Phospholipids and protein determination. Phospholipids were assayed by the Barlett method [30], and protein was determined by the method of Lowry et al. [31], using bovine serum albumin as standard.

Results

Release of carboxyfluorescein from small unilamellar vesicles by Sendai virus

Incubation of Sendai virus particles with carboxyfluorescein-containing PS vesicles at 37°C caused a significant time-dependent increase in the

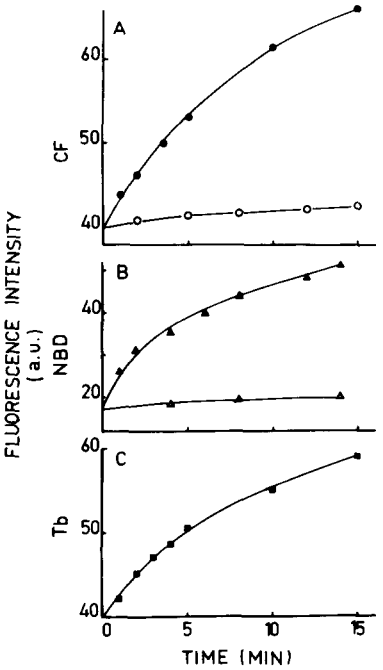

Fig. 1. Time-course of fluorescence intensity enhancement during the interaction of Sendai virus or reconstituted Sendai virus with PS liposomes. (A) Release of carboxyfluorescein (CF). PS liposomes (14 μM) loaded with carboxyfluorescein (80 mM) were incubated at 37°C in a final volume of 1.7 ml solution A in the absence (○———○) or presence (●———●) of Sendai virus (90 μg viral protein). The carboxyfluorescein fluorescence intensity was measured as described in Materials and Methods. (B) Mixing of membrane lipids. PS liposomes (7 μM) labelled with *N*-NBD-PE and *N*-Rh-PE (2 mol %) each were incubated in a final volume of 0.5 ml of solution A in the absence (△———△) and presence (▲———▲) of Sendai virus (150 μg viral protein) and the increase in NBD-fluorescence measured with time. (C) Mixing of membrane contents. PS Liposomes (50 μM) loaded with DPA (150 mM) in 0.5 ml of buffer containing 100 mM NaCl, 4 mM L-histidine and 0.1 mM EDTA (pH 7.4) at 37°C and the enhancement of Tb fluorescence measured with time.

carboxyfluorescein fluorescence intensity (Fig. 1A). When the interaction between the virus and the liposomes was performed within dialysis tubes immersed in excess volume of solution A, almost all the fluorescence of carboxyfluorescein (> 85%) was detected in the external medium (not shown). This clearly indicates that the increase in the fluorescence intensity is a result of dequenching of carboxyfluorescein fluorescence caused by its release from the liposomes and dilution in the external medium [27].

Preliminary results indicate that the interaction of PS liposomes with Sendai virus or reconstituted Sendai virus envelopes (see also 'The effect of reconstituted Sendai virus envelopes on phospholipid liposomes' below) also involve mixing of the virus and liposome membrane lipids (Fig. 1B) as well as mixing of entrapped aqueous contents (Fig. 1C). Mixing of membrane lipids (Fig. 1B) was determined from the release of energy transfer between NBD-PE and Rhodamine-PE present in the membrane of the PS liposomes [41]. Mixing of contents (Fig. 1C) was monitored using Tb-loaded reconstituted Sendai virus envelopes and DPA-loaded PS vesicles [42].

The rate of carboxyfluorescein release was found to be directly proportional to the virus concentration. Thus, for 14 μM PS (5 times the K_m, see below), the specific activity of the virus was 6.9 ± 1.0% (carboxyfluorescein released) per min per 100 μg viral protein. The effect of PS concentration (which is related to the liposome concentration) on the initial rates of carboxyfluorescein release exhibits classical saturation kinetics (Fig. 2). Under the experimental conditions used, the virus-in-

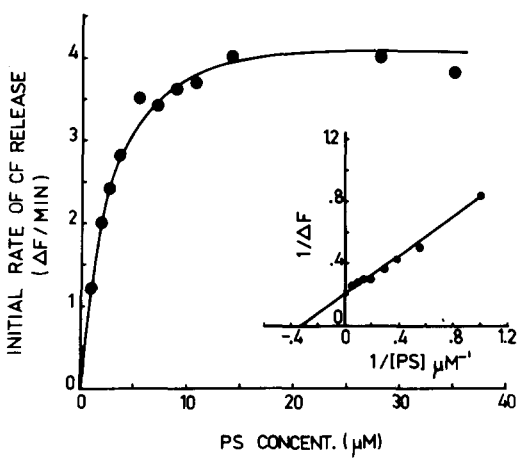

Fig. 2. Virus-induced release of carboxyfluorescein. Effect of PS concentration. PS liposomes, in increasing concentrations were incubated with Sendai virus (90 μg) at 37°C, as described in the legend to Fig. 1. The extent of carboxyfluorescein released was followed with time, and initial rates were calculated from the first two minutes of incubation. Under these conditions, the maximum release obtained did not exceed 50% of total, even at the lowest PS concentration. The virions specific activity and K_m were calculated from the Lineweaver and Burk curve (see inset).

TABLE I

SENDAI VIRUS INDUCED RELEASE OF CARBOXYFLUORESCEIN FROM LIPOSOMES. EFFECT OF LIPID COMPOSITION

Carboxyfluorescein was trapped in liposomes composed of equimolar mixtures of various phospholipids (3.5 μM) with or without cholesterol (50 mol %), as described in Materials and Methods and in the legend to Fig. 1. The release of carboxyfluorescein after the addition of 90 μg viral protein was determined after 10 min of incubation at 37°C. Spontaneous release of carboxyfluorescein after 10 min of incubation at 37°C from the various vesicles used in the absence of virions, did not exceed 4% and 2% in the absence and presence of cholesterol, respectively.

System	Carboxyfluorescein release (% of total)	
	− cholesterol	+ cholesterol
PI : PC	64.9	28.7
PG : PC	48.1	11.3
PS : PC	17.2	5.6
PE : PC	3.3	2.2
PC	4.1	2.0
PS	31.0	14.4

duced release of carboxyfluorescein was characterized by a K_m of 2.8 μM of PS and a $V_{max} = 5$ fluorescence arbitrary units per min (see inset in Fig. 2). As expected, the K_m was independent of the virus concentration, whereas the V_{max} was directly proportional to the virus concentration (up to 100 μg of viral protein; not shown).

Incubation of virus particles with liposomes of various phospholipid compositions showed that among the lipid compositions tested (Table I), only liposomes containing negatively charged phospholipids were affected by the virions. The virus-induced leakage of carboxyfluorescein was significantly reduced when cholesterol was included in the negatively charged vesicles (Table I). No release of carboxyfluorescein was observed upon incubation of Sendai virions and liposomes composed of PC. Neither incorporation of cholesterol nor of phosphatidylethanolamine rendered the PC liposomes susceptible to the lytic action of the Sendai virions (Table I).

Incubation of Sendai virus particles with carboxyfluorescein-containing PS liposomes at different pH values at 37°C, revealed that about the same amount of carboxyfluorescein was released in 30 min at any pH between 7.0 and 9.5 (data not shown). Measurements of carboxyfluorescein release below pH 7.0 are unreliable because of the large effect of pH on carboxyfluorescein fluorescence intensity [40].

The virus-induced release of the entrapped carboxyfluorescein was dependent on the temperature of incubation. It is clear from the results in Fig. 3 that over the entire range of temperatures between 4 and 56°C, the virus-induced release of carboxyfluorescein from the PS liposomes is much faster than the carboxyfluorescein spontaneous release. Arrhenius plots clearly reveal a biphasic behavior, having its break point at 20 ± 1°C for both virus-induced and spontaneous carboxyfluorescein release. Up to 20°C, the virus-induced release is relatively slow and temperature independent, whereas between 20–56°C it is much faster. The energy of activation (for the range of 20–56°C) calculated by linear regression of the Arrhenius plots gave a value of 11 kcal/mol (correlation coefficient $r = 0.946$) for the release caused by the virus, as compared to an energy of activation of almost double, 20.5 kcal/mol (correlation coefficient $r = 0.986$) for the spontaneous release. Noteworthy is our finding that over the temperature range of 10–56°C no thermotropic transition can be detected using the fluorophore diphenylhexatriene [28,29] in neither the PS liposomes' nor in the virions' membrane.

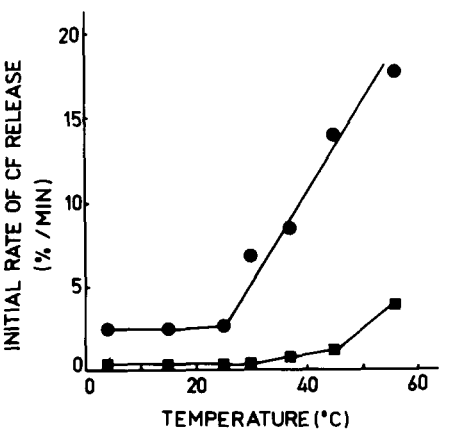

Fig. 3. Effect of temperature on Sendai virus-induced release of carboxyfluorescein (CF) from PS liposomes. PS Liposomes (14 μM) were incubated at the desired temperatures without (■———■) or with Sendai virus (●———●) (90 μg) in a final volume of 1.7 ml of solution A. The determination of initial rates was as described in the legend to Fig. 2.

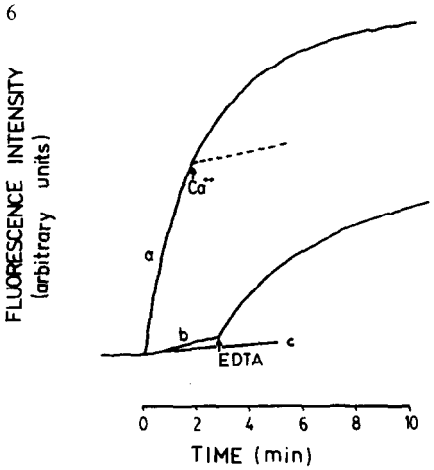

Fig. 4. Effects of Ca^{2+} and EDTA on the Sendai virus-induced carboxyfluorescein release from PS vesicles. Sendai virus particles were incubated with PS liposomes as described in the legend to Fig. 1, in the absence (a) or presence (b) of calcium ions (2 mM), and the carboxyfluorescein fluorescence intensity was measured as a function of time. The arrows indicate addition of Ca^{2+} (2 mM) or EDTA (5 mM) at different periods of time in (a) and (b), respectively. Line c shows the carboxyfluorescein release induced by 2 mM Ca^{2+} in the absence of virus.

Inhibition of virus-induced leakage of carboxyfluorescein by calcium ions

Addition of calcium ions markedly inhibited the viral lytic activity (Fig. 4). As can be seen in Fig. 4, 2 mM of Ca^{2+} suppressed the virus-induced carboxyfluorescein release to the level of the spontaneous release (release of carboxyfluorescein

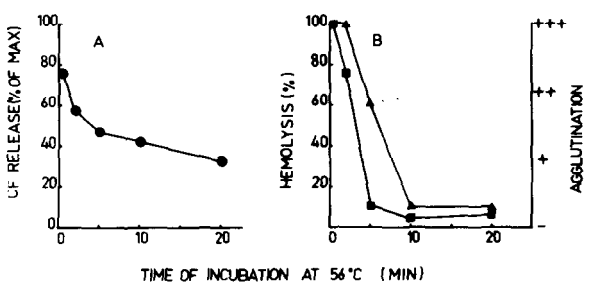

Fig. 5. Time-dependent thermal inactivation of the virus lytic activity on PS-SUV (A) and on the viral-induced agglutination and hemolysis (B). Sendai virus particles (900 μg virus protein in 300 μl of solution A) were heated at 56°C. At different periods of time, samples were withdrawn and incubated at 37°C with carboxyfluorescein-containing liposomes or with human erythrocytes. Hemolysis (■———■) and agglutination (▲———▲) of human erythrocytes, as well as carboxyfluorescein (CF) release (●———●) were determined as described in Materials and Methods.

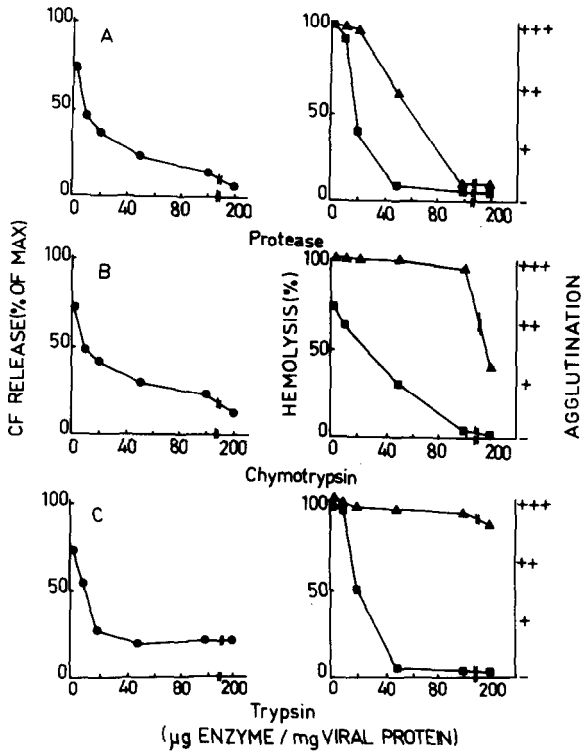

Fig. 6. Inhibition of Sendai virus hemolytic and lytic activities by proteolytic enzymes. Sendai virus particles were digested with streptomyces protease (A), chymotrypsin (B) and trypsin (C) by incubating 300 μg of Sendai virus with the indicated amount of the proteolytic enzyme, in a final volume of 160 μl Solution A, for 30 min at 37°C. Proteolysis was terminated by washing the virus particles with large excess of Solution A. Release of carboxyfluorescein (CF) from PS vesicles (●———●) and virus-induced hemagglutination (▲———▲) and hemolysis (■———■) were performed as described in materials and methods.

from PS liposome in the presence of 2 mM Ca^{2+} but in the absence of virus occurs at a very slow rate, see line c in Fig. 4). The inhibitory effect of Ca^{2+} could be reversed by addition of EDTA (5 mM) to the virus-liposome suspension (Fig. 4).

Comparison between the hemolytic activity of Sendai virus and its ability to release carboxyfluorescein from PS vesicles

(a) Thermal inactivation. The results in Fig. 5A show that incubation of the virus particles at 56°C inhibited their ability to cause release of carboxyfluorescein from PS liposomes (Fig. 5A), as well as the viral hemolytic activity (Fig. 5B). Incubation at

TABLE II

THE EFFECT OF TRYPSIN ON THE VIRUS HEMOLYTIC AND LYTIC ACTIVITIES

For trypsinization, 900 μg of Sendai virus particles were incubated with 60 μg of trypsin for 30 min at 37°C. Proteolysis was terminated by washing the incubation mixture with Solution A. When soybean trypsin inhibitor (STI) was used, it was added to the incubation mixture at a ratio of STI: trypsin (w/w) 10:1 (a 10-fold excess above trypsin). The intact and trypsinized virions were incubated with the PS liposomes for 30 min as described in the legend to Fig. 1. CF, carboxyfluorescein.

Treatment of Sendai virus	Human erythrocytes			PS liposomes	
	Agglutination	Hemolysis (% of total)	Inhibition (%)	CF release (% of total)	Inhibition (%)
None	+	96	0	58	0
Trypsin	+	5	94	17	70
Trypsin + STI	+	94	3	56	3

56°C also impaired the virus' binding activity, namely, its ability to agglutinate human erythrocytes (Fig. 5B).

(b) Proteolytic inactivation. The results presented in Fig. 6 indicate that a virus-associated polypeptide is responsible for the viral lytic activities. Three proteolytic enzymes (trypsin, chymotrypsin and *Streptomyces* proteinase), at relatively low concentrations, substantially reduced the viral lytic activity. Virus particles which were treated with 100 μg or more of these proteolytic enzymes for 30 min caused very little, if any, release of carboxyfluorescein from PS vesicles. The view that inactivation of the viral lytic activity is due to proteolysis of a virus-associated polypeptide is evident from the results in Table II. Soybean trypsin inhibitor completely abolishes the inhibitory effect of trypsin. Virus particles incubated with a mixture of trypsin and soybean trypsin inhibitor which were able to hemolyse hu-

TABLE III

THE EFFECT OF PMSF, DITHIOTHREITOL AND HEAT TREATMENT ON THE VIRUS-INDUCED RELEASE OF CARBOXYFLUORESCEIN FROM PS LIPOSOMES

Treatment of the virus with PMSF was performed by incubation of 100 μl Sendai virus (3 mg protein/ml) with either PMSF (in methanol, 6 mM final concentration) or with methanol (8 μl of methanol/100 μl of Sendai virus) for 30 min at 37°C. Methanol-treated Sendai virus particles caused almost the same carboxyfluorescein (CF) release as non-treated virus (not shown). Reduction of the virus with dithiothreitol (DTT) was performed by incubation of 300 μg Sendai virus particles in 10 mM DTT, in a final volume of 150 μl of Solution A, for 20 min at 37°C. Trypsinization of virus particles was as described in the legend of Table II. All other experimental conditions were as described in Materials and Methods. Carboxyfluorescein release was estimated after 30 min of incubation. n.d., not determined.

Treatment of Sendai virus particles	Human erythrocytes		PS liposomes
	Agglutination	Hemolysis (% of total)	CF release (% of total)
None	+	96	57
PMSF	+	20	48
Methanol	+	96	52
Trypsin	+	10	15
DTT	−	3	87
DTT + trypsin	−	3	38
DTT + trypsin + STI	n.d.	n.d.	86
DTT + heating at 70°C	n.d.	n.d.	18

man erythrocytes, also lysed carboxyfluorescein-containing PS liposomes almost to the same extent as control untreated virus particles (Table II).

Moreover, the results in Fig. 6 confirm previous observations [32] showing that two of the proteolytic enzymes (trypsin and chymotrypsin), at relatively low concentrations, specifically inactivate the viral hemolytic activity, without impairing its agglutination ability (virus-cell binding activity).

(c) The effect of PMSF and dithiothreitol on the viral lytic activity. It has been shown before [33] that the alkylating agent PMSF inactivates the Sendai virus' hemolytic activity but not its agglutination ability. The results in Table III show that PMSF did not affect the viral lytic activity. Virus particles treated with PMSF, although unable to cause hemolysis, induced the release of carboxyfluorescein from PS vesicles almost to the same extent as control untreated virus particles (Table III).

Previously, it has been shown that reduction of virus particles with low concentrations of DTT causes a selective inactivation of the viral binding activity through its HN glycoprotein [34]. Indeed, the results in Table III show that dithiothreitol-treated virus particles did not agglutinate (bind to) human erythrocytes and, therefore, failed to cause any hemolysis. However, reduction with dithiothreitol did not inhibit the viral lytic activity; on the contrary, it even stimulated it (Table III).

TABLE IV

INDUCTION OF CARBOXYFLUORESCEIN RELEASE FROM PS VESICLES BY RECONSTITUTED SENDAI VIRUS ENVELOPES

Reconstituted Sendai virus envelopes (500 μg) were treated with trypsin (30 μg) prior to the incubation with carboxyfluorescein-containing PS liposomes, as described in Table II for intact Sendai virus particles. The non-treated and trypsinized reconstituted Sendai virus envelopes (30 μg viral protein) were incubated with PS liposomes (14 μM) as described in the legend to Fig. 1 for intact Sendai virus particles.

Treatment of reconstituted Sendai virus envelopes	temperature of incubation (°C)	Carboxyfluorescein release in 30 min (% of total)
None	37	56
None	4	5
Trypsin	37	18

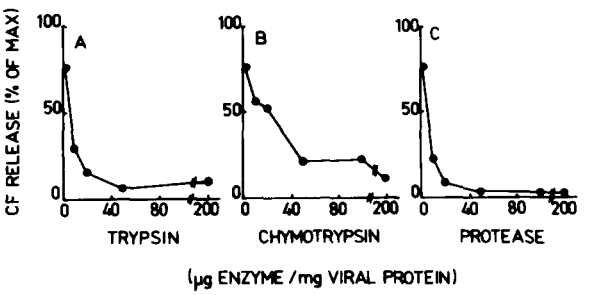

Fig. 7. Inactivation of the lytic activities of dithiothreitol-reduced Sendai virions by proteolytic enzymes. Sendai virus particles were reduced by dithiothreitol as in Table III, and incubated with trypsin (A), chymotrypsin (B), or with *Streptomyces* proteinase (C), as described in the legend to Fig. 6. All other experimental conditions were as described in Materials and Methods. CF, carboxyfluorescein.

The results in Fig. 7 show that the lytic activity of dithiothreitol-treated Sendai-virus particles was inhibited by trypsin, chymotrypsin and proteinases to a similar extent as the lytic activity of control, untreated virions (compare results in Fig. 6 with those in Fig. 7).

The effect of reconstituted Sendai virus envelopes on phospholipid liposomes

The results in Table IV show that reconstituted Sendai virus envelopes, similar to intact virus particles, caused the release of carboxyfluorescein from PS vesicles at 37°C but not at 4°C. The lytic activity of reconstituted envelopes was greatly inhibited after treatment with trypsin.

Discussion

The results of the present work show that Sendai virus particles induce release of carboxyfluorescein entrapped in small unilamellar vesicles composed of negatively charged phospholipids such as phosphatidylserine. Virus-induced lysis of phospholipid vesicles has been demonstrated before, and has been used as quantitative method to follow fusion events between Sendai virus envelopes and liposomes [15]. The results presented in Figs. 1B and 1C indicate that the virus-induced carboxyfluorescein release from PS liposomes is related to virus-liposome fusion, as it is accompanied by mixing of membrane lipids and entrapped contents. Detailed comparison of the three different

events (leakage, mixing of lipids and mixing of contents) is presently under investigation.

In most previous reports vesicles composed of non-charged phospholipids such as PC were used. The interaction of these vesicles with Sendai virions appeared to require the presence of sialic acid-containing components [15,16,19], although in the presence of cholesterol some fusion was observed even in the absence of such components [18].

The virus-induced fusion and lysis of negatively charged liposomes did not require the presence of virus receptors (Fig. 1), despite the fact that a virus-associated polypeptide is responsible for this lytic activity, as virus particles treated with proteolytic enzymes or incubated at high temperatures, lost their lytic activity. Reduction in the viral lytic activity, when tested with PS liposomes, was always accompanied by a reduction of its hemolytic activity. Both activities were inactivated by the same proteolytic enzymes or by heat treatment.

The present work shows that calcium ions, which are known to inhibit the viral hemolytic activity [24], also significantly reduced the viral lytic activity, as measured by its ability to induce release of carboxyfluorescein from the PS liposomes. Calcium ions are known to have fusogenic activity on PS liposomes. This was not the case in our experimental system. No contradiction exists since the Ca^{2+} fusogenic activity is known to be inhibited by the relative high concentration of Na^+ used by us [20]. The presence of 160 mM Na^+ elevate the Ca^{2+} threshold concentration to higher values than the 2 mM used in our system. This is shown by the minimal release of carboxyfluorescein from PS liposomes induced by Ca^{2+} in the absence of virus (see line c in Fig. 4). The inhibitory effect of Ca^{2+} on the Sendai virus-induced carboxyfluorescein release may result from Ca^{2+}-induced non-leaky fusion of the small unilamellar vesicles to form larger vesicles which have lower tendency to fuse with the virions due to the smaller curvature of the resultant vesicles. However, this effect should be irreversible. The observed increase in the rate of carboxyfluorescein release upon addition of EDTA rules out this possibility. An alternative explanation can be based upon previously published data which show that polyvalent cations rigidify the membrane of negatively charged liposomes [43] and in our case such rigidification prevents the leakage of carboxyfluorescein.

This possibility is also consistent with the inhibition of the viral lytic activity observed upon inclusion of cholesterol in the liposome membrane. It is not clear, however, whether the resultant rigidification [35,43] reduces the carboxyfluorescein release by inhibiting liposome-virus fusion or by making this fusion non-leaky.

Based on the present results, it may be argued that negatively charged phospholipids can serve as receptors for Sendai virions. If so, the presence of sialic acid residues which act as receptors for the viral HN glycoprotein, is not essential as previously suggested by Hsu et al. [18]. In this respect, it is noteworthy that recently it has been shown that proteoliposomes, containing the G protein of vesicular stomatitis virus, could fuse only with phospholipid vesicles composed of negatively charged phospholipids such as PS [17]. Negatively charged phospholipid vesicles could also be fused by non-viral positively charged agents, such as Ca^{2+} [20] or polylysine molecules [21]. This process probably involves aggregation of the liposomes to form tetralayer structures, which then collapse into a fused membrane that may be leaky [20].

It is clear that the hemolytic activity of the virus differs from its lytic activity on the negatively charged phospholipid vesicles. Although both processes required viral glycoprotein(s), only the hemolytic activity is sensitive to both dithiothreitol and PMSF treatments. These differences can reflect variability in the composition and/or curvature of the host membrane (compare erythrocyte membrane to small unilamellar vesicles composed of negatively charged phospholipids). Alternatively it is feasible that we are dealing with non-identical processes. It is possible that fusion between Sendai virions and liposomes containing negatively charged phospholipids resembles fusion of these vesicles induced by polypeptides such as polylysine [21], polyhistidine [38,44] or mellitin [39]. Being positively charged at neutral pH, both Sendai viral membrane glycoproteins (F and HN) may interact electrostatically with the negatively charged phospholipid vesicles and induce their fusion with virions in a similar fashion as suggested for the fusion of negatively charged lipo-

somes induced by polypeptides [21,38,39,44]. Studies to clarify which of the two above possibilities is responsible for the vesicles' lysis, as well as the role of F and HN proteins in this process, are currently underway.

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